On the Origin of Type 2 Inflammation in the Intestinal Tract

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The studies described in this work were performed in the Division of Gastroenterology and Nutrition, Boston Children's Hospital and Harvard Medical School, Boston, Massachusetts

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De oorsprong van type 2
inflammatie in het maag-darm kanaal

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Preface

On the 26th of October 2010, I took the 8:00 AM Green Line subway (nicknamed 'the turtle') from Hynes Convention Center to Longwood Avenue, to continue on foot past Brigham and Women's Hospital and Harvard Medical School towards the adjacent, awe-inspiring Boston Children's Hospital. In town for the AASLD's 2010 Liver Meeting, I had taken the opportunity to arrange a meeting with prof. Richard Grand and Edda Fiebiger with the purpose of determining whether we could create a position that would serve as my PhD training in the field of pediatric gastrointestinal immunology.

The architect of today's get-together had been prof. Edmond Rings, whom I had met during my internship at the Beatrix Children's Hospital in Groningen, and who himself was an alumnus of a longstanding exchange program in pediatric gastroenterology for Dutch medical students (spearheaded by prof. Hans Büller and prof. Hugo Heymans) with the laboratory of prof. Grand in Boston (the interested reader is referred to the scientific publication in 2008 by Rings et al. in the *Journal of Pediatric Gastroenterology and Nutrition* volume 46, pages 419–422 that delineates the career outcomes of former students who partook in this program). During the previous months, Edmond and I had sat down a number of times in his office to discuss the options and the obstacles of doing PhD work in the United States, and from the first time we had talked about this I had grown determined to explore all those options and overcome all those obstacles. And this determination turned out to be needed. We soon found out that the 'canonical' pathway – a job in the lab of dr. Stephen Krasinsky who had taken over the reigns in the former Grand lab – was unavailable for at least two more years due to the fact that my colleague (and future friend) Boaz Aronson had recently started his PhD work with Steve's group. Fortunately, Boaz's efforts and dedication had not gone unnoticed, and dr. Edda Fiebiger, a colleague of Steve Krasinsky within the GI Division who was already involved in supervising short-stay Dutch MD students in her lab via an exchange program of her own, had indicated to dr. Grand that she would be interested to meet with me as a potential PhD candidate from The Netherlands.

The meeting went well enough. Dick Grand almost immediately displayed the boundless enthusiasm that, as I would come to learn, so exemplifies his attitude and mentorship, and started drafting a preliminary budget (based on a grossly underestimated monthly expenditure of $1200!). Edda seemed more hesitant; I would be the first PhD student she had ever taken on (would I have what it takes?) and although the financial position of the lab was currently strong it was unclear – as it always is – how long this would remain to be the case. And thus the offer on the table was as follows: I could come for a trial period of one year, with my own funding, and if to mutual satisfaction, I could stay for the length of the PhD and we would then talk about getting my name on the lab's payroll.

I did get to meet Boaz as well that first day at Children's, who at the local Starbucks clearly delineated the contrast between a custom-made PhD program like the one he found himself in (and that I had similarly started plotting for myself that very morning), and the seemingly

smooth, all-expenses-paid-for, experience we believed PhD students in The Netherlands would have. “Basically it is a self-financed endeavor, where you live from grant-to-grant until your PI (‘maybe, fingers crossed!’) one day has sufficient funding to take on your salary”, only to be followed by a vivid recital of the frustrating procedure of procuring the appropriate research visa. However at the end of our talk he also gave me a heartfelt recommendation: “you should do it… it will be a lot of stress and effort, but you should do it”.

And I did, obviously. On the 5th of April 2011 I began working in Edda’s lab in Boston. Not having procured a single grant yet from applications I had written in Holland over the months before, I started those first weeks with writing a research proposal directed at the Ter Meulen Fund of the Royal Netherlands Academy of Arts and Sciences (KNAW), which about a month later generously agreed to pay all my expenses for the first year of my studies in Boston. Furthermore, an application I had sent to the Banning de Jong Fund (part of the Prins Bernhard Cultural Fund) in The Netherlands also got approved, extending my funding period to well over 1.5 years: more than enough to hold my end of the bargain.

The significance of the early support from these two institutions for the work described in this thesis cannot be overstated. During the first years, in which I was still looking for an overarching topic for this project, the privately acquired funding enabled me to pursue a variety of avenues of research that I considered interesting and to collaborate with a great number of people. I was fortunate that Edda has continued to wholeheartedly and generously support this attitude during my entire time in Boston. In search of the research question that would ultimately form the core of my thesis, I was thus allowed to work on several topics. Some of these make up the first part of this thesis; others remain in a drawer, deemed too inconclusive or complicated to continue, and others still eventually did or will get published but fall so far outside the scope of the already broad topic of Th2-type intestinal inflammation that they could not be reasonably included in this thesis.

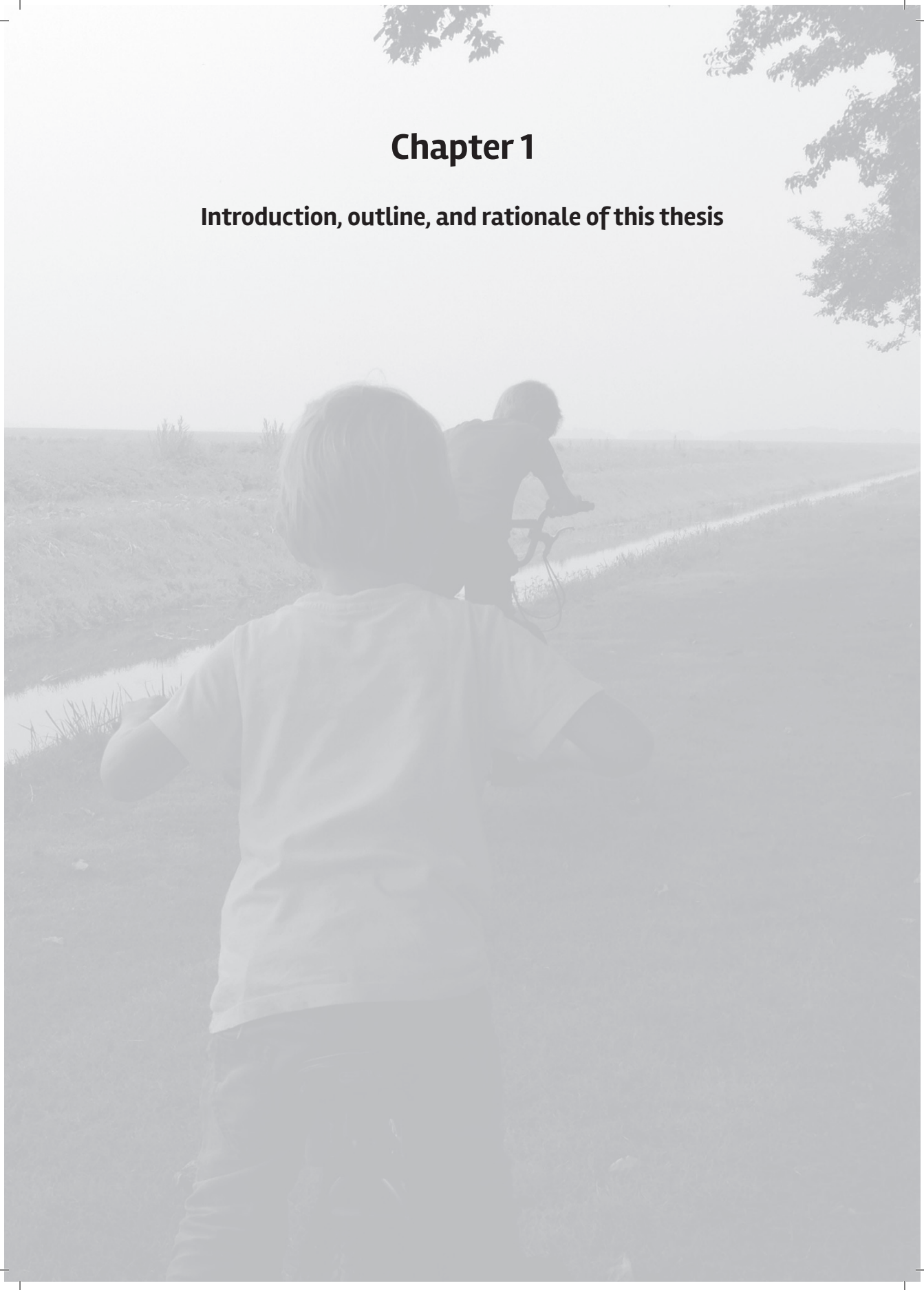
Two years into my time in Boston, on Friday the 10th of May 2013 to be exact, dr. Jeremy Goettel (collaborator, friend, paranimf) and I had an idea that would herald the work described in the second part of this thesis and that I consider to be the core of my scholarly endeavors at the Children’s Hospital. The preliminary data that rapidly accumulated within this line of investigation, through a strike of serendipitous luck, gathered the interest of Dr. Dirk de Hond and Dr. Ric van Tol from the Mead Johnson Company, who had very recently started to get involved with the GI Division at the Children’s Hospital Boston. Their incredible enthusiasm and generous financial support in the form of a tailor-made post-doctoral fellowship has made possible the research delineated in Chapters 7, 8 and 9, and provided the means for me to prolong my stay in Boston up to the 4.5 years that it ultimately took to generate the data described in the thesis as it now lies before you.

I sincerely hope that by reading this work you will experience a fraction of the joy I have gotten out of working on it.

Willem S. Lexmond, June 2017

Chapter 1

Introduction, outline, and rationale of this thesis



These are incredibly exciting times to become involved in the field of mucosal immunology. The mucosal surfaces of our body form our most direct and intimate connection to the outside world, and are therefore at the center stage of the interplay between our rapidly changing environment and our evolutionary (genetic) heritage. We have evolved under circumstances that differed in many ways from our current, affluent living conditions. Any rapid changes in external (environmental) factors[1] have the potential to disrupt the balance that has followed from hundreds of thousands of years of evolution. Such instability between external and internal factors puts us at risk for what are sometimes referred to as evolutionary mismatch diseases. They are complex, multifactorial disorders that are driven by gene-environment interactions and that are only beginning to be understood at the molecular level.

Aberrant type 2 inflammation in the intestinal tract, the topic of this thesis, affects the largest and arguably most complex mucosal surface of our body, and is a prime example of a highly complicated disease state that is directly related to changes in our environment. It underlies several immune-mediated disorders such as food allergy and eosinophilic gastrointestinal diseases that are characterized by rapidly increasing incidence and prevalence in children (and adults) in Westernized societies. **The overarching goal of this thesis is to improve our understanding of the pathophysiology of type 2 intestinal inflammation**. To this end, two complimentary approaches have been applied, and they are described in detail in the two parts that make up this work. In **Part 1**, we have **aimed to obtain novel insights into the pathogenesis of allergic intestinal inflammation from a cohort of patients with established eosinophilic esophagitis**, a type 2 inflammatory disorder of the upper GI tract. Using clinical data in combination with tissue biopsies of these children, we demonstrate considerable immunological heterogeneity, suggesting that distinct mechanisms may underlie a final common pathway of disease. In **Part 2**, we have **approached the problem of aberrant type 2 intestinal inflammation by characterizing a novel experimental mouse model of food allergy that allows for the interrogation of the critical pathways involved in its pathogenesis**. In both these approaches, we have strived towards translational value of our experiments. Multifactorial, immune-mediated diseases at the mucosal surface are defined in humans, but commonly studied in mice. Therefore, in the parts of the thesis where we rely on data from animal models, we have actively investigated the degree of overlap with the human situation and these comparisons form an essential aspect of the experimental work presented in this thesis (**Part 1 Chapter 5, Part 2 Chapters 6-9**). In this introductory chapter, we will first present a brief overview of type 2 inflammation, after which the outline, sub aims, and rationale of both parts of the thesis are sequentially discussed.

1 Environmental factors in this context include physical, biological and behavioral factors, for example diet, family-size and organization, living conditions, birthing habits, availability of therapeutic modalities (antibiotics, immunosuppressants, or anti-inflammatory agents), quality of water, air and soil, etc.

1.1 Type 2 inflammation

More than a hundred years ago, the Frenchman Charles Richet was awarded the Nobel Prize in physiology for the first description of anaphylaxis. In his acceptance speech, he marveled at the "extraordinary phenomenon that so insignificant a quantity of poison can modify the organism to the extent that the succeeding days down long years can not eradicate this indelible modification" (Richet, 1913). Based on transfusion experiments between sensitized and non-sensitized dogs, Richet already concluded that "the anaphylactogen poison is therefore a chemical substance contained in the blood", but it would take an additional fifty years for immunoglobulin E (IgE) to be identified as the mediator of anaphylactic reactions. Historically, IgE-mediated anaphylaxis is classified as the archetypal, type I, hypersensitivity reaction according to the scheme proposed by Gell and Coombs in 1963 (Male et al., 2006). Since the identification of distinct Th1 and Th2 T helper subsets in the 1980s, it has become clear that anaphylaxis can be considered the most extreme manifestation of what are collectively termed type 2 (or Th2-type) immune responses.

Type 2 immunity has evolved to serve a variety of host-defense functions, ranging from protection against parasites and support of epithelial barrier integrity, to regulation of wound healing and control of metabolic homeostasis (Palm et al., 2012; Pulendran and Artis, 2012; Wynn, 2015). Many of these functions are performed by effector cells of the innate immune system, including eosinophils, basophils and mast cells, which in turn are orchestrated by Th2 lymphocytes of the adaptive immune system through the production of type 2 cytokines such as IL-4, IL-5 and IL-13. Th2 help is critical for the production of IgE antibodies from B cells, which through binding to the high-affinity IgE surface receptor FcεRI can bestow antigen-specific effector functions onto mast cells and basophils. Following antigen-mediated crosslinking of surface bound IgE, these IgE effector cells respond by releasing pre-formed inflammatory mediators such as histamine and proteases in the extracellular space, which lead to vasodilation, fluid extravasation and pruritus (Burton and Oettgen, 2011; Gould and Sutton, 2008; Kraft and Kinet, 2007). Severe IgE-mediated immune responses are accompanied by systemic symptoms that can include airway edema and hypotension (anaphylactic shock), and will occasionally result in death if left untreated.

Because of the potency of IgE-mediated immunity, it is no surprise that multiple inhibitory pathways have evolved to control the initiation and magnitude of type 2 immune responses (Wynn, 2015). Failure in these regulatory mechanisms is increasingly common in Westernized societies, and can result in the generation of allergen-specific IgE, type I hypersensitivity reactions and allergic tissue inflammation (Palm et al., 2012; Pulendran and Artis, 2012; Wynn, 2015). Manifestations of allergic diseases depend on the nature of the allergen to which the host has become sensitized: pollen sensitivity will result in hay fever, house dust mite is strongly associated with allergic asthma and food allergies are due to IgE-mediated reactions after ingestion of regular food antigens. However, it is important to stress that the

spectrum of Th2-type atopic disease extends beyond IgE-mediated immune activation. IgE-independent forms of atopic diseases have been described (Novak and Bieber, 2003) and induction of allergen-specific IgE should probably be considered a symptom of type 2 immune disorders rather than its pathognomonic feature. One type 2 inflammatory disease that illustrates this complex relationship is eosinophilic esophagitis, which forms the subject of the first part of this thesis and will therefore be introduced in the following section.

1.2 Eosinophilic esophagitis: a type 2 inflammatory disorder of the upper gastrointestinal tract – an introduction to Part 1

Eosinophilic esophagitis (EoE) belongs to the primary eosinophilic gastrointestinal disorders (EGIDs), which additionally include eosinophilic gastritis, eosinophilic gastroenteritis, eosinophilic enteritis, and eosinophilic colitis (Gonsalves et al., 2016). The unifying feature of all EGIDs is accumulation of an eosinophil-rich inflammatory infiltrate at the mucosal surface. The pathogenesis of these disorders involves a complex interplay of genetic predisposition, exposure to allergens, and Th2-type activation of the immune system (Masterson et al., 2011; Mikhail and Sampson, 2016). EoE is the most frequently diagnosed EGID (Gonsalves et al., 2016), with an incidence of approximately 1 new case per 10,000 persons per year and a prevalence of 0.5-1 cases/1000 persons in Westernized societies (Dellon, 2014; Furuta and Katzka, 2015).

As is clear from its name, eosinophilic esophagitis is characterized by chronic eosinophilic inflammation of the esophagus. It has only been recognized as a distinct clinical entity since the early 1990s (Attwood et al., 1993), and the disorder is strongly associated with allergies to both inhaled as well as food-derived antigens (Hruz et al., 2011; Mulder and Justinich, 2011; Rothenberg, 2009). EoE often manifests in childhood with symptoms that become increasingly localized with a later age of onset; affected infants typically present with failure to thrive and feeding disorders, whereas abdominal pain, vomiting, dysphagia and food impaction are more pronounced in older children and adults (Assa'ad et al., 2007; Brown-Whitehorn and Liacouras, 2007; Straumann et al., 2012). The differential diagnosis of these symptoms is broad, and commonly there is considerable delay between onset of symptoms and diagnosis (Hruz et al., 2011; Schoepfer et al., 2011). The diagnostic gold standard of EoE remains to be histopathological examination of multiple esophageal biopsies for the presence of ≥15 eosinophils per high power field. This finding is, however, not pathognomonic (Liacouras et al., 2011; Straumann, 2009) and diagnostic uncertainty often further delays the start of appropriate treatment. Esophageal inflammation can be controlled with topical corticosteroids, most often in the form of swallowed fluticasone or liquid budesonide, and with dietary interventions. The reported response rates to either dietary interventions or topical steroids vary from 50% to >90%, and best responses are achieved by treatment with

elemental diets (Arias and Lucendo, 2014; Arias et al., 2014; Gonsalves et al., 2012; Lucendo et al., 2013). However, since EoE is a chronic condition, inflammation typically recurs upon discontinuation of treatment. Furthermore, tissue-remodeling events may continue despite installation of appropriate therapy, which in turn can lead to the long-term complications of disease such as strictures of the esophagus (DeBrosse et al., 2011). Dilatation of esophageal strictures is used to relieve EoE-associated symptoms, such as dysphagia, but does not reduce ongoing inflammation (Dellon and Liacouras, 2014). Altogether, EoE results in a reduced quality of life and commonly has persistent symptoms 15 years after presentation (DeBrosse et al., 2011).

The immunologic hallmark of EoE is a Th2-type, allergen-driven immune response that leads to chemotaxis and infiltration of eosinophils into the affected esophagus. The comorbidity of allergic diseases ranges from 42-93% in pediatric and 28-86% in adult EoE patients, which is significantly higher than in patients with reflux esophagitis or in the general population (Liacouras et al., 2005; 2014; Roy-Ghanta et al., 2008; Spergel et al., 2005). Food antigen-specific IgE can be detected in a large percentage of patients, and cross-reactivity between aeroallergens and food antigens has been associated with disease progression (Spergel, 2005; van Rhijn et al., 2013). Despite the clear association between EoE and atopic disease, serum IgE levels are normal in up to 50% of EoE patients, which has led some authors to conclude that eosinophilic inflammation occurs largely independent of IgE (Simon et al., 2016). In support of this notion, anti-IgE treatment with the monoclonal antibody omalizumab does not improve EoE in children or adults (Clayton et al., 2014; Rocha et al., 2011). Instead, it has been suggested that esophageal inflammation is mediated by IgG4 antibodies against food antigens, which are found in higher titers in serum and esophageal tissue of adult EoE patients when compared to controls (Clayton et al., 2014). Importantly, IgG4 and IgE are both Th2-type antibodies because B cells require IL-4 to class-switch to either isotype (Aalberse et al., 2016). *In vivo*, allergen-specific IgG4 and IgE are thought to compete for allergen binding, and increased titers of allergen-specific IgG4 are associated with acquisition of tolerance during oral immunotherapy trials for IgE-mediated food allergies (Akdis and Akdis, 2014b). It is interesting to note that a number of cases have been described in which children undergoing oral immunotherapy developed EoE (Lucendo et al., 2014).

Irrespective of the results of allergenic testing in an individual patient, the type 2 inflammatory nature of EoE is apparent from the esophageal mRNA pattern (Blanchard et al., 2006a; 2006b; Furuta et al., 2007; Noti et al., 2013), the predominant role for IL-13 in pathogenesis (Blanchard et al., 2010; Davis and Rothenberg, 2016) and esophageal infiltration of basophils and mast cells in addition to eosinophils (Abonia et al., 2010; Noti et al., 2013). Much remains to be learned about the interplay between allergic comorbidities and atopic constitution on the one hand, and the evolution of EoE pathophysiology on the other. In **Chapter 5**, we explore one aspect of this interplay by demonstrating that allergic sensitization through an impaired skin barrier promotes the development of EoE via IL-

33 and basophils. This finding links EoE to eczema in early life and suggests that EoE, like asthma, could be regarded another station in what has been termed the atopic march[2]. The large number of clinical and mechanistic reviews that have been published last year alone on the subject of eosinophilic esophagitis (EoE) is indicative of the great interest that allergists, immunologists, and gastroenterologists have in this relatively new disease entity (see for example (Aceves, 2015; Cianferoni and Spergel, 2016; Davis and Rothenberg, 2016; Furuta and Katzka, 2015; Oyoshi, 2015; Rothenberg, 2015)). Given the availability of these recent publications, no attempt will be made to further review the general characteristics of EoE here, since the aim of this chapter is to introduce the specific research questions that are the focus in the upcoming chapters.

1.3 Outline and aims of this thesis pertaining to Part 1

In contrast to asthma, for which analysis of lung biopsies is not part of routine patient care, EoE can currently only be diagnosed through demonstration of an eosinophilic infiltrate in the affected tissue. It is this diagnostic requirement, together with increasing awareness amongst physicians, a relatively high and rising incidence of disease, and technological advances in high-throughput quantification of tissue mRNA levels that has given rise to large cohorts of pediatric and adult patients in whom clinical information can be correlated to histological and functional changes at the level of the esophagus. Examples of such cohorts are the ones at the University of North Carolina (Dellon et al., 2012; 2013), and Cincinnati Children's Hospital (Blanchard et al., 2011; Wen et al., 2015; 2013), as well as the cohort at Boston Children's Hospital that is featured in the following chapters (**Chapter 2, 3, 4,** and **5**). These translational research projects are aimed at (I) reducing the need for (repeated) endoscopy by identifying non-invasive biomarkers of disease; (II) further elucidating EoE pathogenesis; and (III) improving the diagnostic yield of esophageal biopsies that are endoscopically obtained.

With regards to the first aim, in work not included in this thesis, we have explored the possibility of using a soluble form of the high-affinity IgE receptor FcεRI as a serum biomarker for the presence of EoE (Lexmond et al., 2011). In addition, we have tested whether urinary leukotriene E4 (**Chapter 3**) or the soluble IL-33 receptor in serum (**Chapter 5**) could distinguish EoE patients from controls. Others have tried a combination of blood cytokines (Blanchard et al., 2011) or sampling of esophageal luminal contents via a swallowed capsule on a string (Furuta et al., 2013). Despite these efforts, there is still no alternative to upper endoscopy available in daily practice.

2 The World Allergy Organization defines the atopic march (also called the 'allergic march') as the natural history of atopic manifestations, characterized by a typical sequence of immunoglobulin E (IgE) antibody responses and clinical symptoms which may appear early in life, persist over years or decades and often remit spontaneously with age (see www.worldallergy.org).

Research biopsies obtained during routine diagnostic endoscopic evaluation have been studied to shed light on the pathogenesis of EoE. In 2006, the EoE transcriptome was published (Blanchard et al., 2006b), which revealed that the disease is associated with a distinct local gene expression signature, with upregulation of eotaxin-3 and periostin as hallmark features. In **Chapter 2**, we will demonstrate that a mathematical model built around the expression of a combination of 10 genes can identify EoE patients from children with gastroesophageal reflux disease (GERD) or normal controls with up to 94% sensitivity, and can thus provide a diagnosis in a blinded fashion in the absence of evaluation by a pathologist. This high sensitivity and significant diagnostic power can be obtained because the characteristic changes that constitute the EoE transcriptome reflect a final common pathway that appears to be activated in all patients. Indeed, the original description of the EoE transcriptome already delineated that it did not distinguish between food allergen-sensitized or non-sensitized subjects (Blanchard et al., 2006a; 2007). However, given the heterogeneity of the disease in terms of clinical presentation, the age of onset, and association with concurrent allergic diatheses, we asked whether this variation would be reflected in perhaps more subtle differences in the esophageal transcriptome. This hypothesis forms the basis of **Chapter 3**, in which we identified a subset of pediatric EoE patients that shows increased tissue expression levels of leukotriene C4 synthase (LTC4S), an enzyme involved in the synthesis of an inflammatory mediator associated with allergic inflammation. Patients with increased expression of *LTC4S* more frequently suffered from co-existing IgE-mediated food allergies, and as such form a subcategory of EoE patients that can be defined based on both clinical and functional characteristics. Interestingly, treatment with the leukotriene receptor antagonist montelukast showed mixed results in patients with EoE, benefitting some but not others, which allows the tempting speculation that perhaps only LTC4S-positive EoE patients will experience clinical benefit from this treatment strategy.

In addition to potentially informing personalized medicine, identification of EoE patient subsets might shed further light on the pathogenesis of disease. Like other allergic diseases, the incidence of EoE is rapidly rising in Westernized societies, implicating environmental factors as critical determinants in disease pathogenesis (Davis and Rothenberg, 2016; Furuta and Katzka, 2015). As will also be described in **Chapter 7** and **Chapter 9**, signals obtained from commensal microbiota residing in proximity of mucosal tissues have been experimentally demonstrated to play a pivotal role in regulating allergic tissue responses (Berni Canani et al., 2015; Feehley and Nagler, 2014). Particularly dependent upon microbial regulation are invariant chain Natural Killer T (iNKT) cells, which have been shown to accumulate in mucosal tissues in germ-free mice and then confer increased susceptibility to asthma and colitis (Olszak et al., 2012). In **Chapter 4**, we provide evidence that invariant chain Natural Killer T (iNKT) cells are present in the esophagus of pediatric EoE patients. Involvement of the iNKT cell pathway proved most pronounced in patients who developed esophageal inflammation early in life and who showed IgE sensitization against food antigens

and increased *LTC4S* expression, which further attests to the existence of different patient subsets amongst EoE patients. Following allergen avoidance therapy, iNKT cell infiltration and esophageal inflammation resolved, identifying involvement of the iNKT-cell axis as a modifiable factor in disease pathogenesis. Furthermore, we found that EoE was associated with use of antibiotics in early life, which has been corroborated by others (Jensen et al., 2013; Radano et al., 2014), and in combination with beginning efforts to evaluate the esophageal microbiome in patients with EoE (Harris et al., 2015), this line of investigation may ultimately inform preventative treatment strategies.

As stressed before, EoE is a complex, multifactorial disorder, and aberrant microbial colonization and iNKT cell homeostasis is unlikely to explain all facets of the disease. For example, genetic polymorphisms in a variety of genes including *TSLP* and *IL33* have been found to be associated with the disease (Kottyan et al., 2014; Rothenberg et al., 2010; Sherrill et al., 2010; Spergel, 2010), and EoE often occurs in the context of other allergic diseases. In **Chapter 5**, we found that allergen sensitization via the skin of mice with dermatitis (induced either mechanically through tape-stripping, or genetically by using filaggrin-deficient animals) followed by intranasal allergen challenge results in esophageal eosinophilia via increased expression of IL-33 and TSLP. Basophils proved critical mediators of increased disease susceptibility, as basophil-depleted mice showed attenuated EoE and basophils deficient in the IL-33 receptor also failed to mediate esophageal eosinophil accumulation. These findings support the notion that atopic dermatitis, which is commonly the first disease in the atopic march, can directly promote the development of EoE in later life, establishing EoE as a manifestation of an atopic constitution.

In **Chapter 5**, we have used animal models to interrogate cause and effect in the pathogenesis of Th2-mediated intestinal inflammation. The advantages, considerations, and caveats that come with the use of such experimental systems are the focus of the second part of this thesis and will be introduced in the following section.

1.4 Animal models in the study of type 2 intestinal inflammation – an introduction to Part 2

IgE and its effector cells are evolutionarily highly conserved features of the mammalian immune system, and many of our advances in understanding allergic responses have been achieved through animal experiments. Richet already stated that “anaphylaxis has been observed in all animals: the horse, the goat, the ox, the rat, the pigeon, the duck and even recently in frogs” (Richet, 1913), but no animal has played a bigger part in allergy research than the laboratory mouse. As illustrated in the following sections, IgE mediated anaphylactic responses can reliably be induced in mice, rendering them amenable to

therapeutic interventions. However, it is important to note that differences do exist between human and murine IgE systems, which limits the degree to which mechanistic insight can be applied across species. One example is the expression of the high-affinity IgE receptor FcεRI, which in mice is restricted to basophils and mast cells, but in humans can also be found on dendritic cells and eosinophils. The consequences of this disparity have been an important research subject in the laboratory where this PhD thesis has been performed. With the use of transgenic models, FcεRI has been expressed on the surface of murine dendritic cells, and this approach has revealed important functions for dendritic cell-bound IgE in regulating Th2-type intestinal inflammation *in vivo* (Platzer et al., 2015a; 2015b; Sallmann et al., 2011). The ingenuity of this system notwithstanding, bridging one known difference between mice and men does not resolve other disparities between the species, some of which we may not even be aware of as of yet.

An alternative approach to bypass this caveat is to study IgE responses in immunodeficient mice in which the human immune system has been reconstituted with immune cells from human donors. These 'humanized' mice thus not only allow for the investigation of the effects of IgE-mediated activation on human immune cells, but also provide a means to study the regulating mechanism of the entire IgE network on distinct immune cell populations. Multiple techniques are employed to reconstitute a functional human immune system in immunodeficient mice (Shultz et al., 2012), though the most widely studied model in the context of allergy research has been the engraftment of isolated human peripheral blood cells (PBMCs) in NOD/SCID mice deficient in the gamma-chain of the IL-2 receptor (NSG mice). These mice are characterized by arrested lymphocyte development and as such are unable to reject HLA mismatched donor cells. Using this experimental approach, it was shown that the transfer of human allergic PBMCs induced Th2 cytokine production and colonic inflammation upon intestinal challenge with the relevant allergen (Weigmann et al., 2012). Inflammation could be blocked with the anti-human IgE monoclonal antibody omalizumab, implying human IgE as a critical mediator in this model. However, treatment with anti-murine FcεRI antibody MAR1 also reduced inflammation, showing that murine basophils and mast cells may have also contributed to the allergic phenotype. In line with these results, a different study showed that transfer of PBMCs from allergic, but not healthy donors induced airway hyperresponsiveness in NSG mice that were challenged with aeroallergens (Martin et al., 2012).

These findings suggest that human allergic disease can be phenocopied to a certain degree in immunodeficient mice, and several authors have used this model to assess the effect of therapeutic interventions (Bellinghausen et al., 2012; Brightbill et al., 2010). Although such studies highlight the potential of humanized mice in advancing IgE and allergy research, it is important to recognize that reconstitution of NSG mice with human PBMCs is limited by less efficient B cell reconstitution compared to T cells. Furthermore, the immunopathology of interest is likely to be confounded by concurrent GVHD reactions that result from HLA-

mismatch (Shultz et al., 2012). These limitations can be overcome by reconstitution of neonatal NSG mice with human hematopoietic stem cells (HSC). In this setting, human lymphocytes are educated on murine HLA, which eliminates the potential for GVHD and allows for the study of a naïve immune system. Tanaka et al. showed that this strategy gives rise to human mast cells and basophils, but did not report on IgE receptor loading or serum IgE levels (Tanaka et al., 2012). Furthermore, Ito et al. showed that knock-in of transgenes for human IL-3 and GM-CSF in NSG recipients enhanced the populations of FcεRI positive mast cells and basophils after $CD34^+$ HSC transfer (Ito et al., 2013). However, as we shall see in **Chapter 6**, because $CD4^+$ T cells are positively selected on murine MHCII in the thymus, naturally occurring IgE responses in this setting remain modest.

Attempts to humanize mice are relatively recent developments, and the largest contribution by far to our detailed knowledge of downstream effects of allergen-IgE-FcεRI interactions has been derived from *in vitro* experiments using cell lines in combination with experimental models of allergic sensitization in wild-type mice. The body of knowledge generated using these approaches has been instrumental in guiding the development of our currently available therapeutic modalities for allergic diseases, and, as a result, we are not only able to effectively treat anaphylactic shock following accidental allergen exposure, but we can also pharmacologically manage the less dramatic manifestations of IgE–mediated immune activation such as hay fever, atopic dermatitis and allergic asthma. Unfortunately, the advances in our understanding of allergic immune responses have not translated in a reduction of the burden of allergic diseases on our societies. On the contrary, in Western countries, IgE-mediated food allergies alone have a current prevalence of 4-10% in children (Longo et al., 2013; Osborne et al., 2011; Sicherer and Sampson, 2010), and asthma and atopic dermatitis are also among the most frequently occurring chronic diseases in the pediatric population. As long as the prevalence of allergic diseases keeps rising and our therapeutic options remain limited to drugs that only temporarily control symptoms, these disorders will continue to demand an ever-increasing price from public health. Because this allergic epidemic has taken place over the course of one or two generations, it cannot be merely explained by genetic predisposition. Epidemiological studies have given rise to a multitude of hypotheses how environmental factors may contribute to increasing rates of allergic sensitization (Platts-Mills, 2015). Yet despite our advanced understanding of IgE-mediated immunity, the experimental and mechanistic evidence to prove causality of any of these presumed factors has lagged behind.

As will be argued in **Chapter 8**, this discrepancy may at least in part be attributed to the lack of animal models that spontaneously develop allergic diseases. To determine a causal effect of an environmental influence, an ideal experiment would assess the rate of allergic disease between animals exposed or unexposed to that particular factor, with all other factors held equal. Of course, such studies are only feasible when the disease of interest does in fact occur in the experimental animal in a way that faithfully resembles the pathogenic

features of the human disease. Whereas such models do exist for the study of type I diabetes or inflammatory bowel diseaes (Strober et al., 2002; Wirtz and Neurath, 2007), King:2012bs}, there is currently no model that mimics spontaneous sensitization to food antigens as it occurs in humans (Oyoshi et al., 2014; Van Gramberg et al., 2013). Wild-type mice have proven to be highly resistant to the dysregulation of IgE-mediated immune responses to food allergens due to the robustness of 'oral tolerance', the collective of cellular mechanisms that is responsible for tolerance of ingested foreign antigens. In order to overcome the establishment of oral tolerance, murine models of food allergy commonly rely on adjuvant-based strategies to induce systemic production of antigen-specific IgE prior to first intestinal exposure. Alternatively, the induction of oral tolerance can be disrupted by intestinal co-exposure to adjuvants such as cholera toxin or staphylococcal enterotoxin B (Berin and Mayer, 2009). Even less physiological is the passive immunization of animals with exogenous antigen-specific IgE, administered via injection, prior to challenge with an allergen. It is clear that none of these forced sensitization methods resembles the pathogenesis of allergic sensitization in humans. Such models, which recapitulate the symptoms and treatment responses of human disease but not the underlying cause of disease, are termed 'isomorphic'. In contrast, a 'homologous' animal model of food allergy would not only capture the IgE effector phase of disease, but also faithfully mimic spontaneous oral sensitization to food antigens (Oyoshi et al., 2014). It can be argued that our mechanistic insight increases along the temporal sequence of the naturally occurring IgE effector response: we understand the intracellular events following IgE-mediated cellular activation at the deepest molecular level, but we cannot yet explain the origin of the first antigen-specific IgE molecules that are responsible for allergic responses. This difference is reflected in our current treatment strategies for allergic diseases, which are aimed at symptomatic control of IgE effector responses. However, in order to reduce the burden of allergic diseases, curative and preventative strategies are required, and these demand a more thorough understanding of the immunological events that underlie early allergic sensitization. We believe that the lack of homologous experimental models is partly responsible for the paucity of insight into the early stages of food allergy. This argument is summarized in **Figure 1.1**.

1.5 Outline and aims of this thesis pertaining to Part 2

As mentioned in the previous subsection, transfer of human PBMCs to an immunodeficient mouse results in a certain degree of immune reconstitution, which allows the experimental manipulation of human immune cells in an *in vivo* setting. Although IgE-mediated immune activation can occur in this system (Weigmann et al., 2012), HLA-mismatch will ultimately result in graft-versus-host reactions in the recipient with high risk of confounding the interpretation of any spontaneously occurring immune activation. This

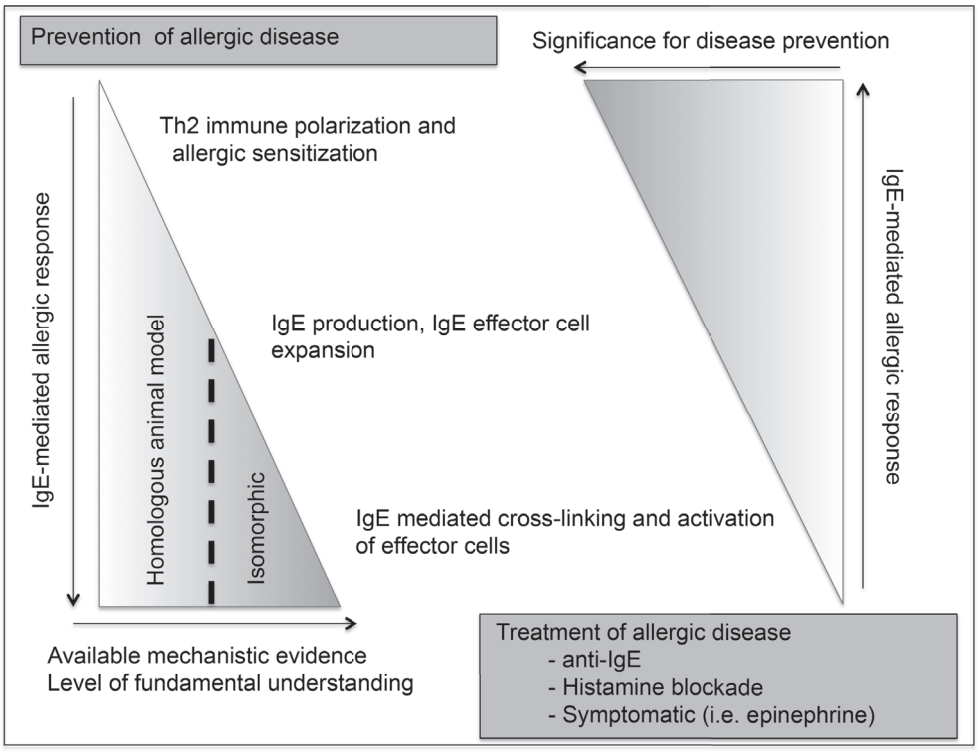


Figure 1.1.

problem is circumvented by reconstitution with $CD34^+$ HSCs, in which education of naïve lymphocytes occurs on murine HLA molecules, but this process is inefficient and adaptive immune responses consequently remain weak (Baenziger et al., 2006). Selection of T cells and IgE-mediated immunity can be enhanced by concurrently transplanting human bone-marrow, liver and thymus-derived tissue as well as autologous $CD34^+$ HSCs, but this approach is limited by high-costs and demand for fetal tissues with its inevitable ethical implications (Shultz et al., 2012). In **Chapter 6**, we have tested whether immune reconstitution from HSCs and adaptive immune responses in immunodeficient NOD.*Rag1*$^{-/-}$*Il2rg*$^{-/-}$ (NSG) mice could be improved by replacing murine MHC II with a human MHC II DR1 allele. We show that this system results in a richer TCR repertoire and higher B cell maturation and class switch recombination resulting in elevated levels of immunoglobulins, including IgE. Furthermore, transfer of human stem cells obtained from a donor suffering from the immune dysregulation, polyendocrinopathy, enteropathy, X-linked (IPEX) syndrome that results from loss of regulatory T cells (Tregs) due to mutation of the Treg-specific transcription factor *FOXP3* led to reconstitution of auto-inflammatory symptoms in animals that expressed the human DR1 allele, but not in those who did not. Based on recently published data, it is tempting to speculate that additional

'humanization' of the model by transgenic expression of IL-3, GM-CSF and stem cell factor will improve mast cell reconstitution in this model as well, which could further augment IgE effector responses (Bryce et al., 2016; Ito et al., 2013). Going forward, this new experimental system could thus serve as an improved pre-clinical model for the study of human allergic diseases and IgE-mediated immunity.

In addition to severe autoimmunity, patients with IPEX almost uniformly develop food allergies, eczema and increased levels of IgE (Akdis and Akdis, 2009; Lin et al., 2005; Suscovich et al., 2012). Similarly, elevated levels of IgE were found in NSG mice reconstituted with IPEX-HSCs (**Figure 6.6, page 135**). These symptoms are the result of unrestrained activation of Th2 immune responses that occur in the complete absence of FOXP3$^+$ Tregs. However, dysregulated Th2 responses, atopy and elevated IgE levels are seen in a variety of primary human immunodeficiencies (Liston et al., 2008; Ozcan et al., 2008). In fact, a number of such diseases have been phenotypically lumped together under the term 'hyper IgE syndromes' (formerly known as Job's syndrome), which share symptoms of extremely elevated serum IgE, eczema and recurrent infections (Yong et al., 2012). It is now becoming clear that hyper IgE phenotypes can result from alterations in distinct immunological pathways. For example, mutations in *STAT3*, *DOCK8, TYK2* and multiple genes involved in TCR signaling such as *LAT, ZAP70* or *RAG* are all associated with aberrant IgE responses. In most of these cases, however, the underlying mechanisms of increased IgE production or the functional consequences of elevated serum IgE have not been studied in detail.

One well-known[3] primary immunodeficiency with increased IgE titers is the Wiskott-Aldrich syndrome (WAS) (Datta and Milner, 2011; Ozcan et al., 2008). WAS is caused by mutations in the Wiskott-Aldrich syndrome (*WAS*) gene on the X-chromosome, which is expressed in all hematopoietic cells and encodes for the Wiskott-Aldrich Syndrome protein (WASP). WASP is the founding member of a family of actin regulators, capable of transducing a variety of signals to mediate changes in the actin cytoskeleton, and has been implicated in a great variety of cellular functions in both lymphocytes and non-lymphocytes (Snapper and Rosen, 1999; Thrasher and Burns, 2010). Over a 100 unique loss-of-function *WAS* mutations have been reported and these give rise to a clinically highly heterogeneous group of patients (Thrasher and Burns, 2010). Males that are most severely affected present early in life with thrombocytopenia, eczema, autoimmune sequelae and recurrent infections, which in the absence of bone-marrow transplantation or gene therapy can be fatal. Milder loss-of-function mutations, in contrast, have been identified in patients who suffer from an attenuated form of the disease that is termed X-linked thrombocytopenia (XLT), which has an excellent long-term survival with medical management alone (Albert et al., 2011). Despite the long-known association between *WAS* mutations and atopy, the antigenic specificity of the expanded IgE pool and the consequences of elevated IgE on the

3 In fact, elevated levels of serum Ig ND (Ig not determined) had been observed in patients with Wiskott-Aldrich syndrome even before Ig ND was formally changed to IgE in Lausanne in 1968 (Berglund et al., 1968).

prevalence of allergic disorders have not been investigated in detail in human patients with WAS.

Since 1998, an animal model has been available in which the murine ortholog *Was* is deleted from the X chromosome (Snapper et al., 1998). Many of the immunological features of human WAS are phenocopied in these mice, and they have been extremely informative in unraveling the immune pathogenesis of WAS-associated autoimmunity and lymphocyte dysfunction. However, aberrant IgE production in WASP-deficient mice had never been examined until very recently in a paper dissecting the role of WASP in B cells (Recher et al., 2012), and no data has been published on the mechanism and consequences of these aberrant IgE responses in mice.

In **Chapters 7**, **8** and **9**, we have studied IgE-mediated immune responses in WASP-deficient mice and in human patients suffering from WAS. These investigations have been driven by the overarching hypothesis that elevated levels of serum IgE could potentially have specificity for ingested food antigens and, if so, that *Was*$^{-/-}$ animals could serve as a novel, perhaps more homologous experimental model of food allergy. In **Chapter 7**, we describe that food allergy occurs in 20% of patients with mutations in the *WAS* gene, which is a markedly higher prevalence than in the general population. We furthermore demonstrate that elevated levels of IgE are found in WASP-deficient mice on a variety of genetic backgrounds, and that IgE in these animals does indeed contain specificity for antigens found in mouse chow. Importantly, anti-food IgE titers were highest for food antigens commonly associated with IgE-mediated food allergy in humans such as wheat and soy. This latter finding suggests that the mechanisms that drive the allergenicity of these particular food antigens in human food allergy are also operative in *Was*$^{-/-}$ mice, which would be an argument in favor of homology of the model.

We dedicated a separate paper to the further characterization of WASP-deficient mice as a novel model of food allergy (**Chapter 8).** Using the model antigen ovalbumin (OVA), we demonstrate that spontaneous induction of OVA-specific IgE occurs following oral OVA exposure, thus bypassing the need for adjuvant-mediated sensitization. Since a homologous model of food allergy should also faithfully recapitulate the IgE effector phase, we assessed the response to OVA challenge, and found that the occurrence of allergic diarrhea was similar to WT mice subjected to a conventional, adjuvant-based model of food allergy.

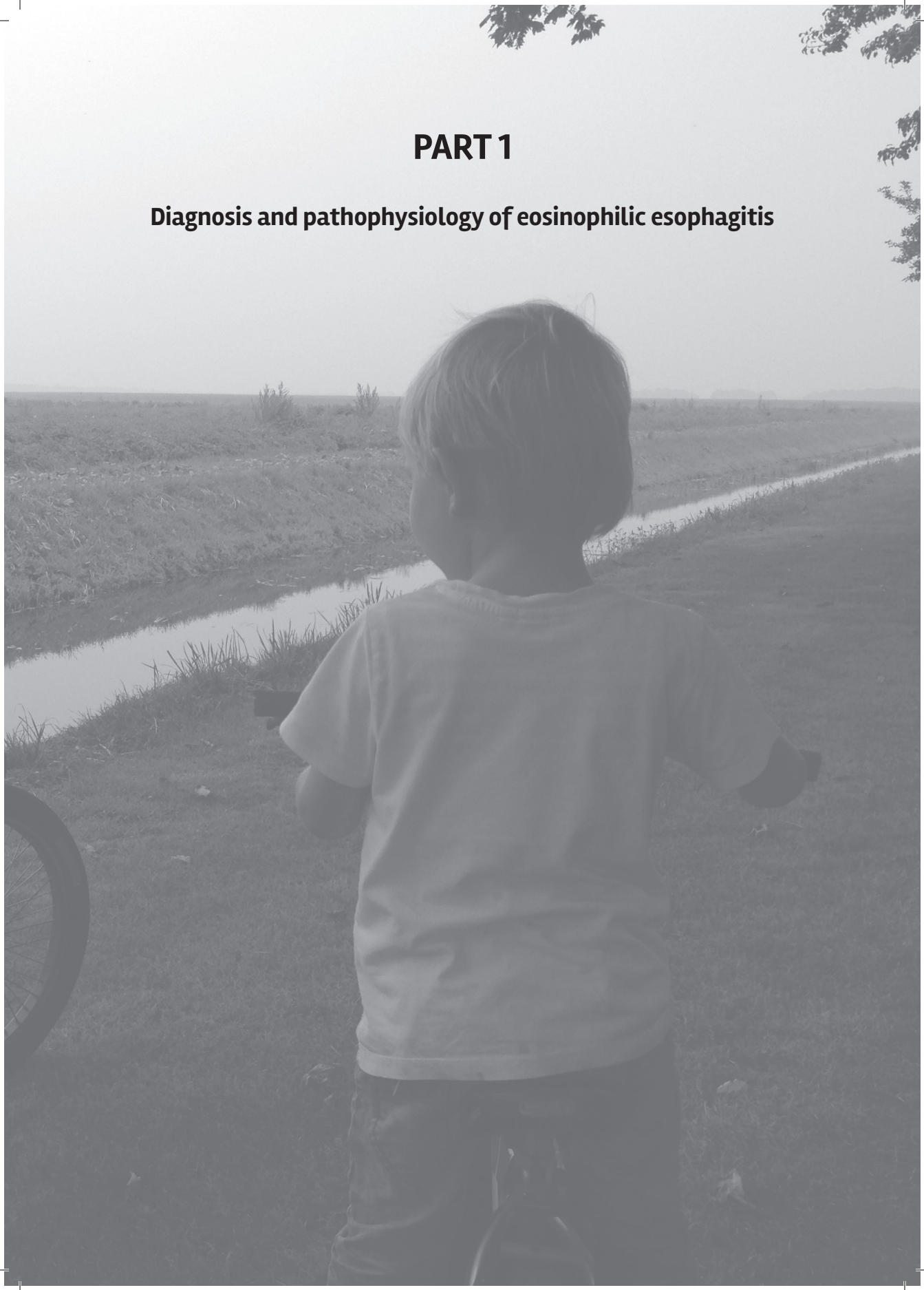
Having established that WASP-deficient mice can be utilized in the study of food allergy, we aimed to further elucidate the pathogenesis of aberrant Th2-mediated intestinal inflammation. First, we ruled out that allergic sensitization resulted from dysbiosis in microbial commensal populations by demonstrating that sensitization to food allergens occurred to a similar degree in WASP-deficient mice raised under germfree conditions (**Figure 7.3, page 163**). Next, using a combination of genetic approaches that allow cell-type-specific deletion of WASP, we show that mice that only lack WASP in FOXP3^{+} Tregs spontaneously develop allergic intestinal inflammation and IgE antibodies against food allergens. Loss of

WASP from Tregs resulted in a selective failure to suppress Th2-type immune responses, whereas Th1 and Th17 subsets remain effectively controlled by WASP-deficient FOXP3$^+$ Tregs. Phenotypically, WASP-deficient Tregs showed increased expression of the Th2 transcription factor GATA3, both in mice as well as human patients. Although the molecular mechanism for this differential transcription factor expression in FOXP3$^+$ Tregs remains speculative, it is important to note that GATA3$^+$ Tregs have recently also been observed in children with food allergy (Noval Rivas et al., 2015), and this finding thus identifies another point of overlap between the pathogenesis of WAS-associated and common food allergy.

As illustrated in **Figure 7.4 (page 164)**, Treg-specific deletion of WASP results in an exacerbated allergic phenotype compared to *Was*$^{-/-}$ mice that lack WASP in all hematopoietic lineages. In **Chapter 9**, this observation is discussed in detail, with a comprehensive review of the known functions of WASP in different cell types of the innate and adaptive immune system. Not limited by journal-specific restrictions to word count or figure numbers, this chapter was written as both a synthesis of the data presented in the second part of this thesis, as well as a means to incorporate additional results from experiments that had not yet been presented in **Chapter 7** and **Chapter 8**. Here we show data that the microbiome and maternal factors affect and shape WAS-associated food allergies, and that WASP-deficient FOXP3$^+$ Tregs can even cause allergic disease in mice that lack WASP in only half of Tregs. In addition, we outline the future directions of this new line of investigation.

PART 1

Diagnosis and pathophysiology of eosinophilic esophagitis



Chapter 2

Accuracy of digital mRNA profiling of esophageal biopsies as a novel diagnostic approach to eosinophilic esophagitis

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* These authors contributed equally to this work.

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2.1 Abstract

Background: Quantification of tissue eosinophils remains the golden standard in diagnosing eosinophilic esophagitis (EoE), but this approach suffers from poor specificity. It has been recognized that histopathological changes that occur in patients with EoE are associated with a disease-specific tissue transcriptome.

Objective: We hypothesized that digital mRNA profiling targeted at a set of EoE-specific and Th2 inflammatory genes in esophageal biopsies could help differentiate patients with EoE from those with reflux esophagitis (RE) or normal tissue histology (NH).

Methods: The mRNA expression levels of 79 target genes were defined in both proximal and distal biopsies of 196 patients with nCounter® (Nanostring) technology. According to clinicopathological diagnosis, these patients were grouped in a training set (35 EoE, 30 RE, 30 NH) for building of a three-class prediction model using the random forest method, and a blinded predictive set (n=47) for model validation.

Results: A diagnostic model built on ten differentially expressed genes was able to differentiate with 100% sensitivity and specificity between conditions in the training set. In a blinded predictive set, this model was able to correctly predict EoE in 14 out of 18 patients in distal (sensitivity 78%, 95% CI 52%-93%) and 16 out of 18 patients in proximal biopsies (sensitivity 89%, 95% CI 64%-98%), without false positive diagnosis of EoE in RE or NH patients (specificity 100%, 95% CI 85%-100%). Sensitivity was increased to 94% (95% CI 71%-100%) when either the best predictive distal or proximal biopsy was used.

Conclusion & Clinical Relevance: We conclude that mRNA profiling of esophageal tissue is an accurate diagnostic strategy in detecting EoE.

2.2 Introduction

Quantification of tissue eosinophils under proton-pump inhibitor (PPI) therapy in combination with appraisal of clinical symptomatology remains the gold standard in diagnosing patients with eosinophilic esophagitis (EoE) (Liacouras et al., 2011). Unfortunately, this approach suffers from poor specificity since a wide variety of gastrointestinal disorders can give rise to esophageal eosinophilia, and EoE-like symptoms such as failure-to-thrive or feeding difficulties are generally non-specific, especially in young children (Brown-Whitehorn and Liacouras, 2007; Liacouras et al., 2011). In daily practice, the distinction between reflux-associated eosinophil infiltration and true EoE remains particularly difficult, because these disease entities have considerable symptomatic and histologic overlap and are typically patchy in nature.(Papadopoulou et al., 2014) Differentiation can furthermore be complicated by poor patient compliance with PPI therapy prior to diagnostic endoscopy, as well as the recent recognition of PPI-responsive EoE variants (Dellon et al., 2013; Papadopoulou et al., 2014). These diagnostic difficulties often cause considerable delay between onset of disease and diagnosis, hence delaying installment of optimal therapy (Hruz et al., 2011; Schoepfer et al., 2011). As such, a need for more refined diagnostic methods to reliably differentiate EoE from reflux esophagitis (RE) and other causes of esophageal eosinophilia clearly persists (Katzka, 2014).

It has been recognized that EoE is associated with a tissue-specific transcriptome and that increased esophageal mRNA levels of genetic markers such as eotaxin-3 and periostin display considerable diagnostic sensitivity and specificity (Blanchard et al., 2008; 2006b). Additionally, we and others have shown that the EoE-transcriptome can be used to identify involvement of specific inflammatory pathways that are operative in subsets of EoE patients (Abonia et al., 2010; Lexmond et al., 2013; 2014). Following the identification of an EoE-specific transcriptome, it has been hypothesized that an esophageal mRNA expression pattern across multiple disease-specific and Th2-inflammatory genes can provide additional diagnostic benefit over the current golden standard of histopathological eosinophil count (Wen et al., 2013). However, analysis of mRNA expression levels of EoE-markers in esophageal biopsies has not yet been introduced into routine clinical practice. This may in part be due to the relatively challenging requirements for large-scale mRNA tissue diagnostics, which in the case of quantitative RT-PCR requires multi-step processing that is both time and manipulation-sensitive. In the current study, we have taken advantage of a digital mRNA expression profiling method (Nanostring technology, nCounter® system) that has been described to have sensitivity comparable to quantitative RT-PCR, yet does not require tissue extraction procedures or generation of cDNA as an intermediate step (Geiss et al., 2008). Using this direct, state-of-the-art mRNA profiling methodology, we here aimed to define an esophageal mRNA pattern stamp that can distinguish patients with EoE from those with RE or normal tissue histology (NH) and lead to improved tissue diagnosis.

2.3 Methods

In the reporting of this study, we have adhered to the guidelines put forth by the STARD initiative (Bossuyt et al., 2003).

2.3.1 Study population

The patients described in this study were included at Boston Children's Hospital as part of a prospective cohort study that focuses on EoE pathophysiology and diagnostics. Details pertaining this study have been previously described (Lexmond et al., 2013). Study inclusion was based on clinical presentation. Children (1-18 years of age) who presented with EoE-like symptoms (such as failure-to-thrive, food aversion, regurgitation or dysphagia) and who were scheduled for diagnostic upper esophagogastroduodenoscopy were invited to participate. In all patients, endoscopy was performed after a minimum of four weeks of PPI therapy (mean duration 10.8 months, with a range of 1-120 months). Additional patient information was collected through a standardized questionnaire at time of enrollment. For each patient, an additional study biopsy was obtained from both the proximal and distal esophagus, defined as lying either ≥10 cm or 1-2 cm from the gastro-esophageal junction respectively. Biopsies were directly stored in RNAlater (Qiagen) for a minimum of 24h and frozen at -80°C. From our cohort, we randomly selected patients to undergo digital transcriptional profiling of both proximal and distal esophageal biopsies. Approval for this study was obtained from the Investigational Review Board of Boston Children's Hospital (Harvard Medical School, Boston, MA, approval number: 07-11-0460). All patients or their legal guardians provided written informed consent prior to enrollment.

2.3.2 Clinicopathological diagnosis (reference standard)

A board-certified pediatric gastroenterologist, who was blinded to the results of pattern profiling, reviewed the clinicopathological diagnosis of every patient by taking into account all available clinical information, which included symptomatology at presentation, laboratory testing, the pathology report of esophageal histology and tissue eosinophil count, as well as the clinical course after initial diagnosis, and the results of follow-up endoscopies if performed. This review took place after a minimum follow-up of two years after the enrollment endoscopy, and was used in our study as the reference standard against which we aimed to validate our novel diagnostic approach (Liacouras et al., 2011). Following consensus guidelines (Liacouras et al., 2011), we considered patients to have *EoE* when they met the following criteria: 1. treatment with PPI for ≥4 weeks prior to diagnostic endoscopy; 2. tissue eosinophil count >15/hpf in at least one biopsy; 3. exclusion of other origins of esophageal eosinophilia. Use of corticosteroids was considered an exclusion criteria. Conversely, patients were classified as *RE* when they showed: 1. histological evidence of esophageal tissue inflammation such as basal zone hyperplasia and an inflammatory cell infiltrate; 2. eosinophil count 1-15/hpf;

3. a clinical history suggestive of reflux-associated symptoms 4. Evidence of pathologic GERD either by abnormal pH/impedance studies, or by erosive esophagitis that healed after antacid therapy, and 5 no evidence of development of EoE after long term follow up. Lastly, *NH* patients were defined as having: 1. normal tissue histology in all routine biopsies, and 2. no evidence of underlying gastrointestinal disease for at least 3 months after endoscopy in the absence of antacid therapy. Patients that did not meet any of these three diagnostic categories were excluded from training and predictive patient set.

2.3.3 Sample processing and mRNA profiling with the nCounter® system

Biopsies were homogenized in RLT buffer (Qiagen) and further processed with the nCounter® Prep Station and Digital Analyzer, following the manufacturer's instructions (nCounter® system, www.nanostring.com). Samples were analyzed using a customized panel that consisted of five housekeeping genes and 79 genes of interest based on previously published microarray data (Blanchard et al., 2006b). This code set is summarized in supplemental **Table 2.S1**. Expression data from separate nCounter® runs were normalized through quantile normalization, and then log2 transformed prior to downstream analysis. Outlying samples with low readout in the internal positive controls were excluded from further analysis

2.3.4 Definition of a training and predictive patient set

A total of 95 unambiguously diagnosed patients were otherwise randomly selected into a training set, which was used to identify differentially expressed genes to include in a diagnostic prediction model. The remaining unambiguous patients were used for the predictive patient test set. For training set patients, both the clinicopathological diagnosis from the reference standard and the mRNA pattern profile were provided to the statistician, who performed differential gene expression analysis and diagnostic model building. For the predictive set, the statistician was blinded to the histopathological diagnosis and only the mRNA profile was provided.

2.3.5 Differential gene expression analysis

Three individual linear statistical models were built (R Bioconductor limma package) to compare training set patients (EoE vs. NH, RE vs. NH, and EoE vs. RE, respectively) to identify genes that were differentially expressed between all three disease conditions ($p\text{-value}<0.05$).

2.3.6 Diagnostic model

A three-class (EoE, RE, and NH) diagnostic model was built with 10-fold cross validation using the random forest method. In each round of the cross validation process, the ratio of EoE, RE, and NH samples was set to be the same as in the complete training set. Once the model for a given biomarker gene set was trained in the training samples, the expression profile of the same biomarker gene set from the predictive set samples was fitted on the trained

model and the EoE/RE/NH classification diagnostic probability, i.e. the probability of having each diagnosis, was calculated for the predictive samples. A predicted probability >50% was considered a positive mathematical diagnosis for that particular condition.

2.3.7 Statistical analysis

Comparison of clinical characteristics and probability scores between diagnostic groups was performed with ANOVA or Kruskal-Wallis test for continuous variables or Fisher's exact test for dichotomous predictors. Correlation analysis was performed using Pearson correlation coefficient. Values are expressed as mean ± SD unless otherwise indicated. Analyses were performed using Stata 12 (StataCorp, TX, USA).

2.4 Results

2.4.1 Patient inclusion

The 196 patients analyzed in this study were randomly selected from a previously described (Dehlink et al., 2011; Lexmond et al., 2013), longitudinal cohort of 429 children who were included at our center between April 2008 and May 2012. For every patient, one proximal and one distal research biopsy was analyzed with the nCounter® system to obtain a digital mRNA expression profile of the esophageal tissue. The collection of two additional research biopsies was not associated with adverse events.

2.4.2 Patient classification

Histological evidence of esophageal inflammation was present in 110 out of 196 patients (56%). In 96 (87%) of those, an unambiguous clinicopathological diagnosis of either EoE or RE could be made based on comprehensive case review after a follow-up time of a minimum of two years. These 96 cases were used to form the basis of a training and a predictive patient set. From those unequivocally diagnosed patients, we randomly selected 35 EoE and 30 RE patients, together with 30 randomly selected NH cases to give rise to the training set. The remaining 31/96 unambiguous patients with esophageal inflammation were combined with another 16 NH patients to generate the predictive test set that we used for validation of the diagnostic model. In 14/110 patients with esophageal inflammation (13%), no unambiguous diagnosis of either RE or EoE could be established with the available clinical and histologic information and were excluded from further analysis. These patients either suffered from non-RE, non-EoE esophageal inflammation (e.g. candida esophagitis), or had undocumented or unclear use of a PPI prior to diagnostic endoscopy. We also excluded patients when a diagnosis of EoE was hampered by prior installment of some form of EoE-specific treatment before study enrollment if biopsies did not have the established number of eosinophils to diagnose them as EoE and 2-year follow-up had not revealed a clear underlying condition.

Table 2.1. Clinical details and characteristics of patients used in training and predictive sets. *comparing patients from all three diagnostic groups in combined training and predictive sets, calculated using Fisher's exact test or ANOVA.

PARAMETER	TRAINING SET			PREDICTIVE SET			p-value*
	NH	EoE	RE	NH	EoE	RE	
n	30	35	30	16	18	13	
Age (years, median, range)	10.3 (1.25-18.0)	10.6 (2-18.5)	10.0 (1.4-18.6)	9.5 (1.6-17.2)	6.8 (2.8-16)	13.5 (4.2-18.5)	0.55
Male gender	10/30 (33%)	20/35 (57%)	20/30 (67%)	5/16 (31%)	11/18 (61%)	9/13 (69%)	**0.003**
Symptomatology in last year							
Dysphagia	10/30 (33%)	16/35 (46%)	6/30 (20%)	4/16 (25%)	9/18 (50%)	4/13 (31%)	**0.04**
Food impaction	0/30 (0%)	4/35 (11%)	0/30 (0%)	1/16 (6%)	2/18 (11%)	1/13 (8%)	0.12
Chest pain	0/30 (0%)	4/35 (11%)	3/30 (10%)	0/16 (0%)	1/18 (6%)	3/13 (23%)	**0.02**
Epigastric pain	13/30 (43%)	5/35 (14%)	11/30 (37%)	6/16 (38%)	5/18 (28%)	3/13 (23%)	**0.04**
Heartburn	2/30 (7%)	7/35 (20%)	6/30 (20%)	2/16 (13%)	2/18 (11%)	4/13 (31%)	0.17
Reflux	2/30 (7%)	14/35 (40%)	16/30 (53%)	3/16 (19%)	2/18 (11%)	7/13 (54%)	**<0.001**
Feeding difficulties	4/30 (13%)	5/35 (14%)	1/30 (3%)	3/16 (19%)	3/18 (17%)	1/13 (8%)	0.22
Vomiting	4/30 (13%)	9/35 (26%)	12/30 (40%)	4/16 (25%)	7/18 (39%)	3/13 (23%)	0.15
Endoscopy							
Pallor	0/30 (0%)	12/35 (34%)	4/30 (13%)	1/16 (6%)	0/18 (0%)	0/13 (0%)	**0.005**
Edema	0/30 (0%)	3/35 (9%)	1/30 (3%)	1/16 (6%)	1/18 (6%)	0/13 (0%)	0.38
Loss of vascularity	0/30 (0%)	13/35 (37%)	4/30 (13%)	2/16 (13%)	5/18 (28%)	1/13 (8%)	**<0.001**
Furrowing	3/30 (10%)	27/35 (77%)	8/30 (27%)	1/16 (6%)	15/18 (83%)	1/13 (8%)	**<0.001**
Exudate	1/30 (3%)	10/35 (29%)	0/30 (0%)	0/16 (0%)	8/18 (44%)	0/13 (0%)	**<0.001**
Allergic diatheses							
IgE (median, range)	21 (2-308)	195 (4-1768)	38 (0-1658)	6 (2-99)	136 (28-714)	69 (7-957)	**<0.001**
Eczema	6/29 (21%)	16/35 (46%)	13/29 (44%)	3/16 (19%)	5/18 (28%)	5/13 (38%)	**0.046**
Asthma	6/30 (20%)	9/35 (26%)	11/29 (38%)	5/16 (31%)	4/18 (22%)	4/13 (31%)	0.39
Seasonal allergies	7/30 (23%)	20/35 (57%)	15/29 (52%)	5/16 (31%)	13/18 (72%)	4/13 (31%)	**0.002**
Food allergy	4/30 (13%)	18/35 (51%)	6/29 (21%)	3/16 (19%)	11/16 (69%)	4/11 (36%)	**<0.001**
Positive RAST or skin prick test against food antigens	0/6 (0%)	22/30 (73%)	9/20 (45%)	1/4 (25%)	16/17 (94%)	1/5 (20%)	**<0.001**
Tissue eosinophilia (peak value)							
Proximal (median, range)	0 (0-0)	30 (5-150)	4 (2-10)	0 (0-0)	40 (2-150)	2 (1-5)	**<0.001**
Distal (median, range)	0 (0-0)	40 (20-150)	3 (1-20)	0 (0-0)	50 (2-150)	1.5 (1-2)	**<0.001**

The flow diagram of patient inclusion and distribution over the training and predictive sets is shown in **Figure 2.1**. The distal biopsy of one RE patient in the predictive set was excluded from analysis due to overall low mRNA counts. Clinical, endoscopic and histological features of patients in both sets are summarized in **Table 2.1.** In line with previous reports (Lexmond et al., 2013; Liacouras et al., 2011; Rothenberg, 2009), statistically significant

differences between diagnostic groups were found in distribution of gender, presenting symptoms, allergic comorbidity and findings during diagnostic endoscopy.

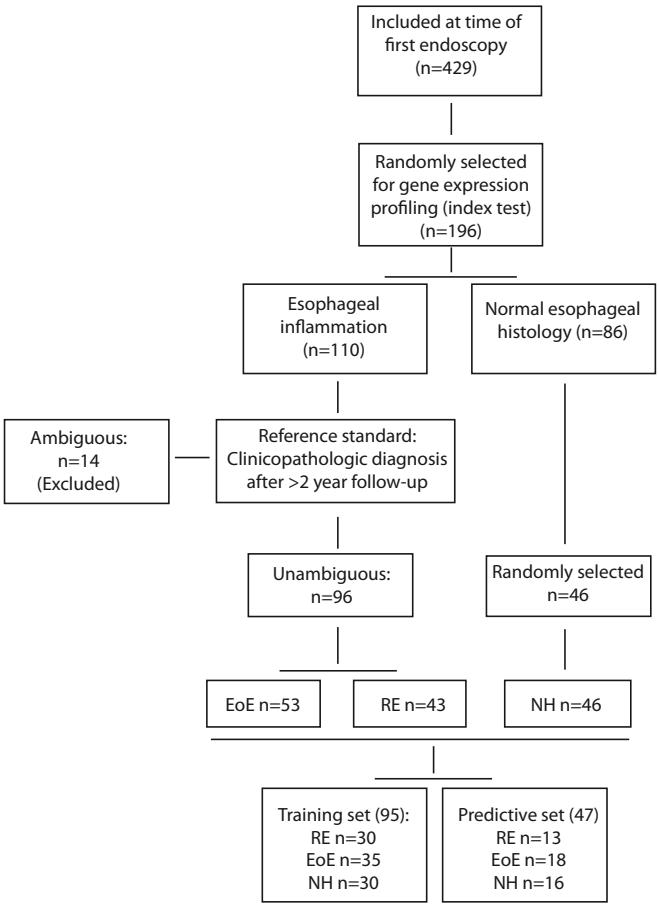

Figure 2.1. Flow diagram of patient inclusion and composition of training and predictive sets.

2.4.3 Selection of predictor genes in the training set of patients

To identify which of the 79 genes in our panel were differentially expressed between disease conditions, we first performed comparative gene expression analysis in patients with EoE, RE or NH that were included in the training set. As expected from previous studies (Blanchard et al., 2006b; 2011; Lexmond et al., 2013), this analysis revealed striking differences between conditions in both proximally and distally-derived biopsies (**Figure 2.2 A, B**). Differential gene expression analysis for EoE vs. NH, RE vs. NH and EoE vs. RE patients identified 38 genes in proximal biopsies and 43 genes in distal biopsies that were differentially

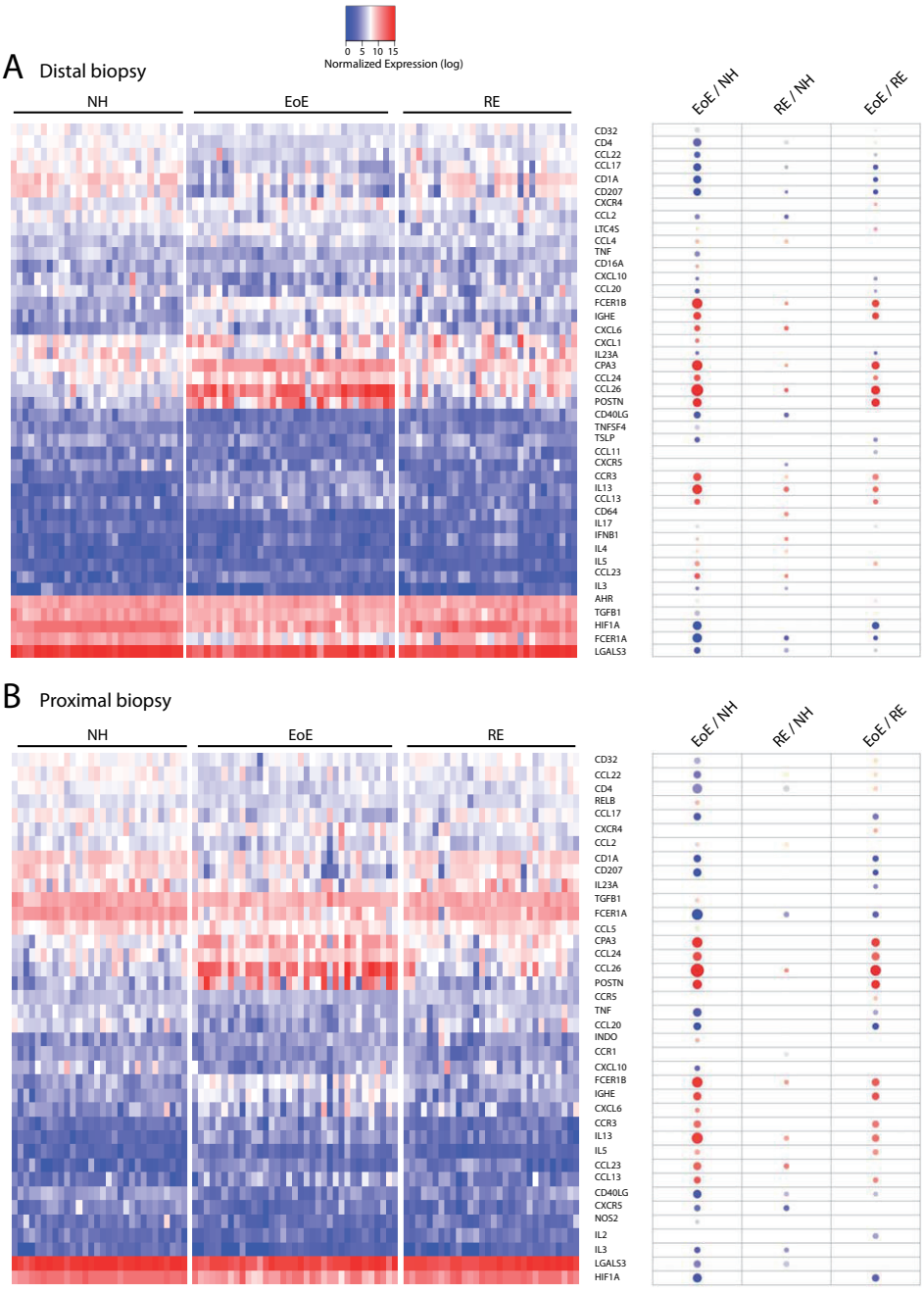

Figure 2.2. Heatmap display of differentially regulated genes in either distal (**A**) or proximal (**B**) oesophageal biopsies by clinicopathological diagnosis. Bubble plots display levels of statistical significance and the direction of regulation (color: blue for down and red for up-regulation), and the statistical significance of the effect (size: larger bubbles signify smaller p-values).

expressed in at least one comparison at α=0.05 (**Figure 2.2 A, B**). We hypothesized *a priori* that most discriminative power would be attained by a model that only included genes that were differentially expressed between all three pathologies. For proximal biopsies, seven genes met this criterion, whereas 10 genes were found to be differentially expressed in distal biopsies at a p-value <0.05 in all three comparisons (**Table 2.2**). Because distal biopsies gave rise to a greater abundance of significantly regulated genes, we selected those biopsies to build our diagnostic model. As such, our strategy resulted in inclusion of the major EoE-specific gene eotaxin-3 (CCL26) as well as the mast cell-marker carboxypeptidase A3 (CPA3) and β-chain of the high-affinity IgE receptor (FcεR1), but not periostin, which although highly upregulated in both biopsies in EoE patients compared to RE or NH patients (t-statistic >5.5, p-value $<10^{-6}$) was not significantly different between NH controls and RE patients in proximal or distal tissue biopsies (p-value >0.05, **Figure 2.2**) Additional upregulated genes were the eotaxin-3 receptor CCR3, which is mainly expressed on eosinophils and basophils, and the Th2-cytokine IL-13. EoE-specific downregulation of genes was noted for CD4, CCL17, CD207, FcεR1α and LGALS3 (galectin-3). In proximal, but not distal, biopsies, statistical significance was reached for CCL22 and CD40 ligand (**Figure 2.2** and **Table 2.2**). These results suggested that disease-specific regulation of these ten target genes could potentially be used to discriminate between patients with EoE, RE, and NH.

Table 2.2. Significantly regulated genes between all three conditions.

Comparison:	**EoE / NH**		**RE / NH**		**EoE / RE**	
	t-value	**p-value**	**t-value**	**p-value**	**t-value**	**p-value**
Proximal						
CD4	-6.61	7.39×10^{-9}	-3.65	0.001	-2.43	0.018
CCL22	-4.72	1.22×10^{-5}	-2.84	0.006	-2.13	0.037
FCER1A	-7.70	7.96×10^{-11}	-3.10	0.003	-3.59	0.001
CCL26	10.56	5.96×10^{-16}	2.13	0.037	7.38	3.00×10^{-10}
FCERIB	7.42	2.51×10^{-10}	2.43	0.018	4.73	1.17×10^{-5}
IL13	8.05	1.85×10^{-11}	2.71	0.009	4.45	3.25×10^{-5}
CD40LG	-5.29	1.42×10^{-6}	-2.54	0.013	-2.45	0.017
Distal						
CD4	-7.90	3.92×10^{-11}	-3.53	0.001	-2.84	0.006
CCL17	-7.13	9.23×10^{-10}	-2.63	0.011	-3.77	3.45×10^{-4}
CD207	-6.80	3.68×10^{-9}	-2.01	0.049	-3.63	5.41×10^{-4}
FCERIB	11.48	2.14×10^{-17}	2.66	0.010	6.55	9.37×10^{-9}
FCER1A	-9.46	6.60×10^{-14}	-3.57	0.001	-3.39	0.001
CPA3	11.43	2.60×10^{-17}	2.29	0.026	7.38	3.17×10^{-9}
CCL26	14.70	1.60×10^{-22}	2.87	0.006	8.66	1.51×10^{-12}
CCR3	7.54	1.74×10^{-10}	2.62	0.011	5.15	2.47×10^{-6}
IL13	10.25	2.69×10^{-15}	4.28	6.80×10^{-5}	4.59	2.02×10^{-5}
LGALS3	-5.40	9.73×10^{-7}	-3.48	0.001	-2.35	0.022

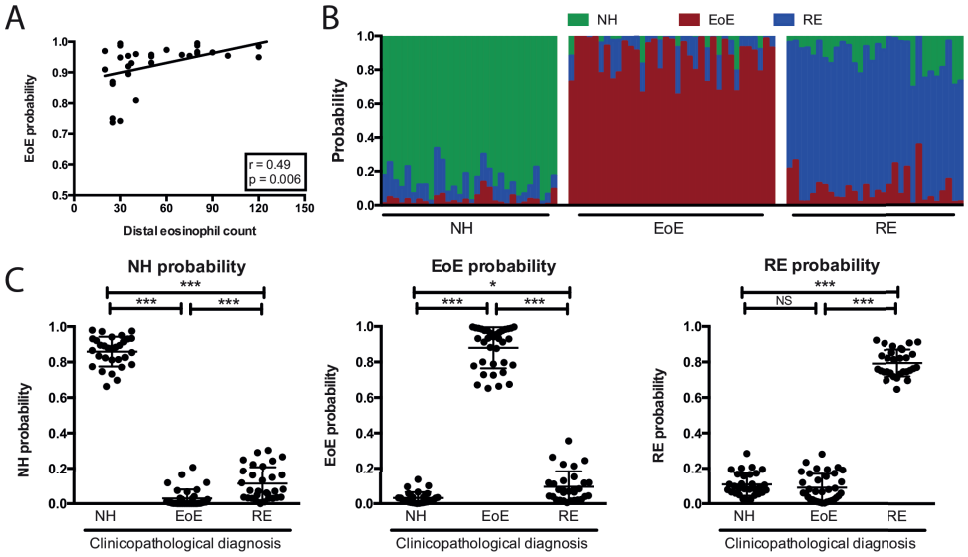

Figure 2.3. (**A**) EoE probability in EoE training set patients correlates positively with the severity of tissue eosinophil infiltration. (**B**) Graphical representation of cumulative disease-specific probability scores in proximal biopsies of training set patients, with summary statistics per diagnosis depicted under (**C**).

2.4.4 Definition of a predictive diagnostic model

A three-class statistical model was built to predict the diagnosis of patients from the expression levels of the ten genes that were identified to be significantly regulated in distal biopsies. The primary output of our model was a probability score per patient for each of the three diagnostic categories (NH, EoE or RE), which allowed for quantification of the confidence of the model in predicting the underlying diagnosis. After 10-fold cross validation on distal training set samples, the disease-specific probability scores discriminated perfectly between all three diagnostic categories: mean predicted EoE-probability in EoE patients 0.92±0.09 (range 0.64-1.0); RE-probability in RE patients 0.81±0.08 (range 0.64-0.94) and NH-probability in NH patients 0.87±0.07 (range 0.67-0.96). In patients with EoE, a positive correlation was observed between distal tissue eosinophil count and the predicted EoE probability, which suggests that a more pronounced eosinophilic infiltrate results in better performance of the diagnostic model (**Figure 2.3A**). However, even in patients with a modest eosinophilic infiltrate in the distal esophagus (20-25 eosinophils/hpf), a clear prediction of EoE over RE or NH etiology could be made with probabilities ranging from 0.74 to 0.97. These results indicated that distal mRNA levels of ten differentially expressed genes are sufficiently dissimilar between disease conditions to allow for clear mathematical differentiation.

This finding, however, does not necessarily guarantee a similar degree of performance on biopsies that do themselves not further shape the algorithmic parameters of the model. In assessing how our pre-defined model performed on an independent subset of biopsies,

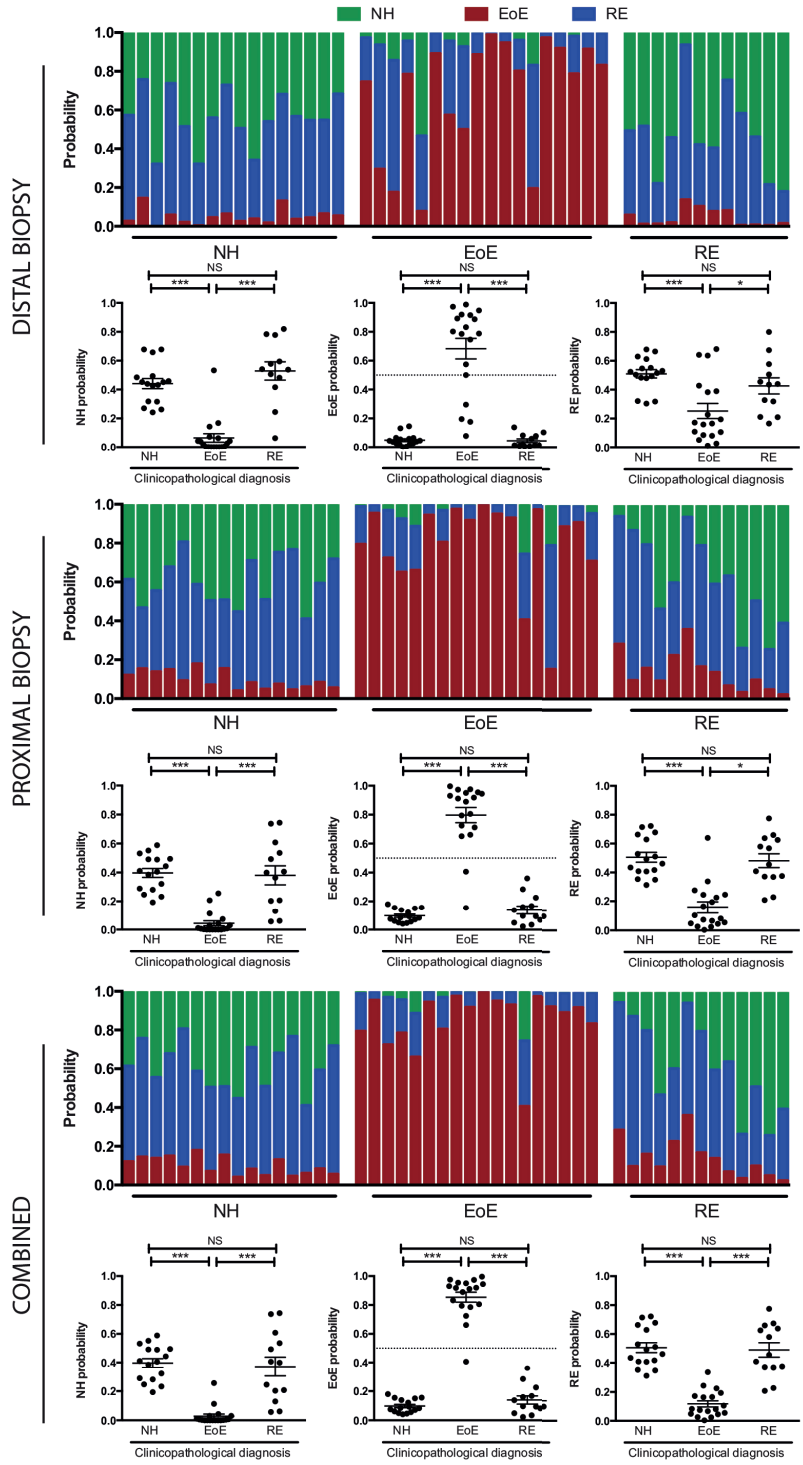
NH
EoE
RE
DISTAL BIOPSY
PROXIMAL BIOPSY
COMBINED
Probability
NH probability
EoE probability
RE probability
Clinicopathological diagnosis
NS
***
*

Figure 2.4. Predicted probability of underlying diagnosis from predictive set patients in distal or and proximal biopsies, with corresponding summary statistics of diagnostic probability per clinicopathological category. Graphs in the combined panel display the highest EoE probability in either proximal or distal biopsy.

we first further validated our prediction model on the paired proximal biopsies from the patients in the training set. This analysis revealed that application of the distally-defined prediction model on proximal biopsies from the same patients resulted in an equally clear differentiation between patients from all three conditions (**Figure 2.3B-C**), and that a diagnostic cut-off of >50% EoE probability identified EoE patients from NH or RE patients with 100% sensitivity and specificity in our training set. Importantly, these results are also indicative of a high degree of test reproducibility as proximal and distal biopsies had been independently collected, stored, and processed prior to digital mRNA profiling.

2.4.5 Performance of diagnostic model in the blinded predictive patient set

To further assess the power of our model in differentiating EoE from non-EoE patients, we next applied it to the patients in the predictive subset (**Figure 2.4).** In 14 out of 18 patients with EoE, the model correctly predicted the presence of EoE with >50% probability in distal biopsies (sensitivity 78%, 95% CI 52%-93%). Conversely, in none of the NH or RE control patients a diagnosis of EoE was made using the model (specificity 100%, 95% CI 85%-100%). For proximal biopsies, similar results were obtained, with 16 out of 18 patients with EoE correctly identified (sensitivity 89%, 95% CI 64%-98%), again with no false positives in the 29 non-EoE control patients. It is well recognized that EoE is a patchy disease and histopathological analysis of multiple biopsies is required for a reliable diagnosis.(Liacouras et al., 2011; Nielsen et al., 2014) When a cut-off of >50% EoE probability in either proximal or distal biopsy was used to identify patients with EoE, sensitivity was increased to 94% (95% CI 71%-100%) without compromising specificity, which remained at 100%. These results indicate that gene expression analysis of multiple biopsies enhances the diagnostic power of this predictive model for EoE.

In contrast to the performance of the model on the proximal biopsies in the training set patients, differentiation between RE and NH patients in this blinded predictive patient set was poor. No significant differences were found in either the NH or RE probabilities between patients with a clinicopathological diagnosis of these conditions (**Figure 2.4**). Our diagnostic model therefore failed to identify patients suffering from RE from the controls within the pool of non-EoE patients. It is, however, conceivable that a gene set for RE diagnostic could be defined with our approach.

2.5 Discussion

In the current study, we have tested the hypothesis that a diagnosis of EoE can reliably be made based on changes in the mRNA expression levels of a limited number of EoE-specific target genes. Using a diagnostic panel of merely ten targets, the analysis of a single research biopsy could detect EoE with a sensitivity of 78% for distal and 89% for proximal biopsies, which was increased further to 94% when results of two investigated biopsies were combined. Importantly, no false-positive EoE predictions were made amongst 29 normal or inflammatory controls.

The increased sensitivity upon analysis of multiple biopsies is not unexpected given the patchy nature of the disease, and is also true in histological assessment of esophageal tissue, where it has been shown that sensitivity increases from 73% in a single biopsy, to 84%, 97%, and 100% with obtaining two, three, and six biopsies respectively (Shah et al., 2009). Our results thus compare favorably to eosinophil count alone, which suggests that the transcriptome signature may be a more sensitive readout of disease involvement than histological evaluation. Although we did observe a positive correlation between eosinophil count and predicted EoE probability, the model still reliably identified EoE at moderate eosinophil counts of 20-25 eosinophils/hpf. One of the main findings of our current study is therefore that an objective, observer-independent diagnosis of EoE could be made using the esophageal transcriptome within our study population. We think that this technique will be a useful adjunct to the clinical evaluation of patients with suspected EoE, and may allow establishment of an accurate diagnosis from fewer biopsies and help in equivocal cases.

A rather surprising observation that can be made from this work is the seemingly paradoxical condition of EoE-specific tissue downregulation of the α-chain of the high affinity IgE receptor *FcεRI* in combination with upregulation of its β-chain. Increased β-chain expression can be readily explained by esophageal infiltration of mast cells, as has been well documented to occur in EoE (Abonia et al., 2010). Less is known, however, about reduced tissue expression of *FcεRIα*. In addition to its expression on mast cells as part of the tetrameric *FcεRI* complex, the α-chain is also expressed in the GI-tract by Langerhans cells to form part of a β-chain-independent, trimeric *FcεRI* (Bannert et al., 2012). As such, it is conceivable that reduced mRNA levels of *FcεRIα* signify lower numbers of Langerhans cells in the esophagus of patients with EoE. In support of this notion, we found significantly lower transcript numbers of CD207 (Langerin, **Table 2.2**) and CD1a (results not shown) in EoE patients compared to non-EoE patients. Since $CD207^+$ dendritic cells have been associated with the establishment of oral tolerance to ingested antigens (Chang et al., 2010), it could be hypothesized that altered numbers of these cells contribute to disease pathogenesis and future experimental studies addressing the role of these dendritic cells in EoE are therefore warranted.

One strength of our study is that we were able to follow the included patients for a minimum follow-up of two years, ensuring that the initial clinicopathological diagnosis that was given initially was accurate. Our strategy allowed us to incorporate additional clinical information

such as response to treatment, progression of symptoms and follow-up endoscopies. Because of this comprehensive follow-up we were able to validate our novel diagnostic approach against an optimized gold standard reference. Mutual blinding of the reviewing clinician and analyzing statistician eliminated the potential of bias during this process.

Our results indicate that the EoE signature is reliably identified in almost all investigated patients. Unfortunately, even though the model performed well in separating EoE patients from those with RE, the sensitivity and specificity of our model was too low to be considered of diagnostic value in differentiating RE patients from NH controls. This distinction is, however, not a difficult problem in daily clinical practice. To more reliably make a transcriptome-based diagnosis of RE-mediated inflammation, it is likely that a broader gene array panel needs to be screened, since our combination of 76 genes only yielded two genes that were differentially regulated between NH controls and RE but not EoE patients.

Our findings are in line with a recently published study that also tested the diagnostic potential of esophageal transcriptome changes in adult and pediatric patients with EoE (Wen et al., 2013). Using a real-time quantitative PCR approach on a panel of 94 EoE-specific genes, of which four (CCL26, CPA3, IL13 and CCR3) are also included in our set of 10 genes, this group attained a 96% sensitivity and 98% specificity rate for a diagnosis of EoE over non-EoE control patients. The results presented here, showing equivalently high degrees of diagnostic accuracy, thus confirm the great diagnostic potential of the esophageal transcriptome in detecting EoE, and further validate this approach using a different technical platform in another patient cohort. In addition, our data demonstrate that a diagnosis of EoE can reliably be made with a greatly reduced number of target genes and that analysis with a novel high-throughput mRNA expression system that eliminates the need for intermediary sample processing steps is a feasible approach to obtaining a tissue diagnosis of EoE. Although restricted availability and costs of this technology limit its current applicability, it is possible that with technological advances these costs will decrease in the future. Furthermore, transcriptome diagnostics with digital mRNA profiling may prove applicable to other disorders as well, which will help further promote distribution of this novel diagnostic technique.

In summary, using a high-throughput digital mRNA expression platform, we show that a statistical model built on changes in expression of 10 EoE-specific genes detects EoE patients from non-EoE patients with high diagnostic sensitivity and specificity. This novel strategy could help stratify patients at time of their first diagnostic endoscopy and lead to rapid installment of appropriate EoE-specific therapy.

2.6 Acknowledgements

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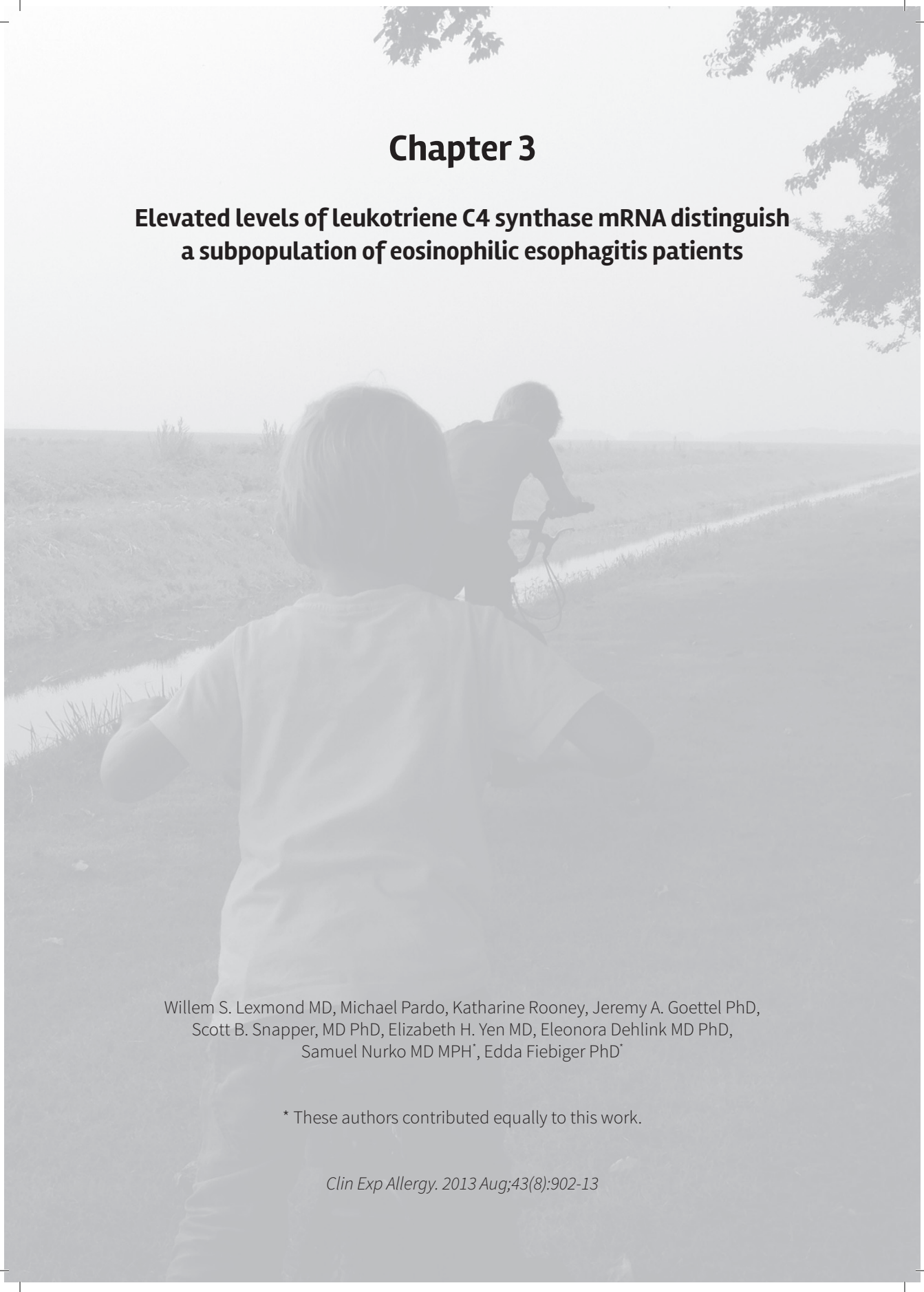
Chapter 3

Elevated levels of leukotriene C4 synthase mRNA distinguish a subpopulation of eosinophilic esophagitis patients



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3.1 Abstract

Background: Cysteinyl leukotrienes contribute to Th2-type inflammatory immune responses. Their levels in esophageal tissue, however, do not distinguish patients with eosinophilic esophagitis (EoE) from controls.

Objective: We asked whether mRNA levels of leukotriene C_4 synthase (LTC_4S), a key regulator of leukotriene production, could serve as a marker for EoE.

Methods: Digital mRNA expression profiling (nCounter® Technology) was performed on proximal and distal esophageal biopsies of 30 paediatric EoE patients and 40 non-EoE controls. Expression data were confirmed with RT-qPCR. LTC_4S mRNA levels were quantified in whole blood samples. Leukotriene E_4 was measured in urine.

Results: LTC_4S mRNA levels were elevated in proximal (2.6-fold, $p<0.001$) and distal (2.9-fold, $p<0.001$) esophageal biopsies from EoE patients. Importantly, increased LTC_4S mRNA transcripts identified a subpopulation of EoE patients (28%). This patient subgroup had higher serum IgE levels (669 U/ml vs. 106 U/ml, $p=0.01$), higher mRNA transcript numbers of TSLP (1.6-fold, $p=0.009$) and CD4 (1.4-fold, $p=0.04$) but lower IL-23 mRNA levels (0.5-fold, $p=0.04$). In contrast, elevated levels of IL-23 mRNA were found in esophageal biopsies of patients with reflux esophagitis. LTC_4S mRNA transcripts in whole blood and urinary excretion of leukotriene E_4 were similar in EoE patient subgroups and non-EoE patients.

Conclusion & Clinical Relevance: Elevated esophageal expression of LTC_4S mRNA is found in a subgroup of EoE patients, concomitant with higher serum IgE levels and an esophageal transcriptome indicative of a more-pronounced allergic phenotype. Together with TSLP and IL-23 mRNA levels, esophageal LTC_4S mRNA may facilitate diagnosis of an EoE subpopulation for personalized therapy.

3.2 Introduction

Eosinophilic esophagitis (EoE) is commonly considered a Th2-type allergic disease of the esophagus (Straumann et al., 2001). As a clinicopathological entity, EoE is characterized by eosinophil-rich, chronic inflammation of the esophagus with symptoms of esophageal dysfunction (Liacouras et al., 2011). The disorder is strongly allergen-driven(Hruz et al., 2011; Mulder and Justinich, 2011; Rothenberg, 2009) and comorbidity with concurrent allergic diatheses ranges from 42% to 93% for paediatric and 28% to 86% for adult patients (Liacouras et al., 2011). Like other Th2-type allergies, elevated serum levels of IgE are commonly encountered in EoE patients and identify allergen-sensitized individuals. However, up to 50% of EoE patients have normal serum IgE levels without evidence of prior allergic sensitization (Erwin et al., 2010; Liacouras et al., 2011; Mulder and Justinich, 2011; Norvell et al., 2007). The extent to which IgE contributes to EoE pathogenesis is therefore not yet fully understood.

mRNA expression analysis of esophageal tissue demonstrated t hat IgE is produced locally in the esophagus of EoE patients, independent of the patient's allergic status(Vicario et al., 2010). Furthermore, the EoE-specific tissue transcriptome fails to distinguish allergic from non-allergic EoE, as increased levels of eotaxin-3 and IL-13 mRNA, signifying Th2-mediated inflammation, are found in all EoE patients(Blanchard et al., 2006b; 2007). Thus, our understanding about the difference between allergic and non-allergic EoE at the site of local pathology is similarly incomplete and characteristics that identify allergic EoE at the tissue level remain to be identified.

Leukotrienes are arachidonic acid metabolites and inflammatory mediators of Th2-type allergies. All leukotrienes are products of the 5-lipoxygenase (5-LO) pathway, which generates leukotriene A_4 (LTA_4) from arachidonic acid by phospholipase A_2. LTA_4 is rapidly converted into either leukotriene B_4 (LTB_4) by LTA_4 hydrolase, or, in the presence of the enzyme leukotriene C_4 synthase (LTC_4S) and glutathione, into leukotriene C_4 (LTC_4), which opens the synthetic pathway for the additional cysteinyl leukotrienes LTD_4 and LTE_4 (Drazen et al., 1999; Murphy and Gijón, 2007; Penrose, 1999). LTC_4S thus functions as the gatekeeper of cysteinyl leukotriene synthesis, which in turn enhances Th2-type inflammation (Drazen et al., 1999).

Despite the contribution of leukotrienes to allergic tissue inflammation, esophageal levels of cysteinyl leukotrienes do not differentiate EoE patients from controls (Gupta et al., 2006). Nevertheless, a number of case series have ascribed therapeutic benefit to the application of leukotriene receptor antagonists in EoE patients (Attwood et al., 2003; Neustrom and Friesen, 1999; Stumphy et al., 2011; Vanderhoof et al., 2003). We hypothesized that mRNA levels of the regulator enzyme LTC_4S, rather than metabolites of the pathway, could serve as a marker to identify esophageal biopsies of EoE patients from patients suffering from other inflammatory diseases of the esophagus. Here we describe that elevated esophageal LTC_4S mRNA levels can be used to identify a subpopulation of EoE patients and that this patient subgroup shows a more-pronounced allergic phenotype.

3.3 Materials and Methods

3.3.1 Patients

Patient material was obtained during an ongoing prospective study on the pathophysiology of EoE at Boston Children's Hospital (Dehlink et al., 2011; 2010; Lexmond et al., 2011; Yen et al., 2010). All children 1-18 years of age whose clinical presentation raised the suspicion of EoE (e.g., dysphagia, feeding intolerance, food aversion, failure to thrive or regurgitation) and who were consequently scheduled to undergo diagnostic upper esophagogastroduodenoscopy were invited to participate. Serum, two esophageal biopsies, whole blood and urine samples were collected at the time of the first diagnostic endoscopy, which was performed after patients had received proton pump inhibition (PPI) for a minimum of 4 weeks (10-40 mg once or twice a day, depending on body weight and symptoms). Information on the subject's past medical history was obtained through a questionnaire. Total IgE levels were assessed in collected serum samples by the hospital laboratory using standardized methods as previously described (Dehlink et al., 2011). Standardized allergic testing in the form of RAST, skin prick testing or antigen-specific IgE levels was not performed in this study. A majority (72%) of EoE patients underwent RAST testing before or after inclusion as part of their diagnostic workup and these results were retrieved from chart review. This study was approved by the Investigational Review Board of Boston Children's Hospital (Harvard Medical School, Boston, MA). Patients or their legal guardians provided written informed consent.

Patients were classified according to routine histopathological analysis of a minimum of 2 esophageal biopsies as: (I) EoE, characterized by the presence of esophagitis with >15 eosinophils per high power field, unresponsive to at least 4 weeks of proton pump inhibitors (Liacouras et al., 2011); (II) Reflux esophagitis (RE), characterized by histological evidence of basal zone hyperplasia, inflammatory cell infiltrate and <15 eosinophils per high power field; or (III) Normal, children with esophageal biopsies without signs of inflammation. The first 30 EoE, 20 RE and 20 normal patients that had undergone digital tissue mRNA expression profiling were selected for this study. None used corticosteroids or leukotriene receptor antagonists (LTRAs) at time of inclusion.

3.3.2 Digital mRNA pattern profiling

Quantification of mRNA transcripts with reporter probes was performed with the *nCounter® system (NanoString* Technologies) according to the manufacturer's instructions (Geiss et al., 2008). Target probes for genes of interest are summarized in supplementary **Table 3.S1**. mRNA counts were collected for all patients, grouped according to anatomical location and normalized following the nCounter® Data Analysis Guidelines (http://www.nanostring.com) against the geometric mean expression of 6 internal positive controls as well as the following 5 housekeeping genes: glyceraldehyde 3-phosphate dehydrogenase (GAPDH), hypoxanthine phosphoribosyltransferase 1 (HPRT1), beta-actin (ACTB), 60S ribosomal protein L13a

(RPL13A) and heat shock protein HSP 90-beta (HSP90AB1). A total of 68 proximal (28 EoE, 40 non-EoE controls) and 63 distal biopsies (26 EoE, 37 non-EoE controls) met the stringency criteria that are recommended by the manufacturer to prevent overcorrection of mRNA counts through normalization and were used for further analysis.

3.3.3 Sample preparation

Proximal (≥10 cm above the gastroesophageal junction) and distal (1-2 cm above the gastroesophageal junction) biopsies were homogenized in 350 µl RLT Buffer (Qiagen) with β-mercaptoethanol (Sigma) in GentleMACS M tubes (Miltenyi Biotec). Homogenates were processed according to the *nCounter® system* manufacturer's protocol. All further processing of samples occurred with the *nCounter® Prep Station and nCounter® Digital Analyzer*.

Whole blood samples were collected in EDTA blood tubes (BD) and processed by incubation in RBC lysis buffer (eBioscience) and centrifugation at 350 RCF. Pellets were re-suspended in PBS (Invitrogen) and centrifuged at 200 RCF to separate thrombocytes. Cells were counted (CASY, Roche) before a final centrifugation at 400 RCF and storage at –80°C. Prior to digital mRNA profiling, total RNA was isolated from pellets with the RNeasy mini kit (Qiagen). RNA concentration was assessed (NanoDrop, Thermo Scientific) and 500 ng was processed for *nCounter®* analysis. Urine was centrifuged at 350 RCF and stored at -80°C till further analysis.

3.3.4 Quantitative RT–PCR

After isolation from tissue homogenates with RNeasy Plus kit (Qiagen), 500 ng of total RNA was reverse transcribed with iScript (Bio-rad). cDNA was diluted 1:5 in H_2O and expression of LTC_4S, eotaxin-3 and periostin was assessed using TaqMan Gene Expression assays (Applied Biosystems) in duplex RT-qPCR reactions with the housekeeping gene GAPDH on a C1000 Thermal Cycler (Bio-rad). Distal biopsies from 15 EoE patients and 5 controls of which enough RNA was available were analyzed; cDNA from the 5 normal controls was pooled and used as an inter-run calibrator on every plate. Results were analyzed with CFX Manager version 3.0 (Bio-rad) by calculating gene expression relative to GAPDH (ΔΔct) (Pfaffl, 2001).

3.3.5 ELISAs

LTE_4 was measured with a commercial ELISA following manufacturer's instructions (Cayman Chemical, 520411). For correction for varying levels of dilution, creatinine content was measured with a urinary creatinine assay kit (Cayman Chemical, 500701).

Table 3.1. Study population. *IgE Z-scores were calculated according to the age-specific reference range. **No information on RAST testing in other centers was available. § Endoscopy report of 1 EoE patient was missing. §§Data on 2 of the 29 EoE patients was missing (7%).

	EoE	Non-EoE: RE	Non-EoE: Normal biopsy	Non-EoE: All	P value EoE vs. Non-EoE	P value RE vs. Normal biopsy
Number of patients	29	20	20	40		
Male/female	22/7	12/8	12/8	24/16	0.20	1.0
Age in years (median, range)	10.3 (2 – 17)	9.3 (1 – 16)	10.3 (1 – 17)	9.5 (1 – 17)	0.70	0.77
Serum IgE in U/ml (median, range)	120 (4 – 1900)	55 (5 – 2446)	24 (4 – 216)	31 (4 – 2446)	0.23	0.31
IgE Z-score (mean, range)*	1.4 (-1.4 – 4.5)	1.23 (-1.22 – 4.1)	0.64 (-1.8 – 2.3)	1.0 (-1.8 – 4.1)	0.31	0.33
IgE Z-score ≥ 2	10/21 (48%)	7/20 (35%)	2/20 (10%)	9/35 (26%)	0.14	0.13
≥1 positive RAST (>0.35 U/ml)**	13/21 (62%)	2/9 (22%)	0/4 (0%)	2/13 (33%)	0.01	1.0
Macroscopic findings on endoscopy §:	28 (100%)	4 (20%)	0 (0%)	4 (10%)	<0.001	0.05
Linear furrowing	23 (82%)	3 (15%)	0 (0%)	3 (8%)	<0.001	0.23
Decreased vascularity	13 (46%)	1 (5%)	0 (0%)	0 (0%)	<0.001	1.0
Edema	5 (18%)	0 (0%)	0 (0%)	0 (0%)	<0.01	1.0
Symptoms in past 6 months §§:						
Painful swallowing	6 (22%)	5 (25%)	4 (20%)	9 (23%)	1.00	1.0
Food getting stuck	12 (44%)	6 (30%)	6 (30%)	12 (30%)	0.30	1.0
Dysphagia	8 (30%)	8 (40%)	5 (25%)	13 (33%)	1.00	0.34
Abdominal pain	11 (41%)	11 (55%)	9 (45%)	20 (50%)	0.62	0.75
Constipation	8 (30%)	8 (40%)	8 (40%)	16 (40%)	0.44	1.0
Diarrhea	10 (37%)	3 (15%)	7 (35%)	10 (25%)	0.41	0.27
Weight loss	3 (11%)	4 (20%)	4 (20%)	8 (20%)	0.50	1.0
Regurgitation	7 (26%)	7 (35%)	12 (60%)	19 (48%)	0.12	0.20
Reported medical history of:						
Eczema	14 (52%)	8 (40%)	5 (25%)	13 (33%)	0.13	0.34
Asthma	7 (26%)	5 (25%)	4 (20%)	9 (23%)	0.78	1.0
Seasonal allergies	16 (59%)	10 (50%)	7 (35%)	17 (43%)	0.22	0.52
Heartburn or chest pain	9 (33%)	12 (60%)	10 (50%)	22 (55%)	0.13	0.75
Food allergies	16 (59%)	8 (40%)	4 (20%)	12 (30%)	0.02	0.30

3.3.6 Statistical Analysis

Data were analyzed with Stata 12.1 (StataCorp) and Prism 5.0 (GraphPad Software). Nominal variables were compared with Fisher's exact test. Distribution of continuous variables was assessed with D'Agostino-Pearson omnibus test and subsequently compared with Student's t test or ANOVA for normally distributed or Mann-Whitney U test for non-parametric variables. Correlation analysis was performed by calculating Spearman's rho for non-parametric variables. ROC analysis was based on logistic regression modeling with proximal and distal gene expression data as individual predictors of diagnosis. Results were considered significant for p-values <0.05 upon two-sided testing.

3.4 Results

The 70 patients included in this study were enrolled between July 2008 and May 2011 (47 boys, 23 girls, mean age 10.1±5.0 years). To investigate the value of LTC_4S mRNA expression as a discriminative marker for EoE at time of diagnosis, our initial analyses focused on a patient set representative of clinical practice. The non-EoE control group thus included children with normal esophageal histology as well as those with reflux esophagitis (RE). One EoE patient was excluded because of low mRNA yield from both esophageal biopsies. EoE patients received an average daily PPI dose of 36 mg (±18 mg), which did not differ from those patients with RE (35mg ±17 mg). Patient characteristics are depicted in **Table 3.1**.

Patient groups did not differ significantly with regards to their GI symptomatology. Frequency of self-reported food allergies was significantly higher in EoE patients compared to the other groups. Normal appearance of the esophagus during endoscopy was reported in 80% of RE patients. In contrast, all EoE patients had a minimum of one macroscopic abnormality, which although not pathognomic for EoE (Liacouras et al., 2011) is found in 93% of EoE patients (Kim et al., 2012). In line with epidemiological data (Noel et al., 2004; Rothenberg et al., 2010), we found a strong male preponderance in the EoE cohort (76%). EoE patients furthermore had higher serum IgE levels than non-EoE controls, though IgE levels were also elevated in the control group. To account for the age-dependent variation in normal serum IgE range, we additionally compared IgE Z-scores expressing IgE levels as the number of standard deviations from the age-dependent mean (Kjellman et al., 1976), which yielded comparable results.

3.4.1 LTC_4S mRNA is upregulated in esophageal biopsies of EoE patients

Comparative analysis of mRNA expression levels showed a significant upregulation of LTC_4S in tissue biopsies of EoE patients when compared to non-EoE controls (2.6-fold in proximal and 2.9-fold increase in distal biopsies, **Figure 3.1A** and **3.1B** respectively). Equal expression levels of normalized GAPDH mRNA counts confirmed that differences were not due to higher mRNA content (**Figure 3.1A** and **3.1B**). To validate the clinicopathological

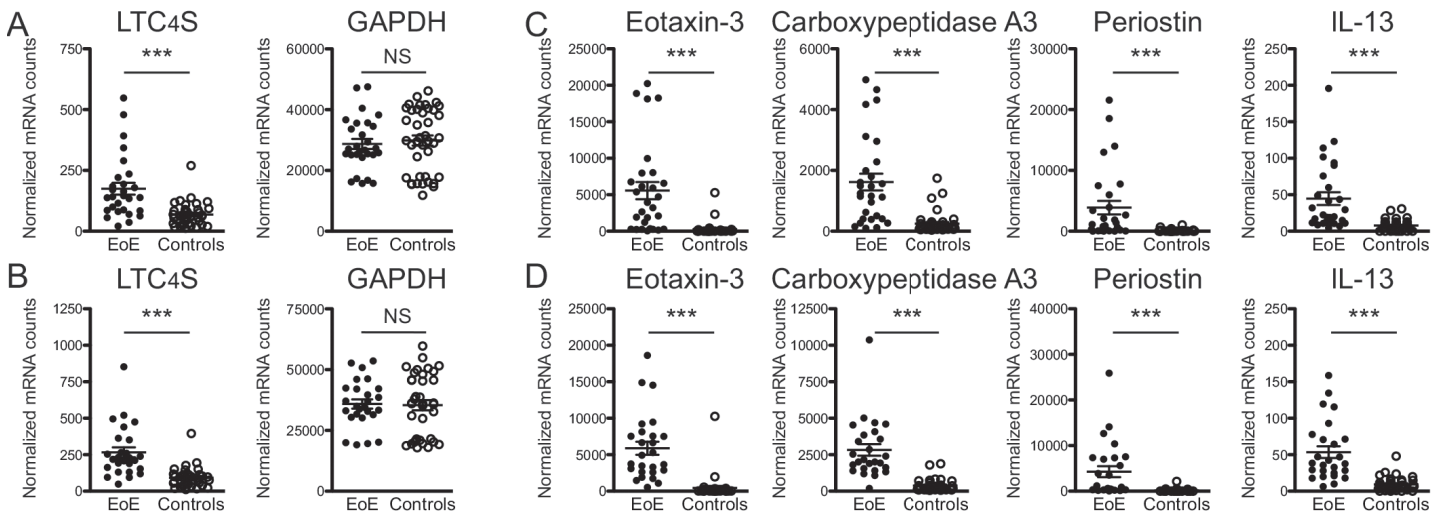

Figure 3.1. Leukotriene C_4 synthase (LTC_4S) mRNA levels are elevated in esophageal biopsies of EoE patients. Digital mRNA profiling was performed with nCounter® analysis. mRNA counts were normalized to internal controls and 5 housekeeping genes and are displayed as normalized counts on the y-axis. (**A**) Proximal as well as (**B**) distal esophageal tissue biopsies of EoE patients (N=28 for proximal and N=26 for distal) show elevated LTC_4S mRNA levels compared to non-EoE controls (N=40 for proximal and N=37 for distal). Expression of housekeeping gene GAPDH does not vary between groups. To confirm clinical diagnosis by mRNA expression profiling, (**C**) proximal and (**D**) distal esophageal biopsies were additionally analyzed for the expression levels of the published EoE markers eotaxin-3, carboxypeptidase A3, periostin and IL-13. ***p<0.0001, NS: not significant.

diagnosis in our patient cohort, we analyzed the expression of 4 EoE marker genes (**Figure 3.1C** and **3.1D**): eotaxin-3 was found to be upregulated 21-fold in proximal and 13-fold in distal biopsies, as were carboxypeptidase A3 (7-fold proximal, 7-fold distal), periostin (41-fold proximal, 27-fold distal) and IL-13 (6-fold proximal, 6-fold distal). These results confirm the diagnostic potential of the published EoE-specific gene expression profile (Abonia et al., 2010; Blanchard et al., 2006b; 2007) in supplementing histopathological EoE diagnosis. To rule out a confounding effect of our normalization method, we applied an alternative normalization strategy that directly divides LTC_4S mRNA counts by GAPDH counts in individual samples, which yielded comparable results (2.1-fold increase in proximal and 2.5-fold increase in distal biopsies, **Table 3.S2**).

We next validated the results obtained with digital mRNA expression profiling using RT-qPCR as an established method to evaluate mRNA expression levels. LTC_4S, eotaxin-3, and periostin mRNA expression in distal biopsies of EoE patients (N=15) and controls with normal distal biopsy (N=5) was compared (**Figure 3.S1A**). Average LTC_4S mRNA levels as determined by RT-qPCR were 4.8-fold higher in EoE patients than controls (p=0.001) and correlated well with digital LTC_4S mRNA counts from nCounter® analysis (**Figure 3.S1B**, r=0.619). These experiments confirm that digital mRNA expression profiling is an alternative to RT-qPCR for analysis of mRNA expression in esophageal tissue (Blanchard et al., 2011). Additionally, we confirmed with a second method that tissue LTC_4S mRNA levels are elevated in EoE patients.

To analyze the sensitivity and specificity of elevated LTC_4S mRNA transcripts for distinguishing EoE from non-EoE controls, we compared the area under the receiver operating characteristic (ROC) curve with those of eotaxin-3 and periostin (**Figure 3.2**). This analysis revealed that expression of LTC_4S mRNA in esophageal biopsies has sensitivity and specificity >80% in predicting EoE (area under the ROC curve 0.89; 95% CI 0.80 – 0.97) but as a single diagnostic tool, LTC_4S expression did not outperform periostin (area under the ROC curve 0.91; 95% 0.84 – 0.99, p=0.56) and was inferior to eotaxin-3 (0.98; 95% CI 0.93 – 1.0, p=0.02). Furthermore, addition of LTC_4S mRNA counts to an EoE prediction model that included eotaxin-3 expression did not significantly increase the area under the ROC curve (0.976 vs. 0.977, p=0.75). Combined, these results indicate good, but non-superior sensitivity and specificity of elevated LTC_4S mRNA levels over known EoE marker genes.

3.4.2 Increased LTC_4S mRNA levels differentiate EoE from RE patients

We next compared EoE patients with those who had RE-associated inflammation and asked whether LTC_4S mRNA levels can specifically differentiate EoE from RE. Comparison of LTC_4S mRNA levels in EoE and RE patients revealed a 2.8-fold increase in proximal and 3.3-fold increase in distal biopsies (**Figure 3.3A** and **3.3B**). This difference was more pronounced than was observed between EoE patients and controls with normal tissue biopsies, which was 2.4-fold in proximal and 2.6-fold in distal biopsies. Within our non-EoE control group, RE patients showed a significantly decreased expression of LTC_4S mRNA in distal biopsies (0.8-fold, p=0.03) compared to normal controls.

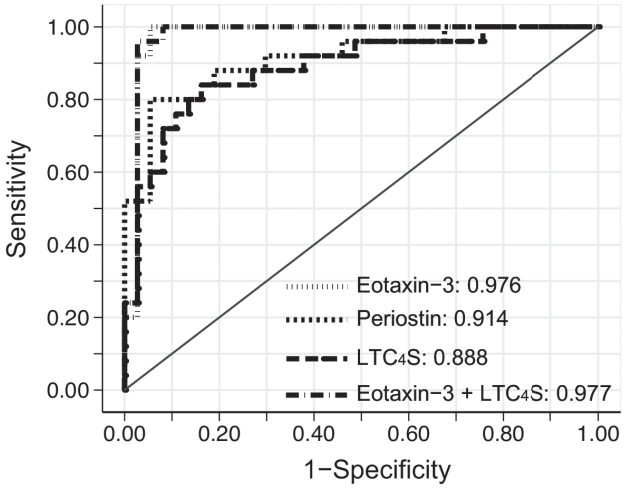

Figure 3.2. Sensitivity and specificity of elevated LTC_4S mRNA levels in distinguishing EoE from non-EoE patients. Receiver operator characteristic (ROC) curves were derived from a logistic regression model in which the proximal and distal esophageal mRNA expression served as individual predictors. Area under the ROC curve was higher for eotaxin-3 than periostin (p=0.09) and LTC_4S (p=0.03). Addition of LTC4S mRNA counts to a model that already included eotaxin-3 did not significantly increase the area under the ROC curve (p=0.75).

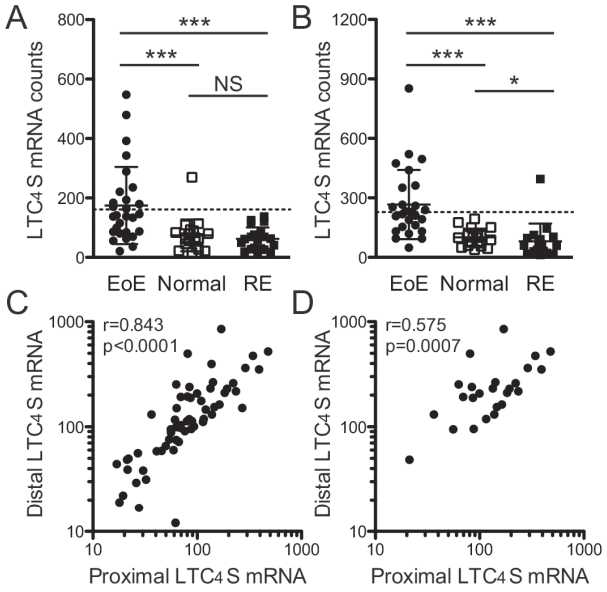

Figure 3.3. Elevated LTC_4S mRNA counts differentiate EoE from RE. Based on the histology of esophageal biopsies, control patients were divided into a group without signs of inflammation (normal, N=20) and inflammation most likely associated with gastroesophageal reflux (RE, N=20). LTC_4S mRNA levels were compared in (**A**) proximal and (**B**) distal biopsies. Horizontal bars mark the mean ± standard deviation (SD). Dotted line indicates the mean LTC_4S

expression + 2 SDs as calculated from all normal and RE control patients. (**C**) Expression of LTC_4S mRNA correlates highly between samples from proximal and distal esophagus in the entire cohort (N=62). (**D**) Subanalysis of LTC_4S correlation in proximal and distal paired biopsies of EoE patients (N=25). *p<0.05; ***p<0.001; NS: not significant.

3.4.3 Increased LTC_4S mRNA levels distinguish a subpopulation of EoE patients

Correlation analysis within the total patient population showed that proximal and distal LTC_4S expression correlated highly in paired samples (Spearman rho = 0.843, **Figure 3.3C**). This correlation remained when only samples from EoE patients were analyzed (Spearman rho = 0.575, **Figure 3.3D**). Of the 6 patients that expressed LTC_4S at the highest level in the distal biopsies, 5 (83%) were found among the 10 patients with the highest expression in proximal biopsies. These findings suggested that the increase in LTC_4S mRNA transcripts in individuals is part of the disease immune phenotype rather than a result of patchiness of the disease.

EoE biopsies were next divided into groups of LTC_4S^{HIGH} and LTC_4S^{LOW} mRNA counts. Low expression levels were defined as ≤200 normalized mRNA counts. This cutoff value was derived from the control population as follows: mean LTC_4S expression in all non-EoE control biopsies + 2 standard deviations. Based on this cutoff, we identified 23 biopsies with high and 31 biopsies with low LTC_4S expression. LTC_4S^{HIGH} and LTC_4S^{LOW} groups differed 3.0-fold in mean LTC_4S mRNA expression but had similar GAPDH mRNA levels (**Figure 3.4A**). The 23 biopsies were derived from a total of 17 patients (59%); biopsies from 8 patients (28%) were exclusive to the LTC_4S^{HIGH} group and 9 patients (31%) had LTC_4S mRNA counts >200 in only one of the biopsies. **Table 3.2** shows the characteristics of EoE patients with high LTC_4S expression in both biopsies versus those with low LTC_4S mRNA counts in one or both biopsies. No difference in the degree of tissue eosinophilia as established by routine histopathological analysis was depicted. Furthermore, neither symptomatology nor comorbidity of allergic diatheses differed significantly between the patient groups; only one LTC_4S^{HIGH} patient was suffering from asthma. We noticed that 7 out of 8 (88%) LTC_4S^{HIGH} patients had been enrolled during the pollen season compared to only 29% of children that classified as LTC_4S^{LOW} (p=0.01). Self-reported seasonal allergies also had a high prevalence in our non-EoE control population (43%). Interestingly, in 17 control patients with seasonal allergies (10 RE and 7 normal controls), the average tissue LTC_4S mRNA counts obtained from 8 patients enrolled during the pollen season was significantly higher than in the 9 patients recruited off-season (normalized LTC_4S mRNA counts of 85±8 vs. 52±9, p=0.02). Importantly, LTC_4S counts were on average still lower in controls than in LTC_4S^{LOW} EoE patients. This set of data argues for an overall correlation of LTC_4S mRNA expression levels with pollen exposure, with an amplified effect in EoE patients.

Because mean serum IgE levels were higher in the 8 patients with the highest LTC_4S expression, we investigated tissue mRNA levels of the constant region of the IgE heavy chain (IGHE). Arguing against a difference in local IgE production by plasma B-cells, no significant difference in IGHE transcript levels was found between the EoE subgroups (**Figure 3.4B**). We

next assessed whether the groups differed in the expression of eotaxin-3, periostin and IL-13. This comparison did not yield significant differences (**Figure 3.4C**). In summary, these results showed that high LTC_4S mRNA counts were only found in a subpopulation of EoE patients and that high expression of LTC_4S was not merely part of a more active EoE transcriptome.

Table 3.2. Characteristics of LTC4S^{HIGH} and LTC4S^{LOW} EoE patients. *Tissue eosinophilia >100/hpf was not further quantified in the pathology reports. **Endoscopy report from 1 LTC4S^{LOW} patient was missing. § Clinical data on 2 of the 21 (10%) LTC4S^{LOW} EoE patients was missing. §§ Pollen season defined as mid March–June and mid August-October according to (Fogg et al., 2003).

	LTC4S^{HIGH}	LTC4S^{LOW}	P value
Number of patients	8	21	
Male/female	6/2	16/5	1.00
Age in years (mean, range)	11.0 (2 – 17)	10.4 (3 – 17)	0.76
Serum IgE in U/ml (mean, range)	669 (5 – 1900)	106 (4 – 377)	0.01
IgE Z-score (mean, range)	2.38 (-1.2 – 4.5)	0.8 (-0.8 – 2.7)	0.04
IgE Z-score ≥ 2	5/8 (63%)	5/13 (38%)	0.38
≥1 positive RAST (>0.35 U/ml)	5/7 (71%)	8/14 (57%)	0.66
Tissue Eosinophil count*	56 (25 – >100)	45 (15 – >100)	0.35
Macroscopic findings on endoscopy **:			
Furrowing	6 (75%)	17 (85%)	0.61
Decreased vascularity	5 (63%)	8 (40%)	0.40
Edema	3 (38%)	2 (10%)	0.12
Reported symptoms in past 6 months §:			
Painful swallowing	1 (13%)	5 (26%)	0.63
Food getting stuck	3 (38%)	9 (47%)	0.70
Dysphagia	1 (13%)	7 (37%)	0.36
Abdominal pain	3 (38%)	8 (42%)	1.00
Constipation	1 (13%)	7 (37%)	0.36
Diarrhea	4 (50%)	6 (32%)	0.41
Weight loss	1 (13%)	2 (11%)	1.00
Regurgitation	2 (25%)	5 (26%)	1.00
Reported medical history of:			
Eczema	4 (50%)	10 (53%)	1.00
Asthma	1 (13%)	6 (32%)	0.63
Seasonal allergies	6 (75%)	10 (53%)	0.40
Heartburn or chest pain	4 (50%)	5 (26%)	0.37
Food allergies	7 (88%)	9 (47%)	0.09
Endoscopy during pollen season §§	7 (88%)	6 (29%)	0.01

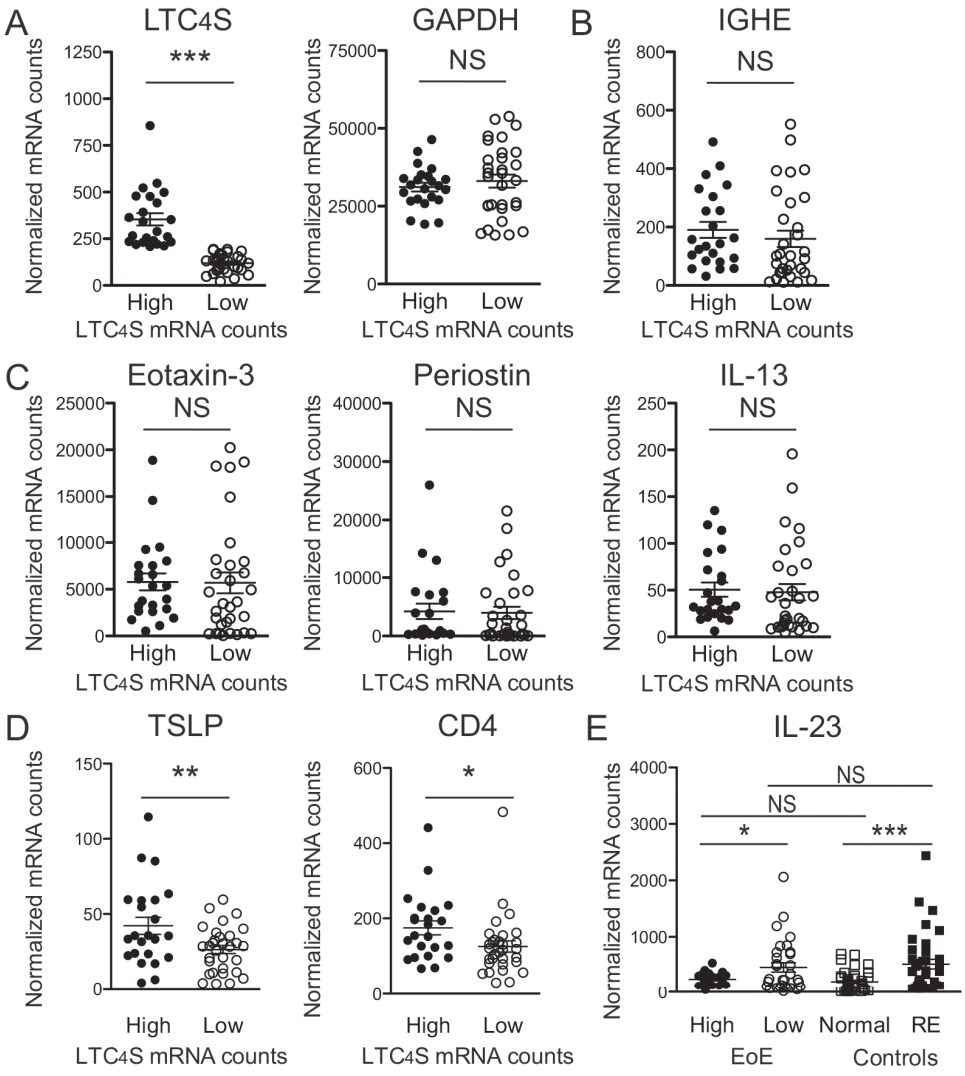

Figure 3.4. Elevated LTC_4S mRNA levels define a subpopulation of EoE patients with a distinct inflammatory transcriptome. (**A**) A subpopulation of 23 EoE biopsies with high LTC_4S mRNA counts was defined (High) using a cutoff of the mean + 2 standard deviations from the LTC_4S mRNA expression in all control biopsies. 31 EoE biopsies (64%) had expression below the cutoff and were classified as low LTC_4S expression (Low). GAPDH expression was similar between LTC_4S^{HIGH} and LTC_4S^{LOW} groups. (**B**) IGHE mRNA transcripts were similar between LTC_4S^{HIGH} and LTC_4S^{LOW} groups. (**C**) LTC_4S^{HIGH} and LTC_4S^{LOW} samples did not differ significantly in expression of the EoE marker genes eotaxin-3, periostin or IL-13. (**D**) LTC_4S^{HIGH} biopsies had significantly higher mRNA levels of TSLP (p=0.009) and CD4 (p=0.01). (**E**) Conversely, mRNA levels of IL-23 were significantly lower (p=0.04) in LTC_4S^{HIGH} compared to LTC_4S^{LOW} biopsies. When compared to control biopsies, IL-23 mRNA was higher in biopsies of patients with RE compared to normal controls (p<0.001). IL-23 expression in LTC_4S^{HIGH} was not different from normal controls and LTC_4S^{LOW} biopsies had IL-23 expression similar to RE patients. *p<0.05, **p<0.01, ***p<0.001, NS: not significant.

3.4.4 LTC_4S^{HIGH} biopsies show increased expression of TSLP and lower levels of IL–23

Leukotriene synthesis is considered part of the tissue Th2 response, but LTC_4S^{HIGH} and LTC_4S^{LOW} biopsies did not differ in their IL-13 mRNA expression levels. We therefore compared LTC_4S^{HIGH} and LTC_4S^{LOW} biopsies for mRNA expression of thymic stromal lymphopoietin (TSLP), a known local regulator of the Th1-Th2 balance. We found a 1.6-fold higher expression of TSLP in the LTC_4S^{HIGH} biopsies and the higher TSLP expression in LTC_4S^{HIGH} biopsies was accompanied by a 1.4-fold increase in CD4 mRNA counts (**Figure 3.4D**). Conversely, we identified a 2-fold lower expression of IL-23 mRNA in LTC_4S^{HIGH} biopsies (**Figure 3.4E**). Since IL-23 is an important early mediator of Th17-type inflammation, high expression of LTC_4S might be the result of a distinct inflammatory pattern of EoE. In support of this interpretation, we found that IL-23 expression was 2.6-fold higher in RE biopsies in comparison to healthy tissue controls (normal controls). IL-23 mRNA counts in the LTC_4S^{HIGH} biopsies did not differ from the levels found in normal controls. Importantly, IL-23 expression in the LTC_4S^{LOW} group was equivalent to that found in RE biopsies. These findings indicate that LTC_4S mRNA overexpression characterizes esophageal inflammation in allergic EoE while the tissue characteristics of the non-allergic EoE group appears to resemble that of RE patients.

3.4.5 Eosinophils and mast cells are both cellular sources of LTC_4S mRNA transcripts

Based on the literature, we hypothesized that the most likely cellular sources of LTC_4S mRNA in esophageal biopsies from EoE patients are eosinophils, mast cells and basophils. Histopathological analysis showed equivalent degrees of eosinophil infiltration in LTC_4S^{HIGH} and LTC_4S^{LOW} patients (**Table 3.2**). No significant correlation between tissue eosinophil numbers and LTC_4S mRNA transcripts numbers was found within the total EoE population (**Figure 3.5A**, r=0.165, p=0.41) or in LTC_4S^{HIGH} patients (**Figure 3.5B**, r= -0.253, p=0.54). In contrast, we found a strongly positive correlation between both parameters in tissue biopsies of patients with low LTC4S mRNA counts in both tissue biopsies (r=0.665, p=0.01). This set of data shows that eosinophils are a likely source of LTC_4S transcripts in the LTC_4S^{LOW} population, but also indicates that an additional cellular source might exist in the LTC_4S^{HIGH} sub-population.

Therefore, we analyzed mRNA expression levels of the β-chain of the high-affinity IgE receptor FcεRI, expression of which is confined to mast cells and basophils. We found increased β-chain transcripts in LTC_4S^{HIGH} when compared to LTC_4S^{LOW} biopsies (**Figure 3.5C**, 428 vs. 256, p=0.01). Analogous to the correlation analysis of LTC_4S expression and eosinophil counts, we found a strong correlation in the LTC_4S^{LOW} but not in the LTC_4S^{HIGH} biopsies (**Figure 3.5D**, r=0.492, p=0.005 versus r=0.260, p=0.25). Similar results were obtained for the mast cell marker carboxypeptidase A3, with higher mRNA levels in LTC_4S^{HIGH} versus LTC_4S^{LOW} patients (mRNA counts 2644 vs. 1880, p=0.06) and correlation coefficients of 0.458 (p=0.01) in LTC_4S^{LOW} and 0.225 (p=0.30) in LTC_4S^{HIGH} biopsies.

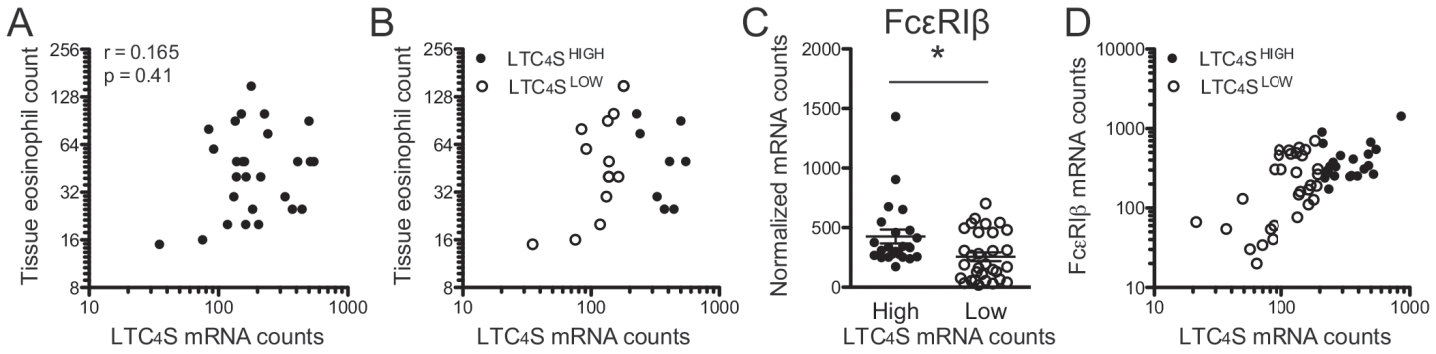

Figure 3.5. LTC_4S mRNA production cannot be ascribed to a single cell type. (**A**) Over the whole EoE population, LTC_4S mRNA tissue counts do not correlate with tissue eosinophil count as obtained from routine histopathological analysis. (**B**) In LTC_4S^{LOW} patients (open circles), a strong positive correlation was depicted (r=0.665, p=0.01) whereas this correlation was lost in the LTC_4S^{HIGH} (full circles) subpopulation (r=-0.25, p=0.54). (**C**) mRNA levels of the mast cell and basophil marker FcεRIβ are higher in LTC_4S^{HIGH} biopsies, (**D**) but a positive correlation with LTC_4S mRNA expression is confined to LTC_4S^{LOW} biopsies (r=0.492, p=0.005 vs. r=0.260, p=0.25 in LTC_4S^{HIGH} biopsies. *p<0.05

Combined, these results suggest that LTC_4S expression levels are not simply a surrogate marker for eosinophil infiltration in LTC_4S^{HIGH} patients and that other cell types such as mast cells and basophils contribute to local LTC_4S mRNA production in this patient group. The correlation analysis indicates that another cell type contributes to the elevated mRNA levels in the LTC_4S^{HIGH} patient group. An alternative explanation would be that LTC_4S mRNA production is upregulated in eosinophils or mast cells of this patient subgroup. In summary, these findings form an additional argument that LTC_4S^{HIGH} EoE patients have a distinct immunologic tissue phenotype.

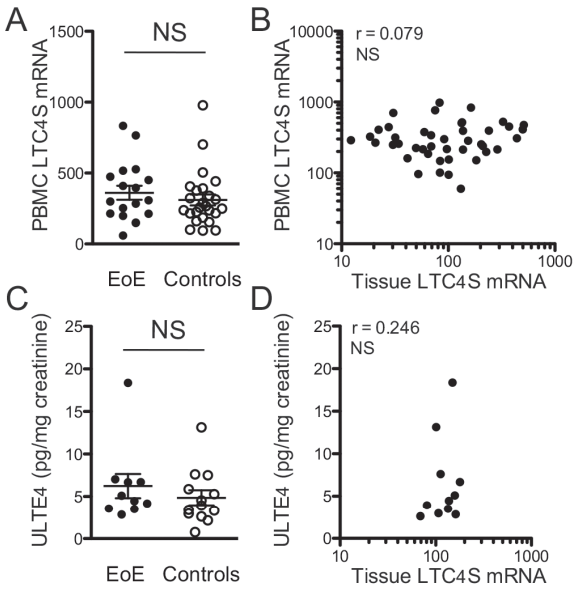

Figure 3.6. LTC_4S mRNA levels in whole blood samples and urinary LTE_4 excretion do not distinguish EoE patients from controls. LTC_4S mRNA levels in whole blood w assessed in 17 EoE patients and 25 controls. (**A**) LTC_4S mRNA expression was similar between EoE patients and controls. (**B**) Expression of LTC_4S mRNA in whole blood did not correlate with the average tissue mRNA levels from proximal and distal biopsies. Urinary excretion of leukotriene E_4 ($ULTE_4$) per mg creatinine was assessed in 10 EoE patients and 13 controls. (**C**) $ULTE_4$ levels were similar between EoE patients and controls. (**D**) $ULTE_4$ did not correlate with the average tissue LTC_4S mRNA levels in 11 available paired samples. NS: not significant.

3.4.6 The LTC_4S^{HIGH} EoE subpopulation has to be defined based on esophageal LTC_4S mRNA transcript levels

We next asked whether LTC_4S^{HIGH} EoE patients could be identified by less invasive methods than tissue biopsy. Therefore, we analyzed mRNA expression in whole blood samples from which erythrocytes and thrombocytes had been removed. Here we found similar LTC_4S mRNA levels between EoE patients (N=17) and non-EoE controls (N=25, **Figure 3.6A**). No

correlation between the average LTC_4S mRNA counts from the proximal and distal biopsy with the patient's LTC_4S mRNA counts in whole blood samples was depicted (Spearman rho = 0.079, **Figure 3.6B**).

Finally, we tested if increased urinary excretion of cysteinyl leukotrienes occurs in EoE patients. Leukotriene E_4 (LTE_4) is excreted in the urine and can be reliably quantified using immunoassays (Kumlin et al., 1995; Rabinovitch, 2007). A total of 10 urine samples from EoE patients and 13 samples from controls were available for analysis. No significant difference in average concentration of urinary leukotriene E_4 was found between patient groups (**Figure 3.6C**). Comparable to our results with mRNA analysis in whole blood, we did not detect a significant correlation between the average tissue LTC_4S expression and urinary LTE_4 concentration (Spearman rho = 0.246, **Figure 3.6D**). Combined, these results stress the importance of local diagnosis in distinguishing LTC_4S^{HIGH} EoE patients.

3.5 Discussion

Our study demonstrates that mRNA expression of LTC_4S, a key regulator of cysteinyl leukotriene synthesis, is elevated in esophageal biopsies of EoE patients. More importantly, we found that tissue LTC_4S mRNA levels identify a subpopulation of EoE patients. LTC_4S^{HIGH} EoE patients were not distinguished from LTC_4S^{LOW} patients by age, sex, tissue eosinophil count or expression of known EoE marker genes, but did show elevated mRNA levels of TSLP and CD4 and significantly lower levels of IL-23. Serum IgE levels in the LTC_4S^{HIGH} subpopulation were significantly higher than in LTC_4S^{LOW} patients. Since IgE levels commonly correlate with the severity of an allergic phenotype (Cheng et al., 2010; Novak and Bieber, 2003), this finding suggests a higher degree of allergic sensitization in patients that overexpress LTC_4S. As such, mRNA levels of LTC_4S, TSLP and IL-23 could potentially discriminate an EoE subpopulation with a more pronounced allergic phenotype at the tissue level.

Our study did not include standardized allergen testing and therefore the antigenic specificity of the allergic tissue phenotype in LTC_4S^{HIGH} patients remains unknown. However, increased secretion of LTC_4S protein has been documented in nasopharyngeal secretions of ragweed-pollen sensitive children during the pollen season (Figueroa et al., 2003; Volovitz et al., 1988) as well as in isolated blood leukocytes after *in vitro* pollen-stimulation (Kopp et al., 2007). Accordingly, increased tissue eosinophilia after exposure to pollen has been described in EoE (Fogg et al., 2003; Onbasi et al., 2005; Spergel, 2005) and experimental EoE could be induced through aeroallergen exposure (Mishra et al., 2001). In line with these published data and our findings, it is well conceivable that aeroallergen exposure during the pollen season results in increased LTC_4S mRNA levels in sensitized EoE patients. Our findings that LTC_4S mRNA levels were also mildly elevated in sensitized, non-EoE control patients enrolled during the pollen season could be used as an argument that upregulation of esophageal

LTC_4S mRNA expression in response to pollen is not confined to EoE patients. However, expression levels were markedly higher in EoE patients, suggesting amplified leukotriene production in those patients with ongoing eosinophilic inflammation.

How LTC_4S mediated inflammation contributes to disease severity in EoE cannot be determined by the present study. We did not identify any differences in GI symptomatology as reported by LTC_4S^{HIGH} vs. LTC_4S^{LOW} patients at time of enrollment. EoE, however, is a relatively new, chronic disorder, in which the clinical course is still impossible to predict at time of diagnosis. One potential sequela of EoE is esophageal fibrosis, which is associated with local collagen deposits in the esophagus that likely contributes to clinical symptoms of food impaction and dysphagia (Aceves and Ackerman, 2009). Recently, LTC_4 has been described to promote skin collagen deposition and thickening in an atopic dermatitis mouse model (Oyoshi et al., 2012). It is thus conceivable that LTC_4S^{HIGH} EoE patients may be prone to local collagen deposition and therefore are at increased risk for the development of fibrosis. This concept is further supported by a 15-year follow-up study of paediatric-onset EoE which showed that allergic rhinitis during childhood was the single factor most strongly associated with dysphagia during early adulthood (OR 3.5, $p<0.001$), closely followed by food allergies (OR 2.7, $p<0.01$) (DeBrosse et al., 2011). Combined, these findings support a model in which LTC_4S mRNA in esophageal tissue in sensitized EoE patients is increased in response to allergen-exposure, resulting in increased collagen deposition and ultimately progression of dysphagia and food impaction.

The published EoE transcriptome, with upregulation of eotaxin-3, periostin, carboxypeptidase A3 and IL-13 as hallmark features, was found not to divert between allergic and non-allergic EoE patients (Blanchard et al., 2006b; 2007). This is confirmed by our data that show similar mRNA levels of these four target genes in LTC_4S^{HIGH} and LTC_4S^{LOW} biopsies. Furthermore, our LTC_4S^{HIGH} samples were not characterized by higher local IgE production, which is in line with data by Vicario et al. who showed that local IgE production occurs to the same extent in both allergic and non-allergic EoE patients (Vicario et al., 2010).

Identification of distinct features of an allergic EoE transcriptome may have important consequences for our understanding of individual disease phenotypes. LTC_4S mRNA was not only found to be upregulated in a subpopulation of EoE patients but also distinguished RE patients from those with normal tissue histology. This disease-associated regulation of baseline tissue LTC_4S expression may reflect two separate pathways of immune activation. Since IL-23 was found to be significantly higher expressed in LTC_4S^{LOW} biopsies as well as in RE patients, it is conceivable that its induction is part of a distinct inflammatory transcriptome that overlaps with the inflammation signature of RE. Within a heterogeneous population of EoE patients, this may potentially identify those with PPI-responsive EoE.

To our knowledge, this study is the first report on mRNA expression of LTC_4S in esophageal tissue of EoE patients. Previously, quantification of the cysteinyl leukotrienes LTC_4, LTD_4 and LTE_4 in esophageal tissue yielded no differences between 12 EoE patients and 10 controls

(Gupta et al., 2006). Only 25% of the EoE patients in this study showed elevated serum IgE levels. Since we found elevated LTC_4S mRNA levels mostly in the EoE group with classical allergic diagnosis, a possible explanation for the equivalent protein levels of LTC_4S between EoE and control patients in the study by Gupta et al. was a relative underrepresentation of allergic EoE patients with elevated serum IgE. Recently, Dellon et al. reported that protein levels of LTC_4S and LTA_4 hydrolase as determined by immunohistochemical staining failed to identify EoE patients (Dellon et al., 2012). These enzymes might be highly stable proteins and the question of how LTC_4S mRNA expression correlates with tissue protein levels in EoE requires clinical follow-up studies. Such studies are important because based on our results, it is conceivable that patients with high expression of LTC_4S mRNA will benefit most from pharmacological treatment with the leukotriene receptor antagonist montelukast, which thus far has been described to yield mixed clinical results in EoE (Attwood et al., 2003; Daikh et al., 2003; Lucendo et al., 2011; Stumphy et al., 2011; Vanderhoof et al., 2003), but showing clear improvement in some patients. Prospective clinical studies to address this hypothesis are now of the utmost importance.

In summary, we here describe the identification of a subpopulation of EoE patients that is characterized by elevated mRNA levels of LTC_4S and TSLP, combined with lower expression of IL-23 in esophageal tissue. Our results stress the importance of local diagnosis of this allergic disease. Incorporation of our findings into clinical practice could advance current EoE classification and direct personalized treatment strategies.

3.6 Acknowledgements

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3.7 Supplemental data

Table 3.S1. Probesets for genes of interest as designed by nCounter® for NanoString mRNA expression analysis. Italics represent the 5 housekeeping that were used for normalization.

Gene ID	Accession number	Targeted region	Target sequence
Carboxypeptidase A3	NM_001870.2	220-320	ACCCACCACGTAGCTGCTAATATGATGGTGGATTTCCGAGTTAGTGAGAAGGAATCCCAAGCCATCCAGTCTGCCTTGGATCAAAATAAAATGCACTATG
CD4	NM_000616.3	835-935	AGACATCGTGGTGCTAGCTTTCCAGAAGGCCTCCAGCATAGTCTATAAGAAAGAGGGGGAACAGGTGGAGTTCTCCTTCCCACTCGCCTTTACAGTTGAA
Eotaxin-3	NM_006072.4	184-284	GGAGTGACATATCCAAGACCTGCTGCTTCCAATACAGCCACAAGCCCCTTCCCTGGACCTGGGTGCGAAGCTATGAATTCACCAGTAACAGCTGCTCCCA
FcεRIβ	NM_000139.3	661-761	TTCTCACCATTCTGGGACTTGGTAGTGCTGTGTCACTCACAATCTGTGGAGCTGGGGAAGAACTCAAAGGAAACAAGGTTCCAGAGGATCGTGTTTATGA
IGHE	AK130825.1	702-802	TGCTGACCGTCTCGGGTGCGTGGGCCAAGCAGATGTTCACCTGCCGTGTGGCACACACTCCATCGTCCACAGACTGGGTCGACAACAAAACCTTCAGCGT
IL13	NM_002188.2	516-616	TTTCTTTCTGATGTCAAAAATGTCTTGGGTAGGCGGGAAGGAGGGTTAGGGAGGGGTAAAATTCCTTAGCTTAGACCTCAGCCTGTGCTGCCCGTCTTCA
IL23	NM_016584.2	411-511	CAGGGACAACAGTCAGTTCTGCTTGCAAAGGATCCACCAGGGTCTGATTTTTTATGAGAAGCTGCTAGGATCGGATATTTTCACAGGGGAGCCTTCTCTG
LTC4S	NM_145867.1	31-131	AGACGGGGCTAAGCGTTCCCCAGCTCGCCTTCACACACAGCCCGTGCCACCACACCGACGGTACCATGAAGGACGAGGTAGCTCTACTGGCTGCTGTCAC
Periostin	NM_006475.2	487-587	GAGGAGATCGAGGGAAAGGGATCCTTCACTTACTTTGCACCGAGTAATGAGGCTTGGGACAACTTGGATTCTGATATCCGTAGAGGTTTGGAGAGCAACG
TSLP	NM_033035.3	395-495	CCGTCTCTTGTAGCAATCGGCCACATTGCCTTACTGAAATCCAGAGCCTAACCTTCAATCCCACCGCCGGCTGCGCGTCGCTCGCCAAAGAAATGTTCGC
GAPDH	NM_002046.3	35-135	TCCTCCTGTTCGACAGTCAGCCGCATCTTCTTTTGCGTCGCCAGCCGAGCCACATCGCTCAGACACCATGGGGAAGGTGAAGGTCGGAGTCAACGGATTT
HPRT1	NM_000194.1	240-340	TGTGATGAAGGAGATGGGAGGCCATCACATTGTAGCCCTCTGTGTGCTCAAGGGGGGCTATAAATTCTTTGCTGACCTGCTGGATTACATCAAAGCACTG
ACTB	NM_001101.2	1010-1110	TGCAGAAGGAGATCACTGCCCTGGCACCCAGCACAATGAAGATCAAGATCATTGCTCCTCCTGAGCGCAAGTACTCCGTGTGGATCGGCGGCTCCATCCT
RPL13A	NM_012423.2	720-820	AGTCCAGGTGCCACAGGCAGCCCTGGGACATAGGAAGCTGGGAGCAAGGAAAGGGTCTTAGTCACTGCCTCCCGAAGTTGCTTGAAAGCACTCGGAGAAT
HSP90AB1	NM_007355.2	1880-1980	AGCCAATATGGAGCGGATCATGAAAGCCCAGGCACTTCGGGACAACTCCACCATGGGCTATATGATGGCCAAAAAGCACCTGGAGATCAACCCTGACCAC

Table 3.S2. Alternative normalization strategy for mRNA levels in our patient population confirms the upregulation of LTC4S as a distinguishing parameter for EoE. Differential gene expression was verified using an alternative normalization strategy in which direct mRNA counts were divided by direct mRNA counts of GAPDH in that sample.

Gene		**nCounter® Normalization**		**Alternative Normalization**	
		Fold Upregulation	P-value	Fold Upregulation	P-value
LTC4S	Prox	2.6	<0.0001	2.1	<0.0001
	Dist	2.9	<0.0001	2.5	<0.0001
Eotaxin-3	Prox	21	<0.0001	22	<0.0001
	Dist	13	<0.0001	12	<0.0001
Periostin	Prox	41	<0.0001	26	<0.0001
	Dist	27	<0.0001	23	<0.0001
Carboxypeptidase A3	Prox	7	<0.0001	5	<0.0001
	Dist	7	<0.0001	7	<0.0001
IL-13	Prox	6	<0.0001	4	<0.0001
	Dist	6	<0.0001	4	<0.0001
GAPDH	*Prox*	*1.0*	*0.64*		
	Dist	*1.0*	*0.96*		
HPRT1	*Prox*			*0.9*	*0.63*
	Dist			*0.9*	*0.16*

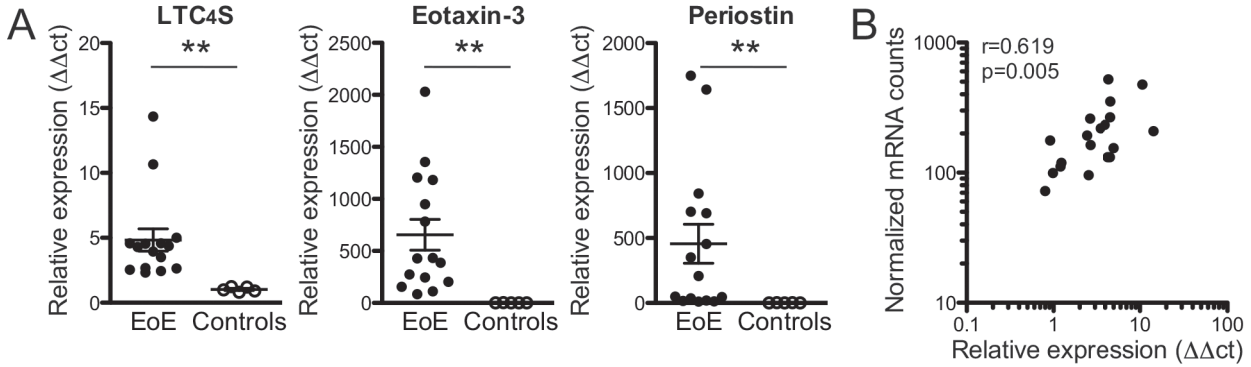

Figure 3.S1. Quantitative RT-PCR results confirm higher expression of LTC_4S in esophageal tissue of EoE patients. (**A**) Digital mRNA profiling for LTC_4S, eotaxin-3 and periostin was validated by RT-qPCR in a subset of EoE patients (N=15) and controls with normal tissue biopsy (N=5). (**B**) LTC_4S expression as determined by RT-qPCR correlates with normalized mRNA counts obtained from digital mRNA profiling. **p<0.01

Chapter 4

Involvement of the iNKT cell pathway is associated with early–onset eosinophilic esophagitis and response to allergen avoidance therapy

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4.1 Abstract

Objectives: Recent experimental evidence suggests that environmental microbial factors early in life determine susceptibility to allergic diseases through inappropriate chemotaxis and local activation of CD1d-restricted, invariant chain natural killer T (iNKT) cells. In this study, we analyzed the involvement of these pathways in pediatric patients with eosinophilic esophagitis (EoE) before and after dietary allergen elimination.

Methods: mRNA expression levels of components of the CXCL16-iNKT-CD1d axis were compared in esophageal biopsies from EoE patients vs. normal or inflammatory controls, and before and after treatment.

Results: CXCL16, iNKT cell associated cell marker Vα24 and CD1d were significantly upregulated in esophageal biopsies from EoE patients and correlated with expression of inflammatory mediators associated with allergy. Upregulation of each of these factors was significantly more pronounced in patients <6 years of age at diagnosis and this early-onset EoE subpopulation was characterized by a more prominent food allergic disease phenotype in a cohort-wide analysis. Successful, but not unsuccessful, treatment of early-onset EoE patients with dietary elimination of instigating allergens led to reduction in infiltrating iNKT cells and complete normalization of mRNA expression levels of CXCL16 and CD1d.

Conclusion: Our observations place iNKT cells at the center of allergic inflammation associated with EoE, which could have profound implications for our understanding, treatment and prevention of this and other human allergic diseases.

4.2 Introduction

A wide variety of allergic and immune-mediated inflammatory disorders are rapidly and globally increasing (Eder et al., 2006; Molodecky et al., 2012), suggesting that environmental factors are critical mediators of these changes (Garn et al., 2013), Moreover, the increasing incidence of these diseases is especially apparent among children (Branum and Lukacs, 2009; Cherian et al., 2006; Noel et al., 2004), which implicates a role for the environment in the processes of education and development of the immune system that follow the acquisition of a commensal microbiota along mucosal surfaces early in life (Blumberg and Powrie, 2012; Ege et al., 2011).

Recently, direct evidence has emerged from animal models of asthma and inflammatory bowel disease (IBD) that microbially-derived signals during a critical neonatal time frame are important regulators of later-life susceptibility to immune-mediated diseases (Olszak et al., 2012). Furthermore, environmentally-induced disruptions of these signals, such as those that result from the use of antibiotics, conferred increased susceptibility to experimental asthma and IBD (Olszak et al., 2012; Russell et al., 2012). These rodent models have focused particular attention on the role played by microbial-induced expression of chemoattractant chemokines that promote infiltration of mucosal tissues with invariant natural killer T (iNKT) cells. iNKT cells respond to host and microbial lipid antigens when presented by CD1d and rapidly express a variety of mediators that regulate downstream immune events and effector cells (Brennan et al., 2013; Kronenberg, 2005). In the absence of microbiota during neonatal, but not adult life, as in germ-free or antibiotic-treated mice, such tissues express increased quantities of CXCL16, a chemokine involved in iNKT cell trafficking (Germanov et al., 2008; Olszak et al., 2012). When these early-life microbial signals are not provided, an excessive and persistent accumulation of iNKT cells occurs in the colon and lungs. Consequently, these mucosal tissues are rendered more susceptible to later-life environmental triggers of iNKT cells, which are potent and rapid producers of Th2-type cytokines such as IL-4, IL-5 and IL-13, which mediate allergic sensitization and tissue inflammation (Brennan et al., 2013; Olszak et al., 2012; Scanlon et al., 2011). These model studies suggest that microbially-regulated immune events during early-life, or lack thereof as a consequence of, for example, antibiotic administration, are critical determinants of later-life susceptibility to allergic disease. In addition, given that components of CD1d-restricted T cell pathways and their regulating factors such as CXCL16 serve as mediators of this susceptibility, it is reasonable to expect that they have a potential role as markers for disease risk in humans.

To test the relevance of the hypothesis that early-life events determine the tone of CD1d-restricted T cell pathways and susceptibility to immune-mediated diseases in humans, we turned our attention to pediatric patients with eosinophilic esophagitis (EoE). EoE is characterized by chronic eosinophilic inflammation of the esophagus and is strongly associated with allergies to inhaled and food-derived antigens (Mulder and Justinich, 2011;

Rothenberg, 2009). Like other allergic diseases, the disorder often manifests during childhood (Assa'ad et al., 2007) and is undergoing a rapid increase in incidence and prevalence worldwide (Hruz et al., 2011; Noel et al., 2004; Prasad et al., 2009), suggesting a pathogenic role of yet-to-be defined environmental factors (Mulder and Justinich, 2011).

In addition to their previously established role in asthma and IBD (Fuss et al., 2004; Umetsu and DeKruyff, 2010), iNKT cells have recently been implicated in the pathogenesis of experimental EoE (Rajavelu et al., 2012). To study the involvement of this pathway in EoE, we here analyzed mRNA expression levels of CXCL16, as well as iNKT cells and their markers in serum and esophageal biopsies that were obtained before and after treatment with an allergen-elimination diet.

4.3 Materials and Methods

4.3.1 Study population

The patients included in this study are part of an observational longitudinal cohort study at Boston Children's Hospital that aims at understanding the pathophysiology of EoE and is approved by the institutional Investigational Review Board (Harvard Medical School, Boston, MA) (Dehlink et al., 2011; Lexmond et al., 2013; Yen et al., 2010). Parents of children 0-18 years of age whose clinical presentation indicated possible EoE (e.g. feeding intolerance, food aversion, dysphagia, failure-to-thrive) were invited to participate. Prior to routine diagnostic endoscopy, caregivers were asked to fill out a questionnaire regarding the child's past medical history, current and past symptomatology, allergic comorbidity, and dietary habits. We only considered children to be food allergic when foods were avoided based on the results of RAST, skin prick, or patch testing. Upon written informed consent, serum and additional esophageal biopsies were obtained. EoE was diagnosed according to current consensus guidelines (Liacouras et al., 2011). After diagnosis, patients were treated independently of this study, but were again asked to participate when admitted for follow-up endoscopy. Since 2008, 451 children have been included in the cohort, 160 of whom received a diagnosis of EoE. The 291 non-EoE patients had either normal esophageal tissue biopsies (N=225) or inflammation that was classified by the hospital pathologist as consistent with RE (N=66). Within this patient cohort, we focused on 31 biopsies available from patients <6 years of age (18 EoE, 5 RE and 8 normal histology) as well as 16 biopsies from patients ≥6 years old (10 EoE, 6 normal histology). Twelve follow-up biopsies from EoE patients <6 years were available for analysis.

4.3.2 Sample preparation and assay

Study biopsies from the distal esophagus (approximately 2 cm from the gastro-esophageal junction) were collected in RNA*later* (Qiagen, Valencia, CA) and stored at -80°C prior to

processing. Tissue was homogenized in 350 µl RLT Buffer (Qiagen) with β-mercaptoethanol (Sigma, St. Louis, MO) in GentleMACS M tubes (Miltenyi Biotec, Auburn, CA) and RNA was extracted using RNeasy plus kit (Qiagen). RT-qPCR was performed using Taqman gene expression assays (Applied Biosystems, Foster City, CA) for CXCL16, CD1d, CD1a, CXCR6, TRAV10 (Vα24), CD3ε, leukotriene C_4 synthase (LTC_4S), eotaxin-3 and periostin in duplex RT-qPCR reactions with the housekeeping gene GAPDH (C1000 Thermal Cycler, Bio-rad, Herculus, CA). Gene expression relative to GAPDH (ΔΔct) was calculated. Pooled cDNA from 5 control patients was used as an inter-run calibrator. CXCL16 protein levels were measured in serum using a commercially available ELISA according to the manufacturer's instructions (R&D Systems, Minneapolis, MN).

4.3.3 Statistical analysis

Distribution of serum and mRNA expression values was assessed with D'Agostino and Pearson omnibus normality test and patient groups were accordingly compared with either Student's t test for normally distributed or Mann-Whitney U test for non-parametric values unless otherwise indicated. Correlations were expressed as Pearson's or Spearman's rho. Epidemiological data retrieved from questionnaires was analyzed with Fisher's exact test. For logistic regression analysis, potential predictors of EoE that were estimated to relate directly to the report of early-life antibiotic use were analyzed with univariate logistic regression, and subsequently included in a multivariate model, regardless of statistical significance. Analyses were performed with Stata 12.1 (StataCorp, College Station, TX) and Prism 5.0 (GraphPad Software, La Jolla, CA). Reported values represent mean±SEM. All statistical testing was two-sided, with an alpha of 0.05.

4.4 Results

4.4.1 The CXCL16–iNKT–CD1d axis is prominently involved in early–onset EoE patients.

To investigate the contribution of CXCL16 to local disease in EoE, we analyzed mRNA expression levels of CXCL16 in esophageal biopsies at time of diagnosis in 47 children. Patients were grouped according to age at diagnosis: (1) patients <6 years (18 EoE vs. 13 non-EoE, of whom 5 with histologic evidence of reflux esophagitis (RE)) and (2) 6-18 years (10 EoE vs. 6 non-EoE with normal tissue histology). CXCL16 was expressed in all esophageal biopsies analyzed (Cycle threshold range 26.6 – 32.7), but expression was significantly higher in EoE patients when compared to controls (3.2±0.3 vs. 1.2±0.2, $p<0.001$, **Figure 4.1A**). Furthermore, expression levels were higher in early-onset EoE patients, which we defined as children diagnosed before 6 years of age, compared to their older EoE counterparts (3.7±0.4 vs. 2.2±0.2, $p<0.05$). No age-dependent difference was seen in the non-EoE control patients (1.3±0.3 vs. 1.0±0.1, $p=0.52$, **Figure 4.1A**).

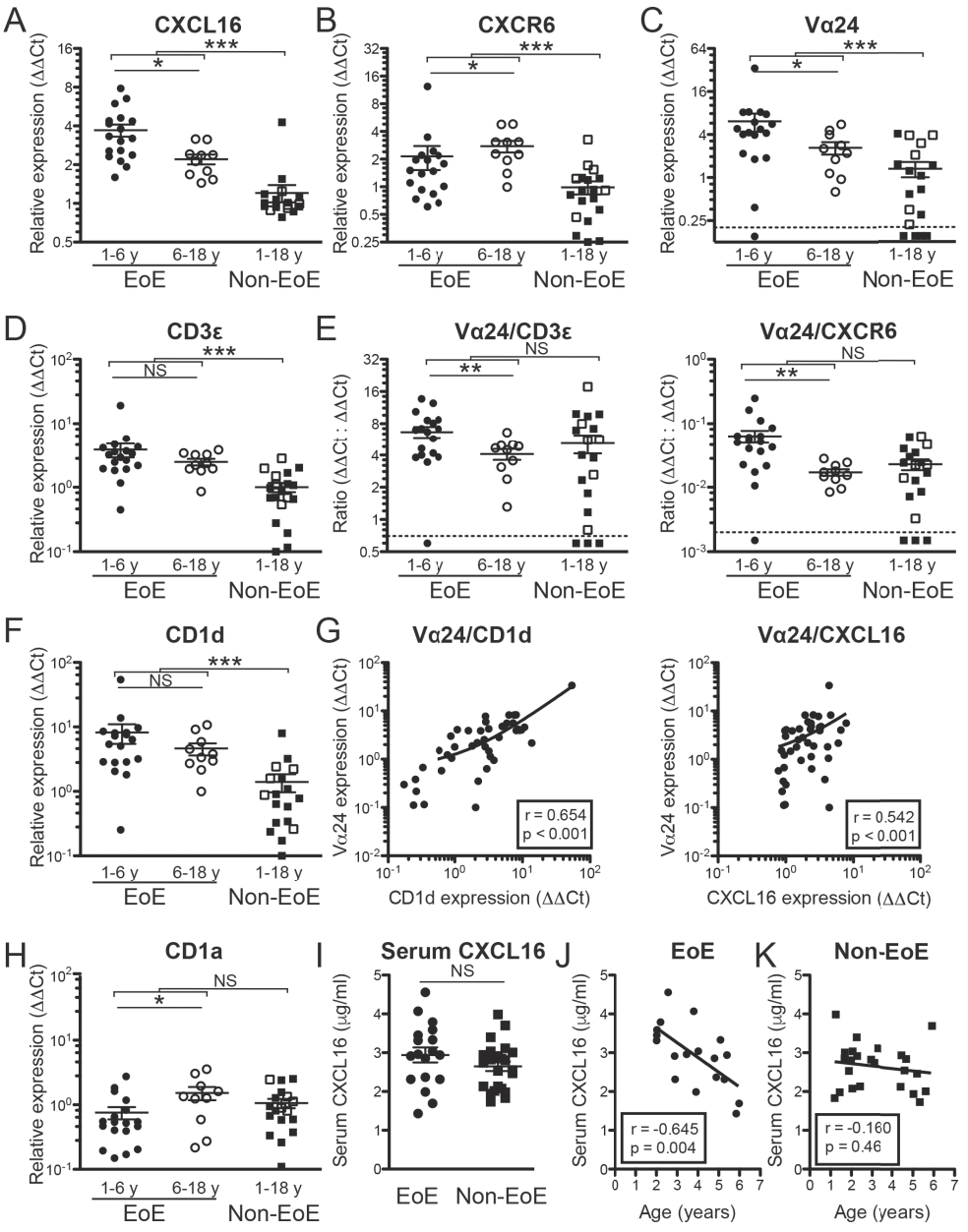

Figure 4.1. Components of the CXCL16-iNKT cell-CD1d pathway are most prominently expressed in early-onset EoE patients. Gene expression relative to housekeeping gene GAPDH (expressed as ΔΔCt values) is shown in early-onset EoE patients (<6 years of age, full circles), EoE patients 6-18 years (open circles), and non-EoE controls (patients <6 years shown as full squares and patients 6-18 years as open squares) (**A-F** and **H**). mRNA expression levels were elevated in EoE patients compared to non-EoE controls for CXCL16 (**A**), CXCR6 (**B**), Vα24 TCR (**C**), CD3ε (**D**), CD1d (**F**), but not for CD1a (**H**). Early-onset EoE patients express significantly higher levels of CXCL16 (**A**), CXCR6

(**B**), and Vα24 TCR (**C**) when compared to older EoE patients. Ratio of expression is plotted for Vα24/CD3ε and Vα24/CXCR6 (**E**). Vα24 TCR mRNA levels correlated significantly with CD1d and CXCL16 (**G**). Expression values under the detection limit or the corresponding expression ratios are plotted under the dotted line when transcripts were not detected. Serum CXCL16 levels of early-onset EoE patients were compared with age-matched non-EoE controls (**I**). Serum CXCL16 was correlated to age at diagnosis (**J** and **K**). *p<0.05, **p<0.01, ***p<0.001, NS: not significant.

We analyzed the same biopsies for mRNA expression of CXCR6, the canonical receptor for CXCL16 that is expressed on a variety of T cell subsets, including Th1-polarized $CD4^+$ and $CD8^+$ T cells(Kim et al., 2001), as well as iNKT cells (Kim et al., 2002). Although higher expression levels were seen in EoE patients compared to non-EoE controls (**Figure 4.1B**), the highest expression levels were observed in EoE patients 6-18 years of age. In light of the elevated CXCL16 levels observed in EoE patients <6 years old, these results are consistent with observations that CXCR6 in T cells is down-regulated upon activation (Koprak et al., 2003) and suggest that CXCL16 engagement is highly active in early-onset EoE patients.

To determine whether CXCL16 overexpression was indeed associated with infiltration of iNKT cells, we analyzed gene expression of Vα24, an essential component of the invariant T cell antigen receptor (TCR) of iNKT cells (Vijayanand et al., 2007) Vα24 TCR mRNA transcripts were detected in 17 out of 18 early-onset EoE patients, all 10 older EoE patients, and in 14 of 19 control patients. In line with CXCL16 expression results, the highest levels of Vα24 TCR mRNA were detected in EoE patients <6 years (**Figure 4.1C**). Increased Vα24 expression did not result from more pronounced overall T cell infiltration, because expression levels of the ε chain of the TCR (CD3ε) were equivalent in both young and older EoE patients (**Figure 4.1D**) and normalization of Vα24 expression against CD3ε or CXCR6 did not resolve the difference between old and young EoE patients (**Figure 4.1E**). These results suggest the existence of distinct differences in infiltrating T cell subsets between early-onset and older EoE patients.

iNKT cells are restricted by recognition of lipid-antigen presented through CD1d on antigen-presenting cells (Brennan et al., 2013). We therefore examined CD1d mRNA and observed increased expression in EoE patients compared to non-EoE control patients, indicating that at the site of inflammation, there is enhanced capacity to activate infiltrating iNKT cells (**Figure 4.1F**). Indeed, we depicted a positive correlation between tissue mRNA expression of CD1d and Vα24 in esophageal biopsies (Spearman rho=0.654, p<0.001, **Figure 4.1G**). Similarly, expression of CXCL16 and Vα24 correlated positively (Spearman rho=0.542, p<0.001, **Figure 4.1G**). Conversely, expression levels of CD1a, a related glycoprotein on antigen presenting cells, did not differ between EoE patients and controls (1.0±0.2 vs. 1.2±0.2, p=0.38), arguing that increased CD1d expression in EoE is part of the disease-specific immune phenotype rather than the result of overall increase in tissue myeloid or inflammatory cells (**Figure 4.1H**). Combined, these data show local involvement of the CXCL16-iNKT-CD1d axis in EoE patients and that this involvement is significantly more prominent in early-onset EoE patients.

4.4.2 Serum CXCL16 levels in early–onset EoE patients are consistent with an age–dependent role in EoE pathogenesis

CXCL16 is present as a membrane-bound protein on epithelial and myeloid cells, as well as a soluble form that can be retrieved from patient serum (Diegelmann et al., 2010). We hypothesized that early-onset EoE patients might also be characterized by higher levels of CXCL16 in serum when compared to non-EoE control patients, as was observed in tissues (**Figure 4.1A**). If so, this might serve as a convenient biomarker for disease. Serum levels were similar between patient groups (2.9±0.2 µg/ml for EoE (N=18) vs. 2.6±0.1 µg/ml for non-EoE (N=24), p=0.19, **Figure 4.1I**). Interestingly, there was a significant negative correlation between serum CXCL16 levels and age of diagnosis in EoE (r=-0.65, p=0.004, **Figure 4.1J**). Conversely, this relationship between age and serum CXCL16 levels was absent in the non-EoE patients (r=-0.16, p=0.46, **Figure 4.1J**) and EoE patients 6-18 years of age (N=8, r=0.31, p=0.45). These results further support an age-dependent role for CXCL16 in EoE pathophysiology, which is detectable in the serum.

4.4.3 Involvement of the CXCL16–iNKT–CD1d axis is associated with a more pronounced food allergic phenotype

Since CXCL16 overexpression and iNKT cell infiltration were significantly more pronounced in the younger EoE patients, we asked whether early-onset EoE differs from EoE that becomes apparent during later childhood. Within our entire cohort of 451 patients, 44 of 160 EoE patients (28%) were <6 years at time of diagnosis. In line with data from multiple patient populations (Hruz et al., 2011; Noel et al., 2004), we saw a strong male preponderance in EoE patients, as well as a higher occurrence of all investigated allergic diatheses compared to control patients (**Table 4.1**). Comparison between EoE patients <6 and ≥6 years of age, however, revealed striking differences in the distribution of allergic comorbidity. Whereas older patients were more likely to be asthmatic (50% vs. 20%, p=0.003), early-onset EoE patients had significantly more often been diagnosed with food allergy against milk (32% vs. 14%, p=0.02) or any specific food allergen (48% vs. 25%, p=0.008) through skin prick, patch, or RAST testing. These epidemiological data are suggestive of a particularly dominant role of food allergic sensitization in early-onset EoE.

To further test this hypothesis at the tissue level, we again turned to tissue biopsies. We have recently demonstrated that elevated mRNA levels of leukotriene C4 synthase (LTC_4S) mark a subpopulation of EoE patients with a more pronounced local allergic phenotype (Lexmond et al., 2013). No difference was found in tissue eosinophil counts between early and later-onset EoE patients (**Figure 4.2A**). However, in line with our epidemiological data, we found that early-onset EoE patients had significantly higher levels of LTC_4S mRNA expression (**Figure 4.2B**).

In addition, we analyzed tissue biopsies for EoE marker genes eotaxin-3, periostin, and IL-13, which were among the first genes to be identified as part of an EoE-specific esophageal transcriptome (Blanchard et al., 2008; 2006b; 2007). We observed that all were increased in patients with EoE relative to the non-EoE subjects (**Figure 4.2C-E**). Although no age-

Table 4.1. Symptomatology and allergic diatheses in early-onset vs. later onset EoE patients. *added later to the questionnaire, responses available for 383 patients (132 EoE). P-values are based upon two-sided testing with Fisher's exact test.

	EoE		Non-EoE		EoE vs. non-EoE	EoE <6 vs. ≥6 years
	<6 years	**≥6 years**	**<6 years**	**≥6 years**		
N	44	116	84	207		
Male Sex	32 (73%)	79 (68%)	45 (54%)	89 (43%)	***<0.001***	0.70
Age (5^{th} – 50^{th} – 95^{th} percentile)	0.18 – 4.0 – 5.8	6.7 – 12.7 – 17.6	0.1 – 2.3 – 5.7	6.1 – 12.9 – 17.2	0.89	
Preterm delivery (<36 weeks)	10 (23%)	33 (29%)	18 (21%)	55 (27%)	0.74	0.55
GI symptomatology						
Pain with swallowing	8 (19%)	31 (27%)	15 (18%)	42 (21%)	0.23	0.41
Food getting stuck	12 (28%)	53 (46%)	20 (24%)	67 (34%)	***0.02***	0.07
Dysphagia	10 (23%)	43 (38%)	19 (23%)	66 (33%)	0.39	0.09
Abdominal pain	14 (33%)	58 (51%)	36 (44%)	141 (69%)	***0.002***	***0.049***
Constipation	17 (40%)	29 (26%)	40 (48%)	82 (41%)	***0.008***	0.11
Diarrhea	16 (37%)	34 (30%)	38 (45%)	44 (22%)	0.45	0.44
Weight loss	11 (25%)	24 (21%)	20 (24%)	36 (18%)	0.54	0.67
Regurgitation	12 (29%)	34 (30%)	29 (35%)	109 (55%)	***<0.001***	1.0
Allergic diatheses						
Milk allergy (skin or RAST tested)	14 (32%)	16 (14%)	15 (18%)	2 (1%)	***<0.001***	***0.02***
Any food allergy (skin or RAST tested)	21 (48%)	29 (25%)	17 (20%)	5 (2%)	***<0.001***	***0.008***
Wheezing*	14 (39%)	49 (50%)	26 (36%)	65 (35%)	***0.03***	0.33
Asthma*	7 (20%)	49 (50%)	7 (10%)	53 (29%)	***<0.001***	***0.003***
Hay fever*	15 (44%)	40 (45%)	6 (9%)	34 (20%)	***<0.001***	1.0
Eczema*	20 (56%)	46 (48%)	33 (46%)	45 (25%)	***<0.001***	0.56

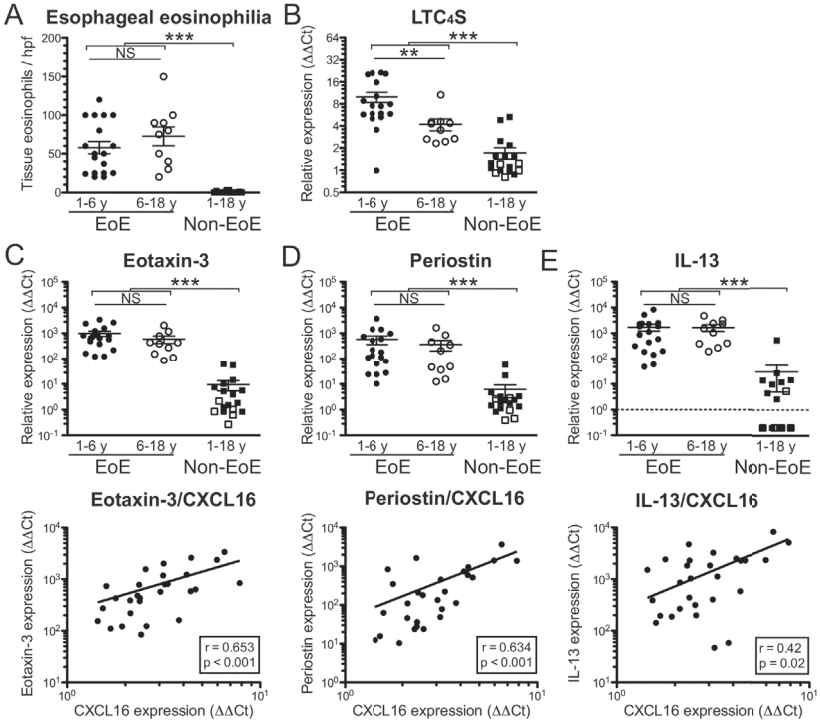

Figure 4.2. CXCL16 correlates with allergic mediators in early-onset EoE. Early-onset EoE patients do not show more severe tissue eosinophilia (counts/hpf, **A**) or increased expression of EoE-specific marker genes eotaxin-3 (**C**), periostin (**D**) or IL-13 (**E**) but show a more pronounced allergic tissue phenotype as signified by increased expression of leukotriene C4 synthase (LTC_4S, **B**). In EoE patients, CXCL16 expression correlates with IL-13 and disease-specific, IL-13-dependent tissue markers eotaxin-3 (**C**) and periostin (**D**). **p<0.01, ***p<0.001, NS: not significant.

dependent differences in expression were found, we observed a strongly positive correlation between tissue mRNA expression levels of CXCL16 and these marker genes (**Figure 4.2C-E**). Eotaxin-3 has been identified as one of the key eosinophil attractant cytokines in EoE (Blanchard et al., 2006b) and periostin is described to facilitate eosinophil tissue infiltration (Blanchard et al., 2008). Expression of both effector molecules has been described to occur downstream of IL-13 signaling in EoE (Blanchard et al., 2008; 2007). Although IL-13 is produced by iNKT cells (Brennan et al., 2013), we did not observe a significant correlation between expression of IL-13 and Vα24 (r=0.13, p=0.50), indicating that iNKT cells are unlikely the dominant cellular source of IL-13. These data suggest a link between the local expression of CXCL16 and effector pathways of EoE at the site of tissue inflammation.

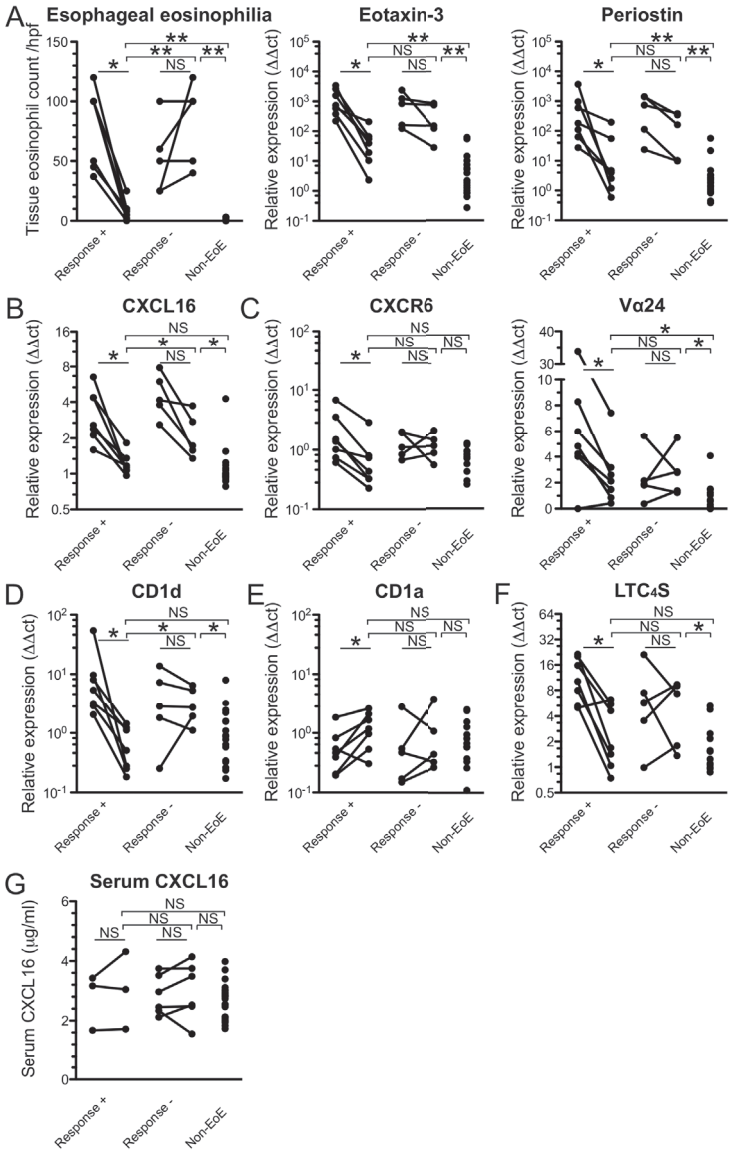

Figure 4.3. The CXCL16-iNKT cell-CD1d pathway responds to treatment with dietary elimination of allergens in early-onset EoE. Tissue eosinophil counts decreased upon successful treatment (Response+, N=7) but remained unaltered or increased in non-responders (Response-, N=5, **A**). CXCL16 mRNA expression levels decreased in all patients upon treatment, but remained significantly elevated in non-responders over responders and controls (**B**). Comparable results were obtained for expression levels of CXCR6 and Vα24 (**C**) and CD1d (**D**). No decrease in CD1a expression was seen upon successful treatment (**E**)**.** Elimination of dietary allergens led to normalization of tissue LTC_4S mRNA levels in responding but not in non-responding patients (**F**). Allergen avoidance does not affect serum CXCL16 levels in EoE patients (**G**). Asterisks represent the result of statistical testing with Wilcoxon signed rank test for before-after expression levels and Mann-Whitney U test for post-treatment expression levels as follows: *p<0.05, **p<0.01, ***p<0.001, NS: not significant.

4.4.4 Treatment with elimination diet leads to normalization of the CXCL16–iNKT–CD1d axis in early–onset EoE patients

To analyze the response of the CXCL16-iNKT-CD1d axis to EoE treatment, we repeated mRNA measurements in biopsies that were obtained during follow-up endoscopies performed to monitor disease progression and therapeutic response. Follow-up biopsies were available from 12 of 18 early-onset EoE patients after a median follow-up time of 10.1 months (range 1.9 – 24.8 months). Based on the degree of tissue eosinophilia obtained from routine histopathological analysis, 7 out of 12 EoE patients had significantly improved with treatment, with median eosinophil counts of 8/high power field (hpf) (range 0–25) compared to the initial biopsies that showed a median of 100 eosinophils/hpf (range 37-120, p=0.02 by Wilcoxon signed rank test, **Figure 4.3A**). Conversely, in the 5 non-responding patients, tissue eosinophilia at follow-up remained high, ranging from 40-120 eosinophils/hpf (median 100/hpf, p=0.25). In line with tissue eosinophil counts and previously published data (Blanchard et al., 2007), mRNA expression levels of eotaxin-3 and periostin at follow-up had decreased significantly in responding patients, whereas this reduction was smaller and not significant in the non-responders (**Figure 4.3A**). Patient and treatment details are summarized in **Table 4.2**.

We next turned our attention to the CXCL16-iNKT cell-CD1d axis in treated patients. We observed that, in the 7 responders, CXCL16 mRNA expression completely normalized to the expression levels encountered in age-matched control patients (1.2±0.1 vs. 1.3±0.3, p=0.88, **Figure 4.3B**). In contrast, CXCL16 expression remained significantly elevated in the 5 non-responders (2.3±0.4) when compared to control patients (p=0.01) as well as responding EoE patients (p=0.03). CXCR6 and Vα24 TCR mRNA levels also decreased significantly in the 7 responding patients (2.7-fold, p=0.02 and 3.3-fold, p=0.03 respectively) but not in the non-responders (p=1.0 and p=0.63, **Figure 4.3C**). Likewise, response to treatment was associated with a 17-fold reduction in CD1d expression (p=0.01) compared to a 1.5-fold reduction in non-responders (p=0.31, **Figure 4.3D**). No decrease, but rather an increase in CD1a expression levels was observed upon successful treatment (2.0-fold increase, p=0.03), in this case showing that reduction of CD1d mRNA levels did not result from decreased myeloid cell infiltration (**Figure 4.3E**). Lastly, we found that LTC_4S mRNA levels only decreased following successful treatment (4.0-fold, p=0.03 compared to 1.3-fold, p=0.81 in non-responders, **Figure 4.3F**). Allergen avoidance therapy did not affect the levels of serum CXCL16 (**Figure 4.3G**), confirming that upregulation of CXCL16 represents a local mucosal event that is not broadcasted systemically. Together, these data show that involvement of the CXCL16-iNKT cell-CD1d axis can be modified by dietary interventions that aim to eliminate common allergens from the diet.

4.4.5 EoE is potentially associated with use of antibiotics during the first year of life

Animal model experiments suggest that CXCL16 overexpression by epithelial cells can result from inadequate instruction from the commensal microbiota at an early age (Olszak et al., 2012). We therefore hypothesized that antibiotic treatment during the first year of

Table 4.2. Patient characteristics and treatment details of 12 early-onset EoE patients. PPI: proton pump inhibitor.

Patient	Sex / Age	Dietary elimination	Pharmacological treatment:	Time to follow-up (months)	Tissue eosinophil response	Reported symptomatology at follow-up	Comments
Responders							
	M, 3.5	Milk, soy, eggs nuts, peas, beans, beef, pork	Swallowed budesonide	17.5	25 → 5	Improved appetite and symptoms	
	M, 2.9	Milk	PPI	5.9	37 → 0	Improvement, normal growth velocity	Negative environmental allergen screen
	M, 2.0	Milk, soy, oat, egg, sweet potatoes, chicken, carrots, grapes, strawberries, pears, string beans	None	11.9	100 → 2	No upper GI symptoms	Soy was reintroduced prior to follow up endoscopy
	F, 4.9	Corn, rye, pork, egg, wheat, dairy, nuts, soy, shellfish, beef	PPI	10.6	120 → 10	Asymptomatic	
	M, 2.2	Elemental diet	PPI	9.6	100 → 25	Improved but not resolved on elemental diet	
	M, 4.0	No restrictions	Swallowed budesonide no PPI	5.7	100 → 8	Asymptomatic	
	M, 2.9	Milk, eggs, wheat, turkey, nuts	PPI	1.9	45 → 10	Clear improvement	Inconsistent use of PPI
Non-responders							
	F, 5.8	Milk, soy	PPI	24.8	25 → 125	Persistent episodes of food getting stuck	Admitted poor compliance with diet
	F, 2.0	Milk, soy, beef, oranges, eggs, wheat	None	7.4	50 → 50	Initially weight gain but recent flare of symptoms	Complete clinical resolution after elimination of corn
	M, 5.3	Eggs, wheat, chicken, turkey, pear, apples, peas, corn, milk, onions, peanuts, tree nuts	PPI	15.5	25 → 40	Less dysphagia and reflux, though symptoms still occur	RAST positive to >50 foods, only 12 of which were excluded
	M, 5.2	No restrictions	PPI	5.3	100 → 100	Persistent symptoms	RAST positive for milk, though no specific dietary interventions
	M, 5.1	None	PPI, 1 course of swallowed budesonide	15.3	60 → 100	Relatively asymptomatic	

life may be associated with the development of EoE, as has been previously suggested for pediatric IBD (Shaw et al., 2010) and asthma (Murk et al., 2011) and, very recently, also for EoE (Jensen et al., 2013). Unfortunately, our questionnaires at time of study enrollment did not directly address the extent of early-life antibiotic exposure or mode of delivery, but instead inquired into medication use during the first year of life, in particular the use of antacids, corticosteroids, and prokinetics. Data on the prescription of antibiotics was obtained through an open-ended question asking parents to list additional medications during the first year of life. Questionnaires of 451 patients (160 EoE) were analyzed. Antibiotics in the first year of life were reported in 29 patients, of whom 20 were subsequently diagnosed with EoE ($p<0.001$). Although it is very likely that underreporting affected the total number of cases, the difference between EoE patients and controls is unlikely the result of recall bias. This is because report of non-antibiotic drug-use during the first year of life did not differ between cohorts (33% of EoE patients vs. 41% of non-EoE controls, $p=0.09$) and questionnaires were filled out prior to diagnosis. Furthermore, a positive association (odds ratio 4.10, 95% confidence interval 1.36 – 12.35) between EoE and reported antibiotics use during the first year of life remained after adjustment for sex, age, prematurity, hospitalization in the first year of life, allergic comorbidities and non-antibiotic drug use in the first year of life (**Table 4.3**). Although statistical significance was reached for this association in our cohort, additional epidemiological studies designed to specifically address this question are needed to confirm a causative link between early-life antibiotic exposure and occurrence of EoE later in life.

4.5 Discussion

In the current study, we provide evidence that supports the involvement of CD1d-restricted iNKT cell pathways in the pathogenesis of pediatric EoE. There are several key elements associated with the findings contained here. First, our results provide a direct human corollary of previous observations in animal models that commensal microbes, in an age-dependent fashion, regulate iNKT cell accumulation in the colon and lung by altering expression of chemokine ligands that promote iNKT cell migration, such as CXCL16. Similar to this previous report, we found that all of the factors associated with iNKT cells such as the associated TCR chain (Vα24), the antigen presenting molecule that regulates their activity (CD1d), and the chemokine ligand that determines their chemotaxis into tissues (CXCL16), are observed at significantly higher levels in the esophageal tissue of EoE patients compared to normal or inflammatory controls. Moreover, the highest levels were observed in children with early-onset EoE (<6 years) and the elements of the CXCL16-iNKT cell-CD1d pathway correlated with the presence of inflammatory mediators associated with EoE-specific inflammation (eotaxin, periostin), pointing towards a direct role in disease pathogenesis. Indeed, successful dietary therapy was associated with resolution of disease, together with a synchronous and

Table 4.3. Predictors in a cohort-wide logistic regression analysis for a diagnosis of EoE versus non-EoE prior to diagnostic endoscopy.

	Univariate analysis		Multivariate analysis (N=345)	
Predictor	**Odds ratio (95% CI)**	**p-value**	**Odds ratio (95% CI)**	**p-value**
Male sex	**2.65 (1.77 – 3.99)**	**<0.001**	**2.47 (1.42-4.28)**	**0.001**
Age (per year increase)	1.00 (0.97 – 1.04)	0.89	1.00 (0.94-1.06)	0.93
Preterm delivery (<36 weeks)	1.08 (0.69 – 1.67)	0.73	1.23 (0.67-2.34)	0.51
Hospitalization in the first year of life	0.70 (0.45 – 1.10)	0.13	0.59 (0.31 – 1.11)	0.10
Reported asthma	**2.35 (1.50 – 3.69)**	**<0.001**	1.45 (0.80-2.63)	0.22
Reported eczema	**2.22 (1.44 – 3.42)**	**<0.001**	1.60 (0.91-2.79)	0.10
Reported seasonal allergy	**4.08 (2.50 – 6.68)**	**<0.001**	**2.90 (1.63-5.18)**	**<0.001**
Any food allergy	**5.56 (3.21 – 9.62)**	**<0.001**	**4.58 (2.19 – 9.57)**	**<0.001**
>1 annual ear infection requiring antibiotics	1.41 (0.95 – 2.09)	0.09	1.04 (0.61-1.80)	0.87
Reported antibiotic allergy	1.54 (0.96 – 2.48)	0.08	1.77 (0.91-3.48)	0.09
Non-antibiotic drugs in the first year of life	0.70 (0.46 – 1.04)	0.08	**0.48 (0.27-0.87)**	**0.02**
Reported antibiotics in the first year of life	**4.48 (1.98 – 10.1)**	**<0.001**	**4.10 (1.36 – 12.35)**	**0.01**

proportionate decrease in the levels of CXCL16, CD1d and Vα24, suggestive of a cause-and-effect relationship. Given the observations in animal models, these findings in humans with EoE support the hypothesis that early-life events that affect CD1d-restricted T cell pathways at mucosal surfaces play an important role in the pathogenesis of EoE. Indeed, we detected an association between antibiotic use in the first year of life and the development of EoE, drawing additional and provocative similarities to the aforementioned animal studies. Together, these studies support the concept that a pathway involving environmentally induced changes in early life, in this case caused by antibiotic administration, cause perturbations in mucosal iNKT cell homeostasis in esophageal tissues and determines susceptibility to EoE.

Prior translational studies on the role of iNKT cells in human allergy have predominantly been performed in asthmatic patients, after it was shown that iNKT cells are required for the induction of experimental airway hyperreactivity in mice (Akbari et al., 2003; Brennan et al., 2013; Lisbonne et al., 2003). Since efforts to demonstrate increased levels of iNKT cells in bronchoalveolar lavage fluid (BALF) of asthmatic patients have not yielded clear differences with control populations (Mutalithas et al., 2007; Thomas et al., 2006; Vijayanand et al., 2007), such a role has remained a matter of debate (Thomas et al., 2010; Umetsu and DeKruyff, 2010). These analyses of BALF have however been performed in adult patients with a longstanding history of asthma and therefore do not rule out a potential role for iNKT cells in the early phase of disease pathogenesis. This concept is further supported by recent experimental work that suggests that rather than being the main effector cell in advanced asthma, iNKT cells are critically involved in the allergic sensitization phase of disease. Specifically, Scanlon *et al.* showed that intratracheal exposure to bacterial lipid antigens resulted in rapid peribronchiolar infiltration of iNKT cells and formation of eosinophil-rich granulomas. Upon intratracheal co-administration of ovalbumin, a Th2 immune response was mounted against ovalbumin, including synthesis of anti-ovalbumin IgE. Allergic sensitization persisted until months after the initial exposure and, once established, was independent of iNKT cells (Scanlon et al., 2011). These observations thus point towards iNKT cells as a crucial instigating cell-type in allergic forms of inflammation and demonstrate that once allergic sensitization has occurred, cytokine production by iNKT cells is dispensable for disease pathogenesis. The latter finding likely explains the absence of a correlation between IL-13 and Vα24 expression in our cohort.

By studying pediatric EoE patients at the time of diagnosis, we were able to obtain affected mucosal tissues to directly correlate the involvement of iNKT cells and their mediators with disease phenotype. Like asthma, EoE is a chronic, immune/antigen-driven condition characterized by aberrant Th2 immune responses and allergic sensitization (Liacouras et al., 2011). Both food-derived and inhaled allergens have been implicated in its pathogenesis (Liacouras et al., 2011; Spergel, 2005; Spergel et al., 2012), which is reflected in high response rates upon initiation of an elemental or 6-food elimination diet (Gonsalves et al., 2012; Kagalwalla et al., 2006). As a corollary, increased esophageal eosinophilia is observed during the pollen season in pollen-sensitized patients (Fogg et al., 2003; Onbasi

et al., 2005). A likely role for iNKT cells in the process of allergic sensitization was suggested from studies that showed that food-allergen dependent, experimental murine EoE, could not be induced in CD1d-deficient mice (Rajavelu et al., 2012). Consistent with this, we have shown that individual components of the CXCL16-CD1d-iNKT axis are overexpressed in our entire pediatric EoE population, but increased expression was significantly more prominent in patients who developed the disease at a very young age. Epidemiological data from our cohort study further indicate that in patients diagnosed when <6 years of age, EoE is characterized by a higher fraction of food-allergic sensitization. In addition, local mRNA levels of LTC_4S, the enzyme that generates the allergic mediator leukotriene C4, were significantly higher in children with early-onset EoE than in their older counterparts. As such, these data suggest that this early-onset disease phenotype is likely to depend more heavily on local sensitization to food-derived allergens. Our results are therefore supportive of an association between local accumulation of iNKT cells and allergic sensitization to food antigens in human EoE. Additional support for our concept was provided recently by Jyonouchi et al., who reported increased numbers of iNKT cells in esophageal biopsies of pediatric patients with active disease. Upon stimulation with milk-derived sphingolipids, iNKT cells from these EoE patients readily responded with increased IL-13 and IL-4, but not IFN-γ production, showing that iNKT cell-mediated responses to food-derived antigens can be responsible for Th2 skewing of immune responses (Jyonouchi et al., 2014).

Despite recent advances in our understanding of iNKT cell biology, the exact molecular mechanism that couples iNKT cell infiltration to allergic sensitization in human EoE patients remains to be further identified. iNKT cells are CD1d-restricted and can be activated in response to exogenous lipid antigens, including pollen and dust mite associated lipids (Agea et al., 2005; Wingender et al., 2011), as well as dietary antigens such as sphingomyelin derived from milk (Jyonouchi et al., 2011) or the allergen *Ber e1* from Brazil nuts in the presence of a naturally-occurring lipid fraction (Mirotti et al., 2013). The study by Olszak *et al.* has furthermore implicated endogenous (self) antigens as a source of iNKT stimulation in a pathogenic environmental context and showed that microbes inhibit this auto-reactivity, at least in part, through dampening CXCL16 expression (Olszak et al., 2012). It is therefore of interest that we identified a relationship between early-life antibiotic use and EoE, suggesting that microbial reduction during early life may also confer risk for the development of EoE as previously demonstrated for asthma (Kummeling et al., 2007; Murk et al., 2011) and IBD (Shaw et al., 2010) in humans. Such antibiotic use may diminish the quantity and/or activity of microorganisms capable of regulating iNKT cells. Interestingly in this regard is evidence that *Bacteroides fragilis* encodes a biosynthetic pathway for the generation of glycosphingolipids capable of inhibiting colonic iNKT cells, suggesting that antibiotics which diminish this or similar types of organisms may predispose to iNKT cell-mediated diseases (55). Our longitudinal cohort study was not designed to specifically address the potential association between antibiotics use and onset of EoE, which has resulted in potential underreporting of early-life antibiotic

use and absence of data on the quantity or types of antibiotics prescribed. Although our data suggest that the association between early-life antibiotic exposure and development of EoE was not due to recall bias, we caution that additional epidemiological studies are needed to prove this important hypothesis. We propose that EoE is a suitable model disease for such studies, since an association with antibiotic exposure is less susceptible to reverse causation, which has hampered inference from pediatric asthmatic cohorts (Kummeling and Thijs, 2008). Indeed, gastrointestinal symptoms are less likely to be treated with antibiotics than upper or lower airway infections, and symptomatology was not more pronounced in EoE patients compared to controls in our cohort. Supportive of our data is a recently published paper that has described an association of the same order of magnitude between EoE and antibiotic use during infancy as found in our study (Jensen et al., 2013). Large European studies have further pointed out that exposure to a wide variety of environmental micro-organisms confers protection against both atopy and asthma (Ege et al., 2011), making it reasonable to surmise that similar mechanisms are operative in EoE.

In summary, we have assessed how recent findings from experimental studies in mouse models translate to human allergic disease in the context of EoE. Based on our results, we propose the following model of EoE pathophysiology: 1. Insufficient immune imprinting by environmental microorganisms results in esophageal upregulation of CXCL16 and chemotaxis of iNKT cells into the esophagus, making the esophagus susceptible to triggers of iNKT cell activation; 2. Lipid antigens derived from food, aeroallergens, microorganisms, and/or potentially self-antigens associated with the esophagus are inappropriately presented to iNKT cells by CD1d in a manner that stimulates their pathologic activation; 3. Activated iNKT cells serve as an early source of IL-4, IL-5, and IL-13, facilitating Th2-mediated allergic sensitization and responses that promote and sustain inflammation. Our observations further suggest that in an esophageal iNKT cell-dominant disease phenotype, as occurs early in life, allergic inflammation is driven primarily by food antigens. Conversely, in EoE that manifests at a later age, esophageal inflammation is potentially less dependent on iNKT cells based upon our findings. This model of disease pathophysiology suggests that pharmacological interference with CXCL16 and CD1d, especially at a young age and at onset of disease, is a potent target in the prevention and treatment of allergic diseases such as EoE and may further serve as diagnostic markers in determining disease etiology and therapeutic response.

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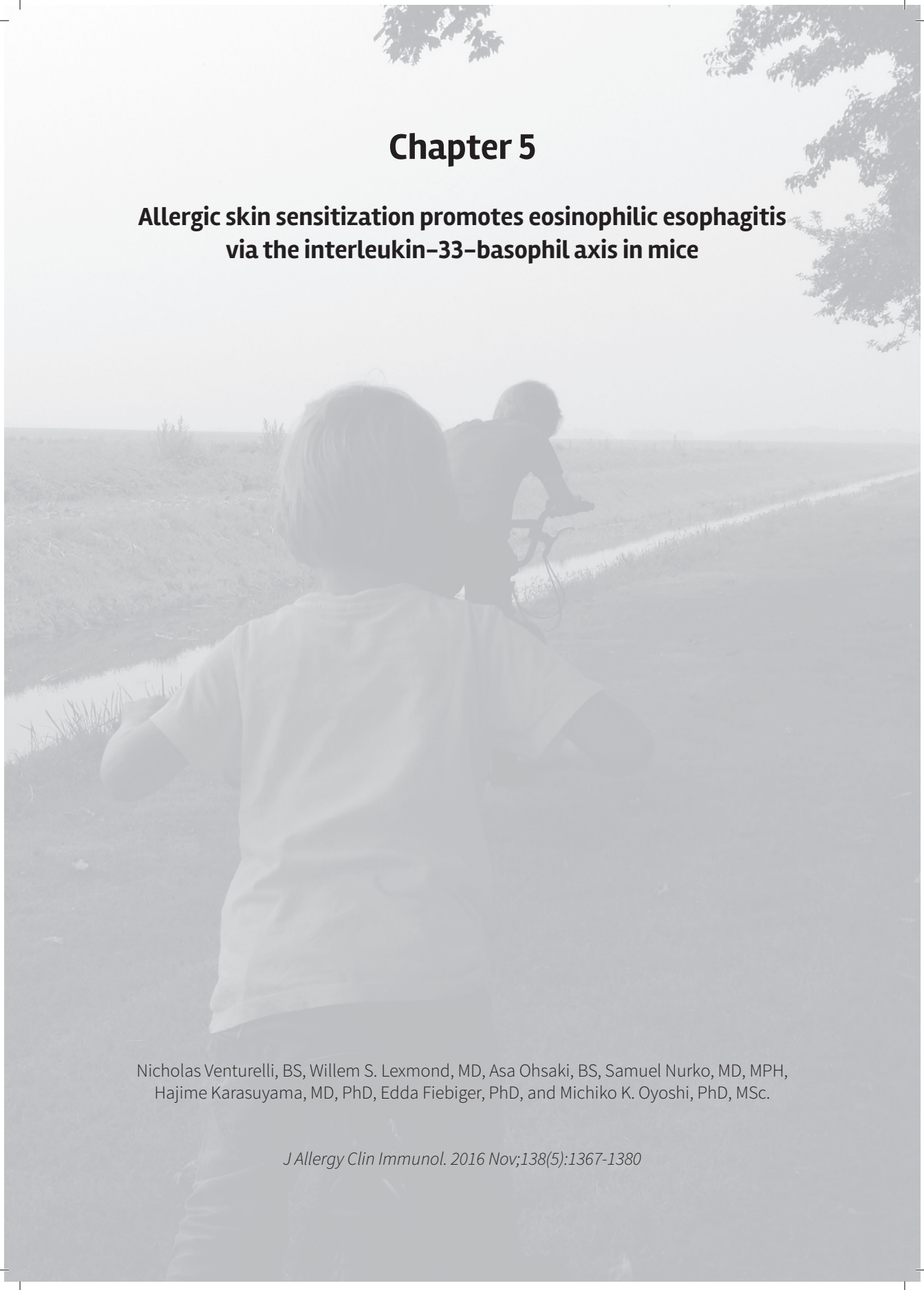
4.7 Author contributions

Project conception and design, data acquisition/analysis/interpretation, drafting of the article, critical revision: Willem S. Lexmond; data acquisition, critical revision: Joana F Neves; Patient identification, inclusion and prospective cohort study design: Samuel Nurko; data interpretation, critical revision: Torsten Olszak and Mark Exley; Project conception and design, data interpretation, drafting of the article, critical revision: Richard S. Blumberg; Project conception and design, data interpretation, prospective cohort study design, critical revision: Edda Fiebiger. All authors approved the final submitted version of the manuscript.

Chapter 5

Allergic skin sensitization promotes eosinophilic esophagitis via the interleukin–33–basophil axis in mice



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5.1 Abstract

Background: Eosinophilic esophagitis (EoE) is an allergic inflammatory disorder characterized by the accumulation of eosinophils in the esophagus. EoE often coexists with atopic dermatitis (AD), a chronic inflammatory skin disease. The impaired skin barrier in AD has been suggested as an entry point for allergic sensitization that triggers development of EoE.

Objective: To define the mechanisms whereby epicutaneous sensitization via a disrupted skin barrier induces development of EoE.

Methods: To elicit experimental EoE, mice were epicutaneously sensitized with ovalbumin (OVA) followed by intranasal OVA challenge. Levels of esophageal mRNA for Th2 cytokines and IL-33 receptor *Il1rl1* (*St2*) were measured by quantitative PCR. Esophageal eosinophil accumulation was assessed by flow cytometry and H&E staining. *In vivo* basophil depletion was achieved by using diphtheria toxin treatment of *Mcpt8*^{DTR} mice and animals were repopulated with bone marrow basophils. mRNA analysis of esophageal biopsies from EoE patients was used to validate our findings in humans.

Results: Epicutaneous sensitization and intranasal challenge of wild-type (WT) mice resulted in an accumulation of eosinophils and upregulation of Th2 cytokines and *St2* in the esophagus. Disruption of the IL-33-ST2 axis or depletion of basophils reduced these features. Expression of ST2 on basophils was required to accumulate in the esophagus and transfer experimental EoE. Expression of *IL1RL1/ST2* mRNA was increased in esophageal biopsies from EoE patients. Topical OVA application on unstripped skin induced experimental EoE in filaggrin-deficient, flaky tail (*ft/ft*) mice, but not in WT controls or *ft/ft.St2*$^{-/-}$ mice.

Conclusion: Epicutaneous allergic sensitization promotes EoE and this is critically mediated via the IL-33-ST2-basophil axis.

5.2 Introduction

Eosinophilic esophagitis (EoE) is a chronic inflammatory allergic disorder characterized by the accumulation of eosinophils in the esophagus (Furuta and Katzka, 2015). EoE affects 1 in 2,000 individuals in the U.S., and has been increasingly diagnosed in the past two decades (Mulder and Justinich, 2011). Patients with EoE can present in infancy with vomiting and failure to thrive. Older patients develop dysphagia, food impactions and esophageal strictures. The mechanism that mediates eosinophil accumulation in EoE is not fully understood. The observations that immune suppression or allergen avoidance ameliorate EoE symptoms indicate that EoE is an allergen-driven disease mediated by an abnormal immune response (Rothenberg, 2015). Several studies support a role of T-helper (Th)-2 cells that express the cytokines interleukin (IL)-4, IL-5, and IL-13 in eosinophil recruitment to tissue, as they regulate the differentiation of eosinophils from their precursors, and induce the production of eotaxin, a major chemoattractant of eosinophils (Rothenberg, 2015). However, modest effects of anti-IL-5 or anti-IL-13 treatment in ameliorating the symptoms in the majority of EoE patients (Cianferoni and Spergel, 2014; Rothenberg et al., 2015; Stein et al., 2006) indicate that additional inflammatory mechanisms involved in the pathogenesis of EoE need to be elucidated.

Approximately 50% of EoE patients have coexisting atopic dermatitis (AD), a chronic pruritic inflammatory skin disease that affects 15-17% of children and 1-3% of adults in the U.S. (Blanchard et al., 2010; Leung, 2013). Patients with AD and EoE are typically characterized by development of allergen-specific Th2 cells, production of immunoglobulin E (IgE), and recruitment of eosinophils into inflamed tissue. The established observation that childhood AD can predispose to the progressive development of allergic diseases suggests AD as a potential risk factor for development of subsequent EoE.

An abnormal skin barrier is a hallmark of AD (Oyoshi et al., 2009a) and may provide an initial entry point for food allergens (Lack et al., 2003), potentially leading to development of EoE. Loss of function mutations in the *Filaggrin* gene (*FLG*) that encodes the epithelial barrier protein filaggrin are associated with increased risk for developing AD (Palmer et al., 2006; Weidinger et al., 2006) and EoE (Blanchard et al., 2010), further supporting the hypothesis that the skin may be a common route of sensitization in the pathogenesis of AD and EoE.

IL-33 is a member of the IL-1 family characterized as a Th2 promoting epithelial cytokine that activates an array of immune cells, including eosinophils, basophils, mast cells, group 2 innate lymphoid cells (ILC2s), and Th2 cells through the receptor IL1RL1 (ST2) (Divekar and Kita, 2015; Molofsky et al., 2015; Schmitz et al., 2005; Siracusa et al., 2011). Levels of IL-33 are increased in the skin lesions and serum of patients with AD (Savinko et al., 2012; Tamagawa-Mineoka et al., 2014) as well as in esophageal tissues from human EoE (Simon et al., 2015). Genetic variants in *IL1RL1/ST2* or *IL33* have been associated with AD (Shimizu et al., 2005) and EoE (Kottyan et al., 2014), suggesting a role of the IL-33-ST2 axis in the pathogenesis of these diseases.

To understand the mechanisms of EoE, we have utilized a mouse model of AD-like allergic skin inflammation to demonstrate that epicutaneous sensitization with egg protein ovalbumin (OVA) on mechanically injured skin of wild-type (WT) mice, or topical sensitization with OVA on FLG-deficient skin of flaky tail (*ft/ft*) mice, followed by repeated intranasal administration of the same allergen results in development of EoE-like inflammation characterized by accumulation of eosinophils and upregulation of Th2 responses in the esophagus. We demonstrate that the IL-33-ST2 axis and basophils are critical for this process. We observed elevated *IL1RL1/ST2* gene expression in esophageal biopsies from EoE patients, substantiating the importance of the IL-33-ST2 axis in EoE. Together, these data suggest that the IL-33-ST2-basophil axis contributes to the pathogenesis of EoE and could be a new therapeutic target for the treatment of EoE.

5.3 Methods

5.3.1 Mice

BALB/c mice were purchased from Taconic. *St2*$^{-/-}$ mice (generated by Dr. Andrew McKenzie, Cambridge, United Kingdom) backcrossed onto the BALB/c background were a gift from Dr. Dale Umetsu (Boston Children's Hospital, currently at Genentech). BALB/c *ft/ft* mice with the matted hair mutation removed were previously described (Oyoshi et al., 2015). BALB/c *ft/ft* mice were subsequently crossed onto BALB/c *St2*$^{-/-}$ background to generate *ft/ft.St2*$^{-/-}$ mice. *Mcpt8*^{DTR} mice (Egawa et al., 2013) were backcrossed onto the BALB/c background. All mice were bred in the animal facility of BCH, and kept in a specific pathogen-free environment and fed an OVA-free diet. All procedures were performed in accordance with the Animal Care and Use Committee of Boston Children's Hospital.

5.3.2 Allergen sensitization

Epicutaneous sensitization of mice was performed as previously described (Nakajima et al., 2012; Spergel et al., 1998) with modification. The dorsal skin of anesthetized four to six-week-old female mice was shaved and tape-stripped six times with Tegaderm (Westnet Inc.). Then 100 µg OVA (Grade V; Sigma) in 100 µl of normal saline, or placebo (100 µl of normal saline), was placed on a patch of sterile gauze (1x1 cm), which was secured to the dorsal skin with Tegaderm. Each mouse had a total of three one-day exposures to the patch separated by two-day intervals. Forty-eight hours after the last sensitization, mice were intranasally challenged with 3 mg OVA every other day for 5 days (see **Figure 5.1A**). The dorsal skin of *ft/ft* mice was shaved and rested for two days for recovery from any injury by shaving. Then OVA or saline was applied in a regimen involving two cycles of daily exposure to OVA for 5 consecutive days prior to allergen challenge (**Figure 5.4A**). For some experiments, animals were treated intravenously with 40 µg of neutralizing anti-ST2 mAb or rat IgG2a isotype

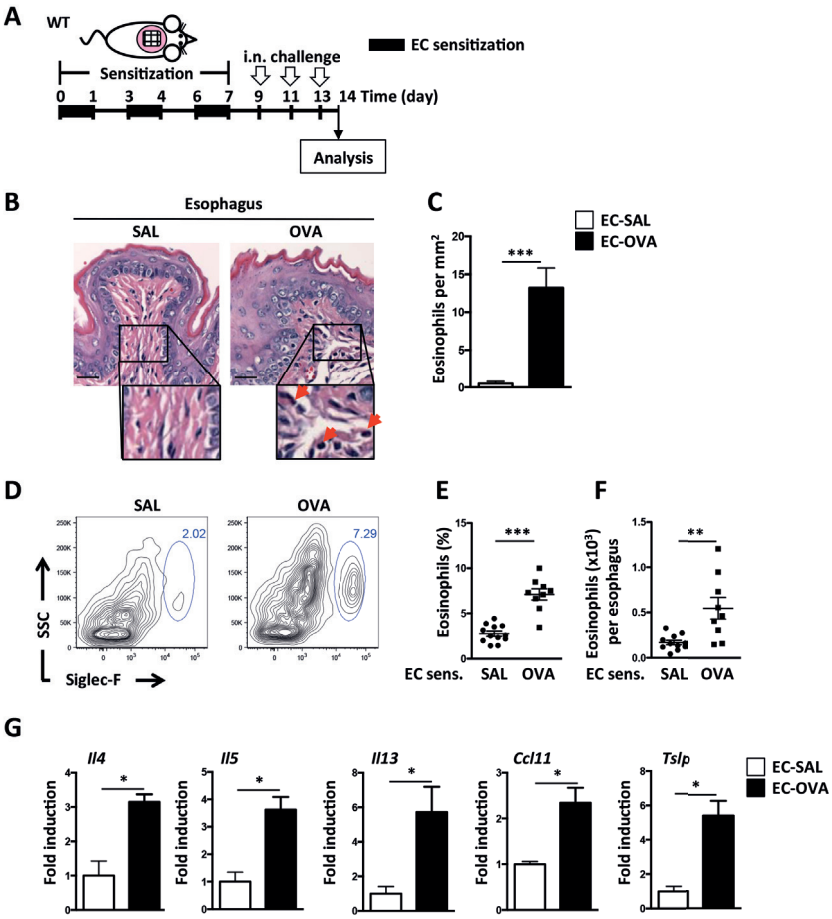

Figure 5.1. Epicutaneous sensitization predisposes to development of experimental EoE. (**A**) Experimental plan. (**B**) Representative H&E stained sections of esophagus. Arrows indicate eosinophils. Magnification, 400X; inset, 800X. Scale bars: 100 μm. (**C**) Number of eosinophils per mm^2. (**D-F**) Representative flow cytometry analysis (**D**), frequencies (**E**), and numbers (**F**) of eosinophils in the esophagus. (**G**) mRNA expression of Th2 cytokines, *Ccl11*/eotaxin-1, and *Tslp* as fold induction relative to saline controls. n = 5-7 per group in C and G. Percentages of eosinophils within live, $CD45^+lin^-$ cells are shown in D. EC: epicutaneous. *P < .05, **P < .01 and ***P < .001.

control (Biolegend). Mice were euthanized 24 hours after the last intranasal challenge to harvest tissues. For basophil depletion by diphtheria toxin (DT), *Mcpt8*^{DTR} mice (Egawa et al., 2013) were treated with 500 ng DT (List Biological Laboratory) intraperitoneally.

5.3.3 Bone marrow (BM) chimeras

BM chimeras were generated as described previously (Jin et al., 2009).

5.3.4 Adoptive transfer of basophils

Bone-marrow-derived basophils were grown in the presence of recombinant mouse IL-3 (Peprotech) for 9 to 14 days as previously described (Siracusa et al., 2011). Basophils were FACS sorted and $3x10^5$ cells were intraveneously injected into DT-treated *Mcpt8*^{DTR} mice.

5.3.5 Histological analysis

Multiple 4 µm sections of mid-esophagus were stained with hematoxylin and eosin (H&E). Eosinophils were counted in a blinded fashion in 5-10 different randomly chosen high-power fields (HPFs) at a magnification of 400X.

5.3.6 Flow cytometry

Esophageal tissue of mice was opened longitudinally, digested in 1 mg/ml collagenase (Worthington Biochemical) and 20 µg/ml DNase (Sigma) for 30 minutes, and mashed through 70 µm nylon mesh filters as previously described (Noti et al., 2013). Single-cell suspensions were incubated with Fixable Viability Dye (eBioscience) for dead cell exclusion and stained with fluorochrome-conjugated mAbs for Siglec-F, IgE, c-kit, CD3, CD11c, CD19, CD45, CD49b, NKp46 (purchased from Biolegend, BD Biosciences, or eBioscience). Mouse eosinophils were identified as live, $CD45^+lin(CD3, CD19, CD11c, NKp46)^-Siglec\text{-}F^+$ side-scatter $(SSC)^{high}$ cells. Mouse basophils were identified as live, $CD45^+lin^-c\text{-}kit^-CD49b^+IgE^+$ cells.

5.3.7 Quantitative PCR analysis

Total RNA was extracted from homogenized mid-esophageal tissue and quantitative real-time PCR was performed as described previously (Oyoshi et al., 2011).

5.3.8 Serum antibody measurement

These assays were performed as described previously (Oyoshi et al., 2011).

5.3.9 Gene expression analysis in esophageal biopsies of human subjects

All patients of this study are part of a longitudinal cohort study at Boston Children's Hospital, which is approved by the Institutional Investigational Review Board. The subset of patients studied in this paper has been previously described (Lexmond et al., 2014) and includes pediatric patients between 2-17 years with either EoE (defined as ≥15 eosinophils per high-power field in at least one esophageal biopsy under proton pump inhibitor treatment) or normal tissue histology as determined by routine pathological examination. RNA was isolated from biopsies taken from the distal esophagus (defined as ~2 cm from the gastro-esophageal junction). RNA processed as previously described (Lexmond et al., 2014) and quantitative RT-PCR was performed

using Taqman gene expression assays for *IL33* and *IL1RL1* (Applied Biosystems). Expression values were normalized against the internal housekeeping gene *GAPDH* using the ΔΔCt method in duplex assays performed in duplicate. The history of AD was determined by self-report from a questionnaire filled out by patients and caregivers at the time of inclusion.

5.3.10 Statistical analysis

A Mann-Whitney's U test was used to compare the distribution of each outcome between two groups. Nonparametric one-way ANOVA was used to compare three or more groups. All analyses were performed using the Graphpad Prism version 5.0 (Graphpad Software).

5.4 Results

5.4.1 Epicutaneous sensitization predisposes to development of experimental EoE

Given the potential role of skin sensitization in EoE, we tested the hypothesis that epicutaneous exposure to allergen results in development of EoE-like allergic inflammation in the murine esophagus. To achieve epicutaneous sensitization, we utilized a mouse model of AD-like allergic skin inflammation elicited by repeated exposure to OVA on tape-stripped skin (Nakajima et al., 2012; Spergel et al., 1998). BALB/c WT mice were subjected to three cycles of epicutaneous sensitization with OVA or saline control. Intranasal challenge with OVA, which results in direct exposure of the esophagus to OVA, was started two days after the last cycle of sensitization (**Figure 5.1A**). Epicutaneous sensitization with OVA resulted in the generation of OVA-specific IgE and IgG1 antibodies (**Figure 5.S1A-B**), as previously reported (Nakajima et al., 2012; Spergel et al., 1998). Following intranasal challenge with OVA, WT mice epicutaneously sensitized with OVA exhibited higher eosinophil infiltration in the esophagus as compared to similarly challenged WT mice epicutaneously exposed to saline, as assessed by histology and quantified by enumeration of eosinophils (**Figure 5.1B-C**), consistent with a previous report (Akei et al., 2005). Flow cytometry analysis also demonstrated higher frequencies and numbers of eosinophils in the esophagus of OVA-sensitized mice compared to saline-exposed mice upon OVA challenge (**Figure 5.1D-F**). Levels of mRNA for *Il4, Il5, Il13, Ccl11/Eotaxin-1, and thymic stromal lymphopoietin* (*Tslp*) were higher in the esophagus of OVA-sensitized mice than those of saline-exposed controls following OVA challenge (**Figure 5.1G**). Importantly, eosinophil accumulation in OVA-sensitized mice was specific to the esophagus since eosinophilia was not observed in the stomach or the jejunum of OVA-sensitized mice or saline controls following OVA challenge (**Figure 5.S2A-B**). Consistent with previous reports of experimental EoE (Akei et al., 2005; Mishra et al., 2001; 2008; Niranjan et al., 2013b), intranasal challenge of OVA-sensitized mice resulted in development of eosinophilia in the lung in addition to the esophageal symptomatic (**Figure 5.S2C**). These results suggest that epicutaneous allergen sensitization promotes development of EoE-like inflammation.

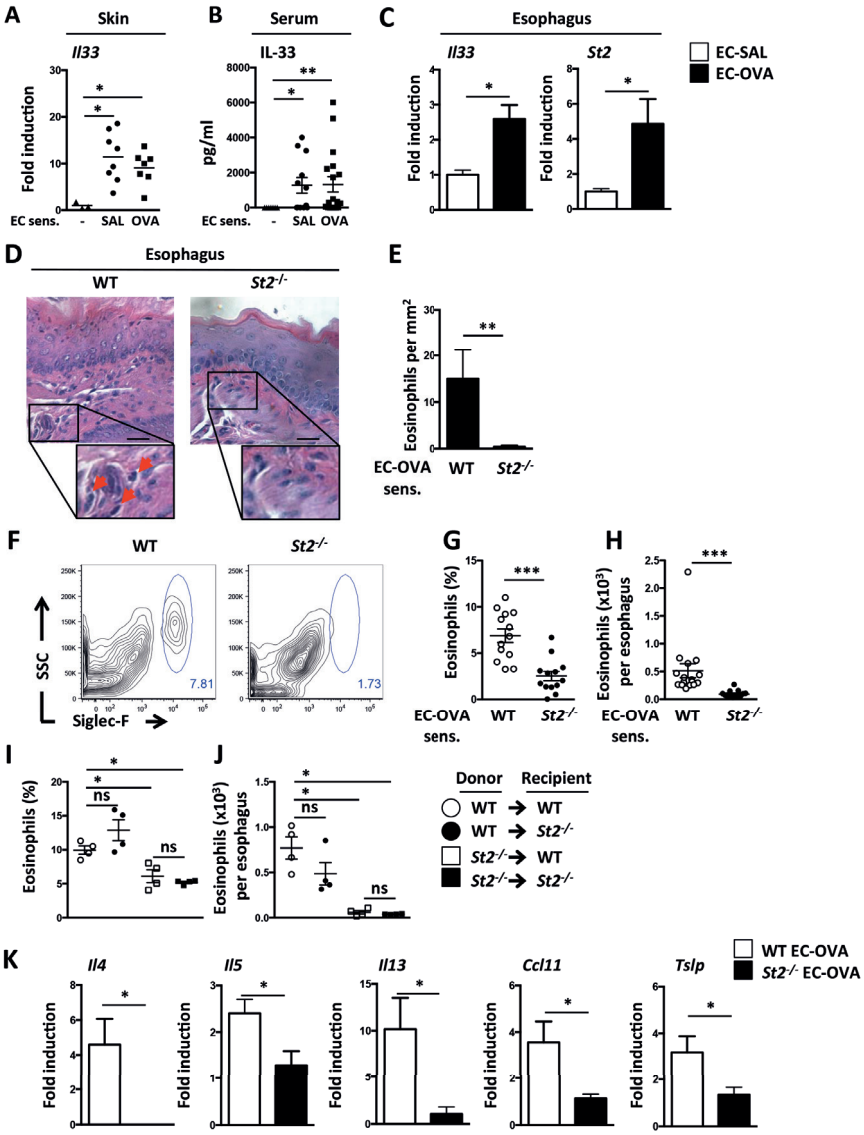

Figure 5.2. The IL-33-ST2 axis mediates development of experimental EoE elicited by epicutaneous sensitization. (**A-C**). Levels of *Il33* mRNA in skin (**A**), IL-33 in serum (**B**), and *Il33* and *St2* mRNA in the esophagus of WT mice (**C**). (**D**) Representative H&E stained sections of esophagus of OVA-sensitized WT and *St2*$^{-/-}$ mice. Arrows indicate eosinophils. Magnification, 400X; inset, 800X. Scale bars: 100 μm. (**E**) Number of eosinophils per mm^2. (**F-H**) Representative flow cytometry analysis (**F**), frequencies (**G**), and numbers (**H**) of eosinophils in the esophagus. (**I-J**) Flow cytometry analysis of frequencies (**I**) and numbers (**J**) of eosinophils in the esophagus of BM chimeras. (**K**) mRNA expression of Th2 cytokines, *Ccl11*/eotaxin-1, and *Tslp*. Fold induction relative to unsensitized (**A**) or saline (**C,K**) controls are shown. n = 5-9 per group in **C**, **E**, and **K**. EC: epicutaneous. *P < .05, **P < .01 and ***P < .001. ns, not significant.

5.4.2 The IL–33–ST2 axis mediates development of experimental EoE elicited by epicutaneous sensitization

The hallmark of AD is skin barrier dysfunction, which results in dry itchy skin (Oyoshi et al., 2009a). IL-33 is upregulated in the skin of patients with AD by external triggers including scratching (Savinko et al., 2012; Tamagawa-Mineoka et al., 2014). We hypothesized that the impaired barrier in AD skin allows allergen entry as well as the release of IL-33 that initiates a cascade leading to development of EoE. We therefore tested whether tape stripping of mouse skin, a surrogate for scratching in human AD, results in elevated IL-33 expression. Levels of *Il33* mRNA in both OVA- and saline-sensitized skin were higher than in unstripped skin (**Figure 5.2A**). Similarly, serum levels of IL-33 were increased in OVA-sensitized and control mice as compared to unstripped mice (**Figure 5.2B**). Importantly, we observed that levels of *Il33* and *St2* mRNA were increased in the esophagus of OVA-sensitized mice than saline-exposed controls following OVA challenge (**Figure 5.2C**), demonstrating a dependence on allergen. These observations prompted us to test the role of IL-33 in development of experimental EoE. We examined the response of IL-33 receptor *St2*$^{-/-}$ mice to epicutaneous sensitization and intranasal challenge with OVA. In contrast to WT controls, OVA-sensitized *St2*$^{-/-}$ mice failed to develop EoE-like inflammation, as indicated by a decrease in esophageal eosinophil accumulation (**Figure 5.2D-H**). To determine the contribution of ST2 expression on non-hematopoietic cells versus hematopoietic cells in eosinophil accumulation in the esophagus, we generated reciprocal BM chimeras using WT and *St2*$^{-/-}$ mice (**Fig. 2I,J**). Lethally irradiated WT or *St2*$^{-/-}$ recipients reconstituted with WT BM showed comparable increases in esophageal eosinophil accumulation irrespective of the ST2 sufficiency of the recipients (**Figure 5.2I-J**). Conversely, esophageal eosinophil accumulation was comparable in WT or *St2*$^{-/-}$ recipients reconstituted with *St2*$^{-/-}$ BM and significantly lower as compared to those in WT or *St2*$^{-/-}$ recipients of WT BM (**Figure 5.2I-J**). These data suggest that ST2 expression on hematopoietic cells plays a key role in development of AD-associated experimental EoE.

OVA-sensitized *St2*$^{-/-}$ mice showed lower levels of cytokine mRNA in the esophagus upon OVA challenge (**Figure 5.2K**). This was not due to an impaired systemic cytokine response to OVA, given that OVA-specific antibody concentrations in *St2*$^{-/-}$ mice as well as Th2 and Th1 cytokine secretion by *St2*$^{-/-}$ splenocytes *in vitro* stimulated with OVA were comparable to those in WT controls (**Figure 5.S3**), consistent with previous reports on *Il33*$^{-/-}$ mice (Morita et al., 2015). As an alternative approach to abrogating the IL-33-ST2 signaling, we intravenously treated WT mice with a monoclonal antibody (mAb) that neutralizes ST2 during epicutaneous sensitization with OVA (**Figure 5.3A**). Multiple systemic treatments of WT mice with anti-ST2 mAb resulted in a decrease in eosinophil accumulation in the esophagus after OVA challenge (**Figure 5.3B-F**). Treatment with anti-ST2 mAb also limited levels of *Il4, Il5, Il13, and Ccl11* mRNA in the esophagus, but not *Tslp* (**Figure 5.3G**). Collectively, these data support our hypothesis that the IL-33-ST2 interactions are required for development of experimental EoE.

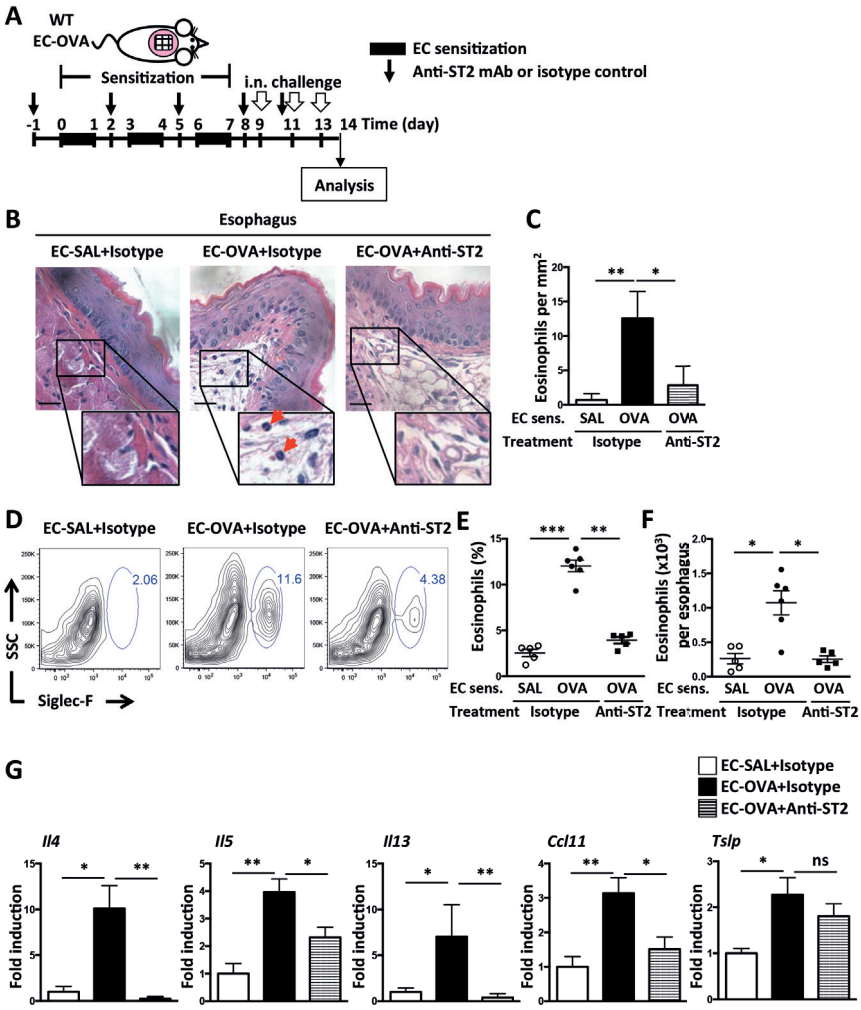

Figure 5.3. Blockade of the IL-33-ST2 responses attenuates development of experimental EoE. (**A**) Experimental plan. (**B**) Representative H&E stained sections of esophagus. Arrows indicate eosinophils. Magnification, 400X; inset, 800X. Scale bars: 100 μm. (**C**) Number of eosinophils per mm^2. (**D-F**) Representative flow cytometry analysis (**D**), frequencies (**E**), and numbers (**F**) of eosinophils in the esophagus. (**G**) mRNA expression of Th2 cytokines, *Ccl11*/eotaxin-1, and *Tslp* as fold induction relative to saline controls treated with isotype control. n = 5-6 per group in C and G. EC: epicutaneous. *P < .05, and **P < .01. ns, not significant.

5.4.3 FLG deficiency promotes IL–33–mediated esophageal eosinophil accumulation

Carriers of *FLG* mutations have an increased risk for multiple allergic disorders including EoE (Blanchard et al., 2010; Irvine et al., 2011). *ft/ft* mice carry a 5303delA frame shift mutation in the *FLG* gene (Fallon et al., 2009; Lane, 1972) and exhibit skin barrier dysfunction (Moniaga et al., 2010). To test whether *FLG* deficiency predisposes to development of EoE, *ft/ft* mice were

topically sensitized with OVA or saline on *unstripped* skin over a 2-week period, then intranasally challenged with OVA (**Figure 5.4A**). *ft/ft* mice topically sensitized with OVA developed more intense esophageal eosinophil accumulation as compared to *ft/ft* mice topically exposed to saline or WT controls sensitized with OVA (**Figure 5.4B-D**). Eosinophil accumulation was minimal in WT mice topically sensitized with OVA or saline (**Figure 5.4B-D**), consistent with our previous reports that mechanical injury by tape stripping is a prerequisite for induction of allergic skin sensitization in WT mice (Oyoshi et al., 2015; 2009b). We next examined whether the IL-33-ST2 axis contributes to the increased susceptibility of *ft/ft* mice to esophageal eosinophil accumulation. To this end, we bred the *ft/ft* genotype onto the *St2* null background and examined the responses to topical sensitization and challenge with OVA. *ft/ft.St2*$^{-/-}$ mice topically sensitized with OVA exhibited less esophageal eosinophil accumulation compared to OVA-sensitized *ft/ft* controls (**Figure 5.4E-G**). The results obtained in *ft/ft* mice reinforce the concept that cutaneous sensitization via an impaired skin barrier predisposes to EoE and this is critically regulated via the IL-33-ST2 axis.

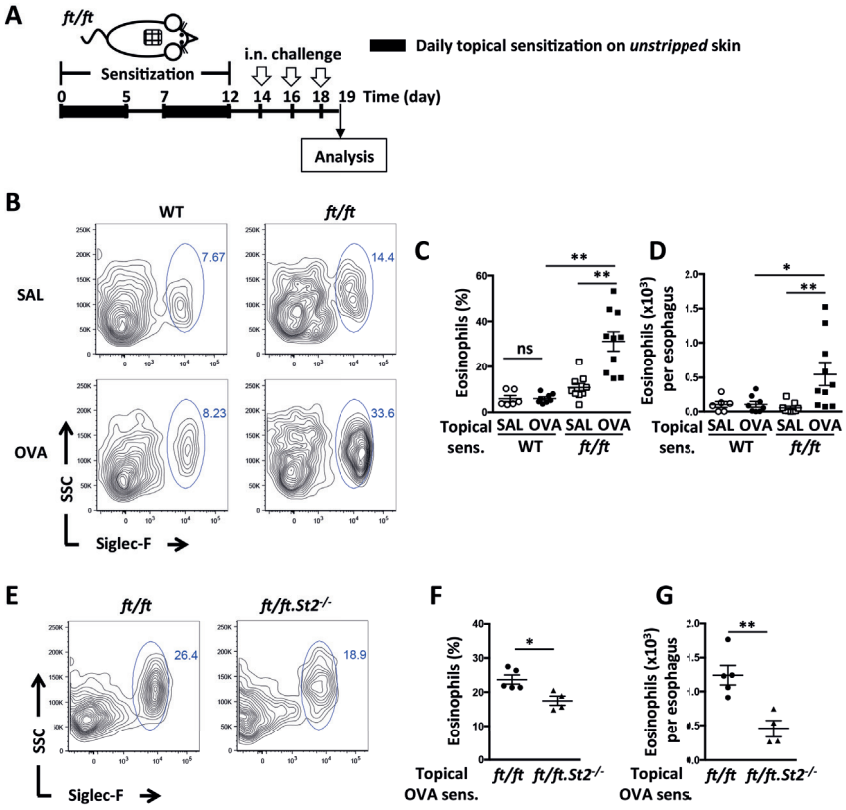

Figure 5.4. FLG deficiency promotes IL-33-mediated esophageal eosinophil accumulation. (A) Experimental plan. Representative flow cytometry analysis (**B**), frequencies (**C**), and numbers (**D**) of eosinophils in the esophagus of WT or *ft/ft* mice. Representative flow cytometry analysis (**E**), frequencies (**F**), and numbers (**G**) of eosinophils in the esophagus of *ft/ft* or *ft/ft.St2*$^{-/-}$ mice. *P < .05, and **P < .01. ns, not significant.

5.4.4 Basophils are required for development of experimental EoE

TSLP-induced basophil responses have been implicated in experimental EoE and in EoE patients (Noti et al., 2013), highlighting the role of basophils in the pathogenesis of EoE. We examined the role of basophils in mediating EoE-like inflammation elicited by epicutaneous allergen sensitization. Flow cytometry analysis revealed that OVA-sensitized WT mice exhibited higher accumulations of basophils in the esophagus compared to saline-exposed controls following OVA challenge (**Figure 5.5A-C**). To test whether basophils promote development of EoE-like inflammation, we used a genetic approach in which lineage-specific expression of the diphtheria toxin receptor gene allows selective basophil depletion by DT treatment (Egawa et al., 2013). *Mcpt8*^{DTR} mice were epicutaneously sensitized with OVA or saline then intranasally challenged with OVA while being treated with DT or PBS (**Figure 5.5D**). OVA-sensitized *Mcpt8*^{DTR} mice treated with PBS exhibited higher eosinophil accumulation and upregulation of Th2 cytokines in the esophagus (**Figure 5.5E-J**) as compared to saline-exposed *Mcpt8*^{DTR} controls. Interestingly, depletion of basophils in OVA-sensitized *Mcpt8*^{DTR} mice decreased these features as compared to OVA-sensitized *Mcpt8*^{DTR} mice treated with PBS (**Figure 5.5E-J**). *Mcpt8*^{DTR} mice treated with DT exhibited no signs of hypersensitivity to DT (data not shown). The decrease in esophageal eosinophilia in OVA-sensitized *Mcpt8*^{DTR} mice treated with DT was likely due to depletion of basophils, but not mast cells, as injection of DT into *Mcpt8*^{DTR} mice resulted in > 97% loss of basophils in the skin with no deleterious effects on mast cells (**Figure 5.S4A**) (Wada et al., 2010). Furthermore, OVA-sensitized mast cell-deficient *Kit*$^{W\text{-}sh/W\text{-}sh}$ mice exhibited comparable levels of esophageal eosinophil accumulation to WT controls after OVA challenge (**Figure 5.S4B-C**), suggesting that mast cells unlikely account for development of esophageal eosinophilia in our model. This is consistent with a previous observation that development of esophageal eosinophilia is independent of mast cells (Niranjan et al., 2013a) based on experiments in mast cell-deficient WW^{v} mice. These results indicate that basophils are required for the pathogenesis of EoE-like inflammation.

5.4.5 Basophils mediate experimental EoE through the IL–33–ST2 interactions

IL-33 activates mouse and human basophils (Pecaric-Petkovic et al., 2009; Schneider et al., 2009). Flow cytometry analysis revealed ST2 expression on basophils in the esophagus of OVA- and saline-sensitized mice (**Figure 5.6A**). Given that the IL-33-ST2 axis, basophils, and ST2 expression on hematopoietic cells play a key role in development of experimental EoE, we sought to test whether basophils promote experimental EoE in an IL-33-ST2 dependent manner. To this purpose, basophils were expanded from BM of WT and *St2*$^{-/-}$ mice in the presence of IL-3 *in vitro*, FACS sorted, then intraveneously injected into *Mcpt8*^{DTR} mice while being sensitized with OVA and treated with DT (**Figure 5.6B**). Adoptive transfer of WT basophils restored esophageal eosinophil accumulation in basophil-depleted *Mcpt8*^{DTR} recipients sensitized with OVA following OVA challenge (**Figure 5.6C-G**). In contrast, adoptive

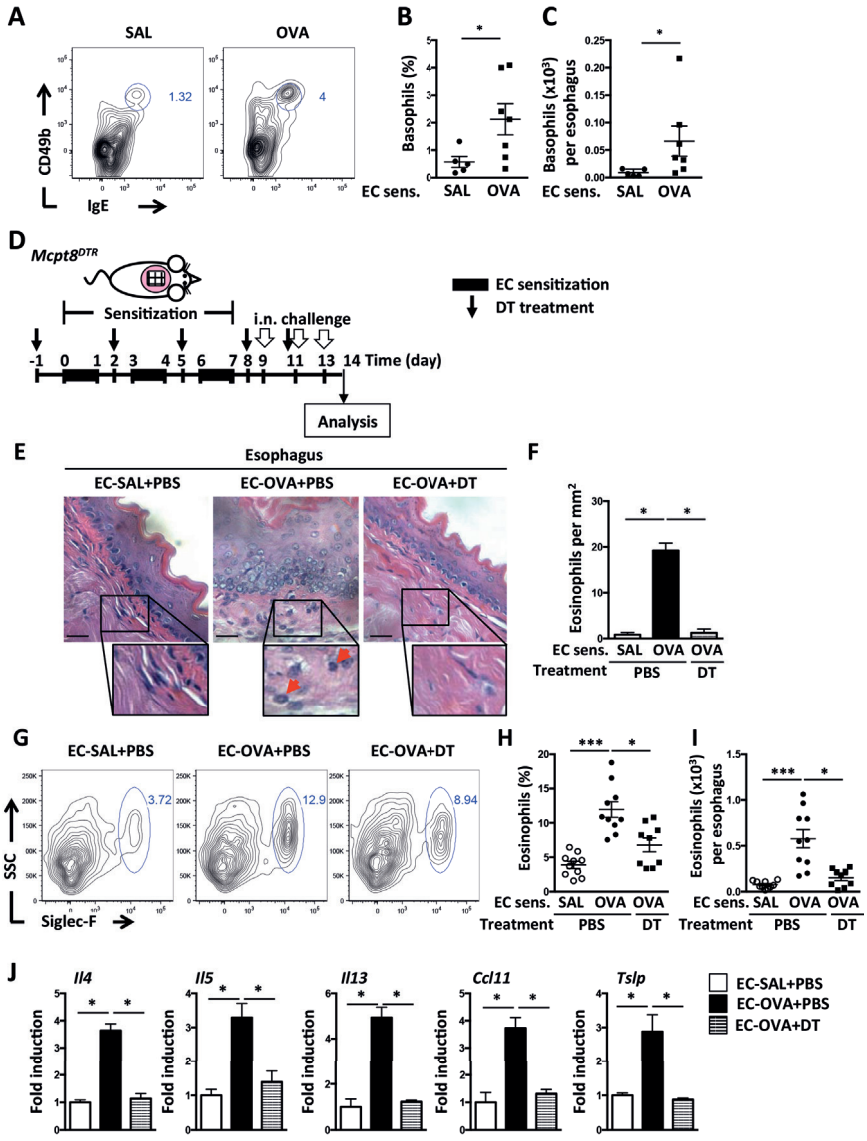

Figure 5.5. Basophils are required for development of experimental EoE. Representative flow cytometry analysis (**A**), frequencies (**B**), and numbers (**C**) of basophils in the esophagus. (**D**) Experimental plan. (**E**) Representative H&E stained sections of esophagus. Arrows indicate eosinophils. Magnification, 400X; inset, 800X. Scale bars: 100 μm. (**F**) Number of eosinophils per mm^2. Representative flow cytometry analysis (**G**), frequencies (**H**), and numbers (**I**) of eosinophils in the esophagus. (**J**) mRNA expression of Th2 cytokines, *Ccl11*/eotaxin-1, and *Tslp* as fold induction relative to saline-exposed *Mcpt8*^{DTR} mice treated with PBS. n = 5 per group in F and J. EC: epicutaneous. Percentages of basophils within live, CD45$^+$lin$^-$c-kit$^-$ cells are shown in A. *P < .05, and ***P < .001.

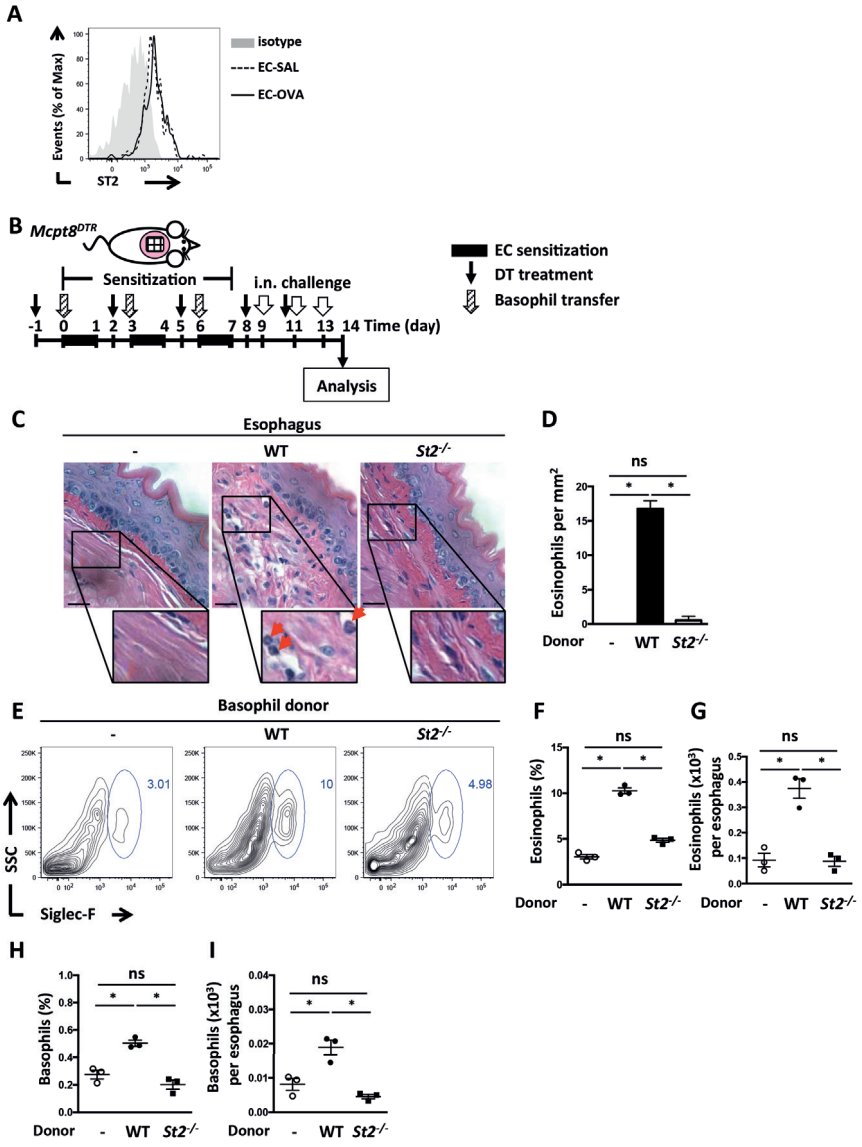

Figure 5.6. Basophils mediate experimental EoE through the IL-33-ST2 interactions. (A) Representative flow cytometry analysis of basophils in the esophagus for ST2 expression. (**B**) Experimental plan. (**C**) Representative H&E stained sections of esophagus. Arrows indicate eosinophils. Magnification, 400X; inset, 800X. Scale bars: 100 μm. (**D**) Number of eosinophils per mm^2. Representative flow cytometry analysis (**E**), frequencies (**F**), and numbers (**G**) of eosinophils in the esophagus. Frequencies (**H**), and numbers (**I**) of basophils in the esophagus. n = 3 per group in C. EC: epicutaneous. *P < .05. ns, not significant.

transfer of *St2*$^{-/-}$ basophils failed to elicit esophageal eosinophil accumulation in basophil-depleted OVA-sensitized *Mcpt8*^{DTR} recipients (**Figure 5.6C-G**). Adoptive transfer of WT basophils into basophil-depleted mice resulted in an increase in basophil accumulation in the esophagus (**Figure 5.6H-I**). In contrast, basophil-depleted mice reconstituted with *St2*$^{-/-}$ basophils showed significantly lower numbers of esophageal basophils, comparable to minimal basophil influx in basophil-depleted mice without basophil reconstitution (**Figure 5.6H-I**). These results suggest that failure of *St2*$^{-/-}$ basophils to restore esophageal eosinophil accumulation is likely due to an inability of *St2*$^{-/-}$ basophils to home to the esophagus, consistent with previous reports that IL-33-ST2 axis promotes migration of basophils (Pecaric-Petkovic et al., 2009; Suzukawa et al., 2008). These results indicate that basophils are key cellular mediators of IL-33-ST2 mediated experimental EoE.

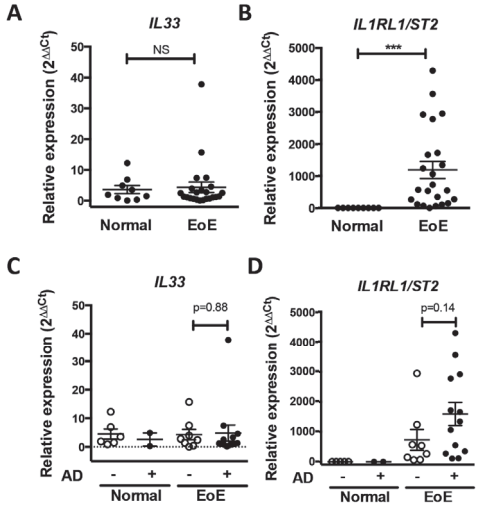

Figure 5.7. EoE patients have elevated esophageal expression of *IL1RL1/ST2* mRNA. Expression of *IL33* (**A**) and *IL1RL1/ST2* (**B**) in esophageal biopsies from normal controls and EoE patients. Expression of *IL33* (**C**) and *IL1RL1/ST2* (**D**) in esophageal biopsies from normal controls and EoE patients with and without AD. A and C, B and D use the same data, respectively. ***P < .001. ns, not significant.

5.4.6 EoE patients have elevated esophageal expression of IL1RL1/ST2 mRNA

Since the IL-33-ST2 axis critically contributes to the pathogenesis of experimental EoE in our model, we assessed the expression of *IL33* and *IL1RL1* (encoding ST2) in esophageal tissue biopsies obtained from pediatric patients (average age 8.0 years, ranging from 2-17) who were undergoing diagnostic endoscopy for suspected EoE. None of the patients had used systemic steroids in the six months prior to inclusion. Quantitative PCR analysis revealed that levels of *IL33* mRNA were comparable in EoE patients and controls, regardless of AD history (**Figure 5.7A,C**). However, expression of *IL1RL1*/ST2 was significantly increased in EoE patients (**Figure**

5.7B). EoE patients who reported a history of AD demonstrated a higher average expression of *IL1RL1/ST2* compared to those who did not suffer from AD (**Figure 5.7D**). No differences in mRNA expression of *IL1RL1*/ST2 were detected in esophageal biopsies from control subjects with and without AD (**Figure 5.7D**). Fifteen patients in this cohort reported a history of AD and only three EoE patients reported rash in the past six months (data not shown). Peak esophageal eosinophil counts were significantly higher in EoE patients with AD compared to those without AD (**Table 5.1**). There was a positive correlation between peak esophageal eosinophil counts and *ST2* mRNA levels (r=0.30, p=0.21)(**Figure 5.S5**). It is likely that significance of this positive correlation was not reached because of the rather small patient numbers. These data demonstrate that elevated *IL1RL1/ST2* gene expression is associated with EoE in a pediatric cohort and further support the concept that the IL-33-ST2 axis contributes to the EoE pathogenesis.

Table 5.1. Characteristics of EoE patients. *Statistical significance of continuous variables was compared using a non-parametric Mann-Whitney U test. Dichotomous data was analyzed using Fisher's exact test.
None of the patients had used systemic steroids in the 6 months prior to inclusion.

Parameter	EoE without AD	EoE with AD	p-value*
N	8	13	n/a
Age (median, range)	6.1 (3.8 – 13.9)	5.3 (2.2 – 17.7)	0.69
Male sex	7 (88%)	9 (69%)	0.61
Asthma	1 (13%)	3 (23%)	1.0
Seasonal allergy	5 (63%)	10 (77%)	0.63
Food allergy	3 (38%)	10 (77%)	0.16
Peak eosinophil count/hpf (median, range)	27.5 (15-100)	80 (25-150)	**0.03**

5.5 Discussion

In this study we demonstrate that allergic sensitization via a disrupted skin barrier promotes development of experimental EoE that is critically mediated by the IL-33-ST2 axis and basophils. The importance of the IL-33-ST2 axis in EoE was corroborated by our findings of elevated *ST2* gene expression in human EoE.

The experimental murine EoE model used in our study shows several key features that parallel human EoE, validating our approach. Mice epicutaneously sensitized with OVA exhibited eosinophil accumulation and upregulation of Th2 cytokines and *Tslp* in the esophagus after intranasal OVA challenge, as shown in human EoE (Blanchard et al., 2011; Rothenberg et al., 2010). In addition, OVA-sensitized mice had an increase in esophageal accumulation of basophils, mast cells, and ILC2s (data not shown), which have been shown to be enriched in human EoE (Abonia et al., 2010; Doherty et al., 2015; Noti et al., 2013). Unlike

another model of EoE elicited by epicutaneous allergen sensitization with the vitamin D analog MC903 (Noti et al., 2013), the mouse models we have used had no change in eosinophilia in the stomach or in the jejunum, consistent with the observations that EoE patients exhibit eosinophilia limited to the esophagus within the gastrointestinal tract (Spergel et al., 2009). Intranasal administration of allergen resulted in development of lung eosinophilia in our model, consistent with previous models of EoE (Akei et al., 2005; Mishra et al., 2001; 2008; Niranjan et al., 2013b). This is in line with the observations that EoE is frequently associated with asthma (Greenhawt et al., 2013; Spergel et al., 2009) and that aeroallergens exacerbate EoE (Ram et al., 2015), suggesting a link between EoE and respiratory allergy. Our model is physiologic in that it is adjuvant free and employs a portal of exposure (the skin) that is important for EoE development. Collectively, our results indicate that allergen sensitization via a disrupted skin barrier predisposes to development of allergic esophageal inflammation upon encountering the same allergen, supporting the hypothesis that impaired skin barrier in AD allows allergen sensitization that leads to development of EoE (Oyoshi, 2015; Oyoshi et al., 2014).

Mechanical skin injury caused by tape stripping induced IL-33 in mouse skin, consistent with previous reports in mice and humans (Dickel et al., 2010; Morita et al., 2015). The link between IL-33 upregulation and a disrupted skin barrier is also supported by elevated IL-33 expression in *ft/ft* skin (Savinko et al., 2012). The mechanisms by which tape stripping induces IL-33 in skin and systemic circulation may include a release of IL-33 from the nucleus into the extracellular environment from necrotic cells during tissue damage (Molofsky et al., 2015). Levels of *Il33* mRNA were upregulated in the esophagus of OVA-sensitized mice, consistent with previous findings in human EoE (Judd et al., 2016). A recent genome-wide association study has identified genetic variants at the *IL33* locus (Kottyan et al., 2014), also associated with allergic sensitization (Bønnelykke et al., 2013; Hinds et al., 2013), accentuating the importance of IL-33 in both EoE and allergic sensitization. Indeed, we demonstrate that the IL-33-ST2 axis is critical in development of experimental EoE, as EoE features were attenuated in $St2^{-/-}$ mice or in WT mice treated with anti-ST2 antibodies. Comparable allergen-specific systemic responses in $St2^{-/-}$ mice and WT controls suggest that the IL-33-ST2 interactions in the esophagus underlie the decreased susceptibility of $St2^{-/-}$ mice to EoE. One possible explanation for the presence of local allergic inflammation in WT mice, but not in $St2^{-/-}$ mice, could be increased esophageal expression of IL-33 that promotes cytokine production by infiltrating basophils, Th2 cells, mast cells, and/or ILC2s via ST2. Increased local expression of TSLP could also amplify Th2 responses (Han et al., 2014; He et al., 2008; Oyoshi et al., 2010; Ziegler, 2012). This is in line with the hypothesis that allergen sensitization followed by esophagus-specific responses may drive EoE (Oyoshi, 2015; Rothenberg, 2015).

Cutaneous allergen sensitization in *ft/ft* mice mimics skin exposure to environmental antigens in patients with AD (Oyoshi et al., 2009b; Scharschmidt et al., 2009). We demonstrate that allergen sensitization through an impaired skin barrier due to congenital FLG deficiency in *ft/ft* mice induces EoE features. The increased susceptibility of *ft/ft* mice to experimental EoE

is consistent with the association of *FLG* mutations with human EoE (Blanchard et al., 2010). We further verified the critical role of IL-33 in promoting EoE in *ft/ft* mice, as OVA-sensitized *ft/ft.St2*$^{-/-}$ mice showed an decrease in esophageal eosinophil accumulation, supporting our hypothesis that IL-33 produced by epithelial cells is an important cofactor in development of EoE by potentiating allergic inflammation to cutaneously introduced allergens. Expression of filaggrin is downregulated in esophageal tissues of EoE patients (Blanchard et al., 2010; Simon et al., 2015). Impaired esophageal barrier function in EoE may allow allergen penetration and exaggeration of esophageal inflammation. Analysis of differential contribution of barrier abnormalities in skin and in esophagus to the pathogenesis of EoE is an important future question to be addressed.

TSLP and basophils have been implicated in promoting Th2 responses and development of EoE (Giacomin et al., 2012; Kottyan et al., 2014; Noti et al., 2013; Rothenberg et al., 2010; Siracusa et al., 2011; Sleiman et al., 2014), however, the capacity of *Tslpr*$^{-/-}$ basophils in promoting EoE has not been directly examined. Experimental EoE in our model was attenuated in basophil-depleted mice, consistent with a previous report (Noti et al., 2013). Our findings that WT, but not *St2*$^{-/-}$, basophils restored esophageal eosinophil accumulation in basophil-depleted mice indicate that basophils are the critical source of IL-33-mediated EoE. These findings may also apply to human EoE because basophil responses and IL-33 levels are increased in patients with EoE (Noti et al., 2013; Simon et al., 2015). Our data suggest that ST2-mediated migration of basophils to the esophagus (Pecaric-Petkovic et al., 2009; Suzukawa et al., 2008) likely accounts for development of EoE, however, further mechanisms by which basophils promote EoE in this model remain elusive. IL-33 directly activates basophils via ST2 (Pecaric-Petkovic et al., 2009; Suzukawa et al., 2008). IL-33 also influences dendritic cells (DCs) and promotes allergic skin sensitization and Th2 response in mouse models of food allergy (Han et al., 2014; Tordesillas et al., 2014). Recent studies have suggested that DCs and basophils cooperate to promote Th2 responses (Han et al., 2014; Leyva-Castillo et al., 2013; Tang et al., 2010). It is possible that IL-33 primes DCs for Th2 responses and activation of local innate immune responses to secrete Th2 cytokines for further recruitment of basophils. ILC2s are enriched in skin lesions from patients with AD and EoE, and respond to stimulation with IL-33, resulting in secretion of Th2 cytokines (Doherty et al., 2015; Kim et al., 2013; Salimi et al., 2013). IL-33-primed basophils may also interact with ILC2s to promote Th2 responses (Kim et al., 2014; Motomura et al., 2014) and regulate eosinophil recruitment to the tissue (Nakashima et al., 2014). The contribution of ILC2s together with innate and adaptive immune pathways to development of EoE requires future investigations. Consistent with a previous report (Noti et al., 2013), esophageal eosinophilia was significantly decreased in OVA-sensitized *Tslpr*$^{-/-}$ mice (data not shown), suggesting that TSLP also plays a key role in our EoE model. We previously reported that TSLP is induced in skin by tape stripping (Oyoshi et al., 2010) and induce allergic skin inflammation (He et

al., 2008). Whether TSLP and IL-33 synergistically or differently regulate basophils and other immune cells in promoting EoE will be an important subject to be addressed in future.

Levels of *St2* gene expression were increased in the esophagus of mice and this was associated with eosinophil accumulation and Th2 responses. The importance of the IL-33-ST2 axis in the pathogenesis of EoE was strongly corroborated by the increased expression of *IL1RL1/ST2* gene in esophageal biopsies from EoE patients, but not in control biopsies. Peak median esophageal eosinophil counts were significantly higher in EoE patients with AD than in EoE patients without AD, potentially implying a more severe EoE phenotype may affect EoE patients with AD. The obvious trend towards elevated *IL1RL1/ST2* gene expression in EoE patients with AD as compared to those in EoE patients without AD supports our concept of the IL-33-ST2 axis mediating the pathogenesis of AD-associated EoE, although the difference in mRNA levels did not reach statistical significance in the patient population analyzed in this study. The positive correlation of peak esophageal eosinophil counts with *ST2* mRNA levels suggests that higher infiltration in the esophagus of EoE patients by ST2 expressing cells including eosinophils, basophils, mast cells, ILC2s, and Th2 cells may contribute to the increased ST2 expression at these sites. Future study with a larger patient cohort and clinically diagnosed atopic dermatitis rather than self-reported disease is needed to confirm the correlation between the disease severity and *ST2* expression levels. Levels of IL-33 mRNA were comparable in esophageal biopsies from control and EoE subjects unlike the previous report (Judd et al., 2016). The reason for the discrepancy is not clear. It could possibly reflect instabilities of cytokine mRNA during the sampling process and/or elevated IL-33 protein levels in EoE biopsies (Simon et al., 2015).

Taken together, we demonstrate that skin barrier dysfunction in AD caused by mechanical injury secondary to scratching or due to *FLG* deficiency plays a key role in allergen sensitization and promotes the progression to EoE. Our study endorses the paradigm that allergic skin sensitization and basophils play a key role in the pathogenesis of EoE and adds new insights into currently underinvestigated role of the IL-33-ST2 axis to the pathophysiology of EoE, leading to a better understanding of mechanisms whereby epicutaneous sensitization promotes EoE. Our findings are novel as we demonstrate the fundamental role of IL-33 in EoE by blocking IL-33 pathway with two independent models of experimental EoE as well as anti-ST2 neutralizing antibodies. We have directly proven impaired ability of $St2^{-/-}$ basophils to elicit esophageal eosinophil accumulation. Our data using filaggrin-deficient mice provide further experimental support for the epidemiologic findings that filaggrin deficiency predisposes to EoE. In conclusion, this work has important implications for the development of new therapeutic strategies since neutralization of IL-33 and/or blockade of ST2 might be an effective target for EoE.

5.6 Acknowledgements

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5.7 Supplemental data

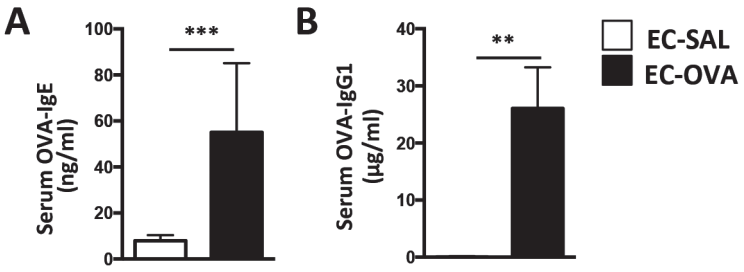


Figure 5.S1. Allergen-specific systemic immune responses in epicutaneously sensitized mice. Serum levels of OVA-specific IgE (**A**) and IgG1 (**B**) in WT mice EC sensitized with OVA or saline. EC: epicutaneous. n = 7-10. **P < .01, ***P < .001.

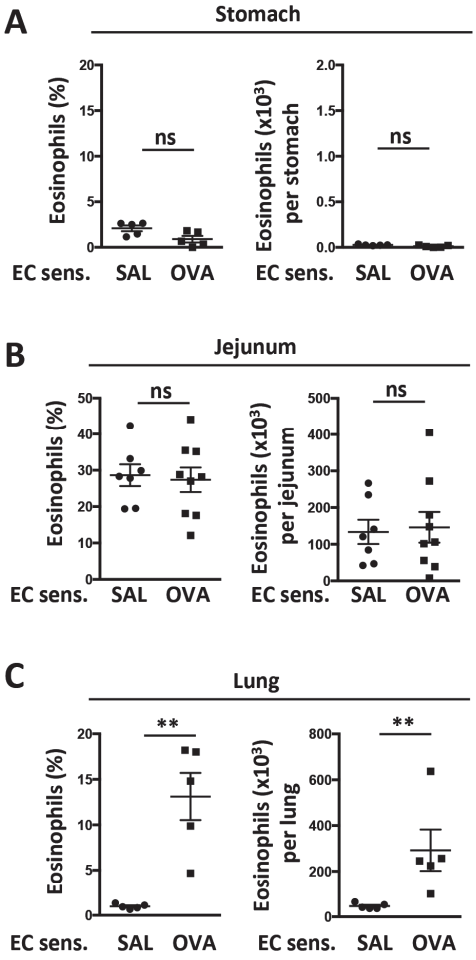


Figure 5.S2. Analysis of tissue eosinophilia in epicutaneously sensitized mice. Flow cytometry analysis of frequencies and numbers of eosinophils in the stomach (**A**), the jejunum (**B**), and the lung (**C**). EC: epicutaneous. ns, not significant.

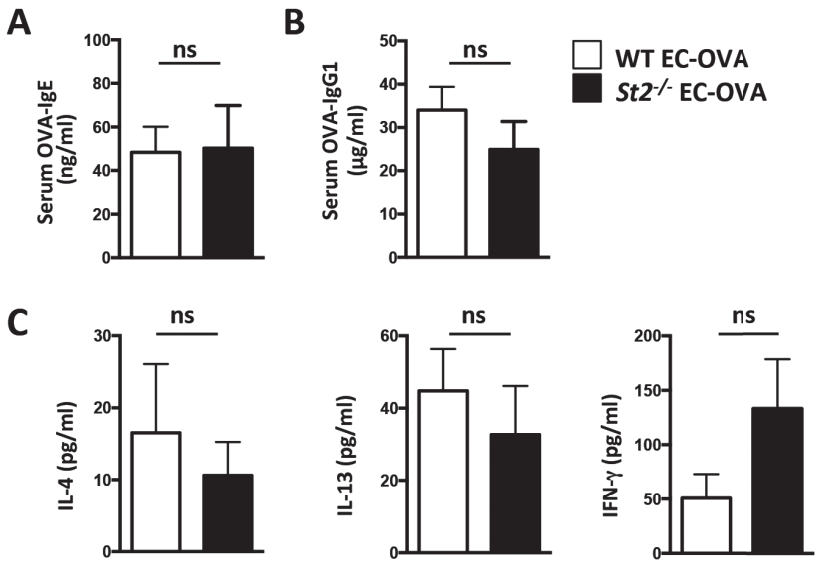


Figure 5.S3. Allergen-specific systemic immune responses in epicutaneously sensitized *St2*$^{-/-}$ mice. Serum levels of OVA-specific IgE (**A**) and IgG1 (**B**) in WT or *St2*$^{-/-}$ mice with OVA. (**C**) Cytokine production by splenocytes *in vitro* stimulated with OVA. n = 5-10 per group. EC: epicutaneous. ns, not significant.

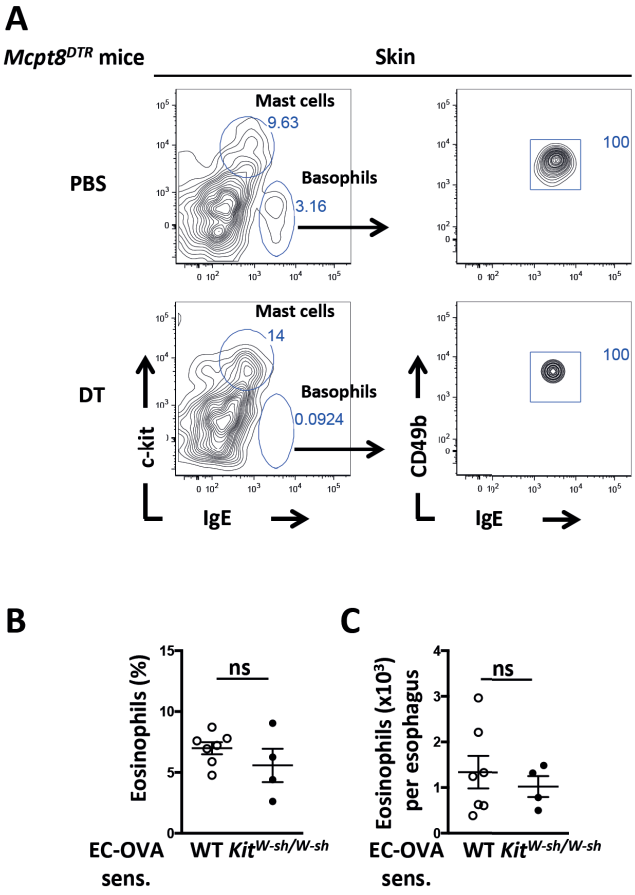


Figure 5.S4. Development of esophageal eosinophil accumulation is independent of mast cells. (**A**) Frequencies and numbers of mast cells and basophils in the skin of *Mcpt8*^{DTR} mice 3 days after DT injection. Flow cytometry analysis of frequencies (**B**) and numbers (**C**) of eosinophils in the esophagus of OVA-sensitized *Kit*$^{W\text{-}sh/W\text{-}sh}$ mice following OVA challenge.

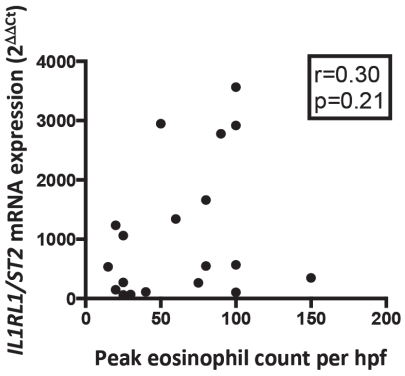
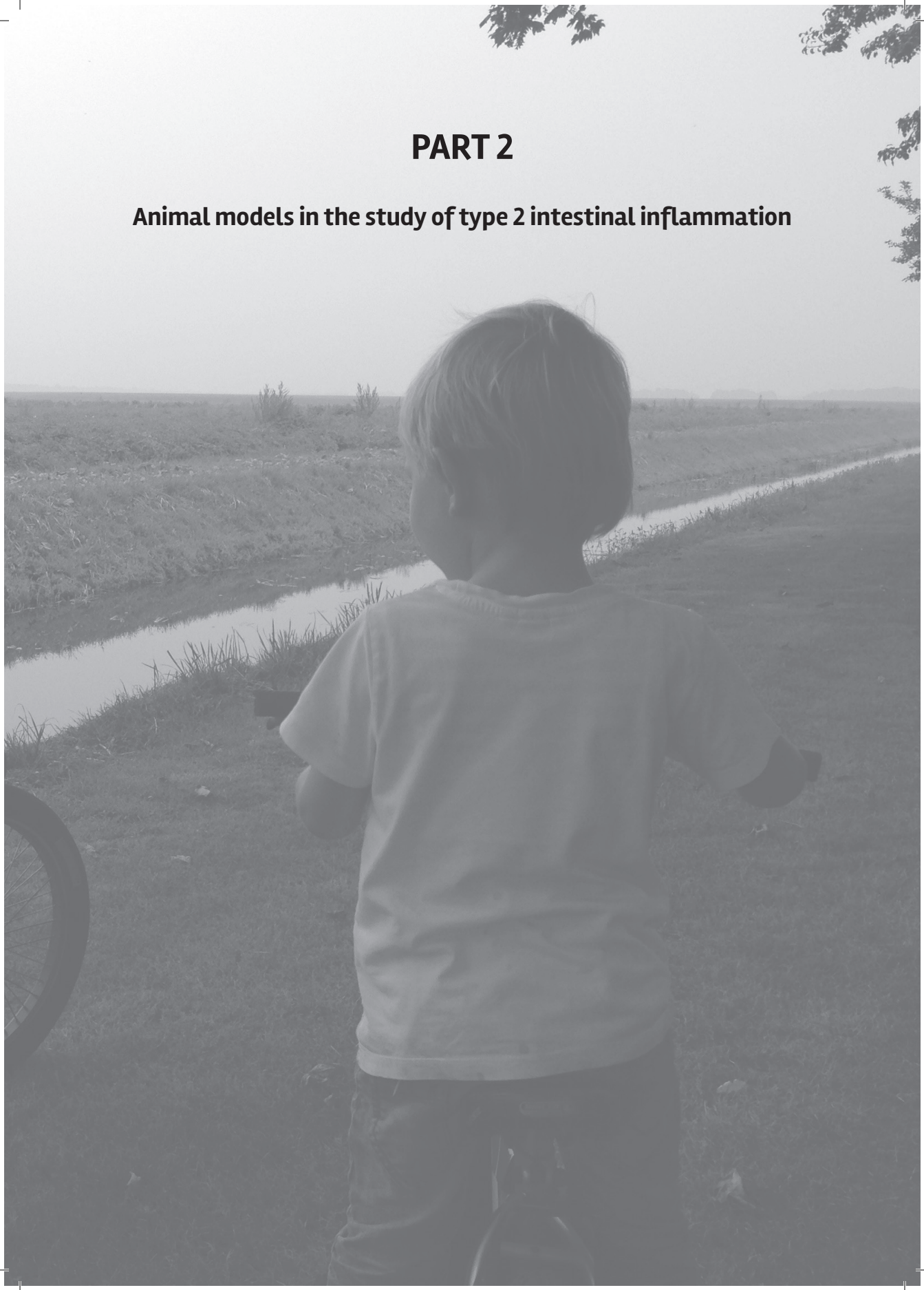


Figure 5.S5. Correlation analysis of peak esophageal eosinophil counts and *IL1RL1/ST2* mRNA expression levels in esophageal biopsies from EoE patients. Pearson's r=0.30. p=0.21.

PART 2

Animal models in the study of type 2 intestinal inflammation



Chapter 6

Fatal autoimmunity in mice reconstituted with human hematopoietic stem cells encoding defective FOXP3

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6.1 Abstract

Mice reconstituted with a human immune system provide a tractable *in vivo* model to assess human immune cell function. To date, reconstitution of murine strains with human hematopoietic stem cells (HSCs) from patients with monogenic immune disorders have not been reported. One obstacle precluding the development of immune-disease specific "humanized" mice is that optimal adaptive immune responses in current strains have required implantation of autologous human thymic tissue. To address this issue, we developed a mouse strain that lacks murine major histocompatibility complex class II (MHCII) and instead expresses human MHCII DR1. These mice displayed improved adaptive immune responses when reconstituted with human HSCs including enhanced T cell reconstitution, delayed-type hypersensitivity responses, and class-switch recombination. Following immune reconstitution of this novel strain with HSCs from a patient with immune dysregulation, polyendocrinopathy, enteropathy, X-linked (IPEX) syndrome, associated with aberrant FOXP3 function, mice developed a lethal inflammatory disorder with multi-organ involvement and autoantibody production mimicking the pathology seen in affected humans. This humanized mouse model permits *in vivo* evaluation of immune responses associated with genetically altered HSCs, including primary immunodeficiencies, and should facilitate the study of human immune pathobiology and the development of targeted therapeutics.

6.2 Introduction

Studies in mice have offered significant insight into the pathogenesis of human diseases; however, animal models have frequently failed to predict the efficacy and safety of novel therapeutics in humans (McKenzie et al., 1995; Seok et al., 2013; Suntharalingam et al., 2006; Xu et al., 2014a). An experimental system allowing direct functional assessment of patient cells *in vivo* could serve as an invaluable intermediate step in the process of drug-development that could increase safety while reducing overall cost of clinical trials. Over the past decade, advanced immunodeficient mouse models have been established to improve engraftment of human hematopoietic stem cells (HSCs) and leukocyte development facilitating *in vivo* mechanistic studies. Though several iterations of humanized mice have been described (Shultz et al., 2012) most strains combine null mutations in *Prkdc* or *Rag* genes with *Il2rg*$^{-/-}$ to impair *de novo* murine lymphocyte maturation and NK cell development respectively, while permitting xenogeneic thymopoiesis in the murine thymus (Melkus et al., 2006). Transfer of human CD34^{+} HSCs in these mice leads to multi-lineage hematopoiesis with variable levels of reconstitution depending on the strain and age of recipient mice and the source of donor HSCs (Lepus et al., 2009; Traggiai et al., 2004). Despite robust lymphoid reconstitution in most models, adaptive immune responses remain incomplete in both the CD34^{+} HSC model as well as advanced models incorporating concurrently implanted human fetal thymic and liver tissue and autologous HSCs (BLT mice) (Ishikawa et al., 2005; Rajesh et al., 2010; Traggiai et al., 2004). This impediment has been postulated to result from inefficient CD4^{+} T cell selection on murine major histocompatibility complex class II (MHCII) in the mouse thymus (Baenziger et al., 2006). In support of this hypothesis, intravenous injection of human HSCs into *adult* NOD.*Rag1*$^{-/-}$*Il2rg*$^{-/-}$ (NRG) mice expressing human MHCII leukocyte antigen (HLA)-DR4 improves CD4^{+} T cell development as well as B cell function (Danner et al., 2011). One potential limitation of this model is that human CD4^{+} T cells can be restricted on either murine MHCII or HLA-DR4 molecules.

In this report, we developed a novel immunodeficient mouse strain lacking murine MHCII and instead express a human MHCII molecule to test whether adaptive immunity would be improved in this model. We show that these mice reconstituted with human HSCs exhibit adaptive immune responses and when reconstituted using HSCs from a patient with immune dysregulation, polyendocrinopathy, enteropathy, X-linked (IPEX) syndrome, recapitulate many aspects of the patient's disease. This humanized murine model has the potential to serve as a pre-clinical tool to screen therapeutic alternatives and ultimately facilitate precision medicine.

6.3 Materials and methods

6.3.1 Human hematopoietic stem cell isolation and HLA typing

Human $CD34^+$ HSCs were obtained by positive selection using CD34 microbeads (Miltenyi Biotec, San Diego, CA) on healthy human cord blood. Screening for HLA-DRA*0101, HLA-DRB*0101-matched donor samples was performed at the tissue typing laboratory of Brigham & Women's Hospital using high resolution LABType SSO kits (One Lambda, Canoga Park, CA). The IPEX patient sample was obtained from a bone marrow aspirate with parental consent and approval from the IRB at Boston Children's Hospital prior to allogeneic hematopoietic stem cell transplantation.

6.3.2 Human immune reconstitution

One-day old pups were pre-conditioned using 150 rads of ^{137}Cs source g-radiation. Pups were injected 5 hours later via the intrahepatic route with $3\text{-}5x10^4$ human $CD34^+$ HSCs in PBS.

6.3.3 Human immunophenotyping and flow cytometry

Human immunophenotyping on reconstituted mice was performed at 20 weeks of age. Cells were blocked in 10% rat serum then incubated with fluorochrome-conjugated antibodies for 20' at 4^oC, washed in 2X FACS buffer, and then analyzed using a 3-laser BD FACS Canto II (BD Biosciences, San Jose, CA).

6.3.4 Delayed–type hypersensitivity

Mice were injected subcutaneously with 200 µL emulsion containing 250 µg ovalbumin (OVA) (Sigma-Aldrich, St. Louis, MO) and 100 µL complete Freund's adjuvant (CFA) (Sigma-Aldrich) at the base of the tail. Seven days later, mice were challenged with 50 µL of 10 $\mu g ml^{-1}$ OVA injected into the left footpad. The right footpad was injected with 50 µL of phosphate-buffered saline (PBS) as a control. Footpad swelling was measured at 24 hours using a digital caliper. DTH was calculated as the difference in swelling between the left and the right footpad.

6.3.5 FOXP3 sequencing

A DNA fragment containing the C-terminal DNA binding domain of the *FOXP3* gene was PCR amplified using For: 5'-TAGTCCTGTCCCTGATTACCTGCCCC and Rev: 5'-TGTGCTTGTGTGTGATTGTGTGATGAT primers, gel purified, and sequenced using a For: 5'-GTCTGGGCTCATAGGCACAT sequencing primer.

6.3.5 Immunohistochemistry

Histopathology was carried out on formalin fixed paraffin embedded tissue sections stained with hematoxylin and eosin. Detection of human T cells was performed using an anti-human CD3 antibody (cat# A0452, Dako, Carpinteria, CA) while human macrophages were detected using anti-human CD68 (clone KP1, Dako).

6.3.6 TCR CDR3 sequencing

Splenic human $CD4^+$ T cells were enriched using negative selection (Miltenyi Biotec) and DNA isolated by ethanol precipitation following an overnight proteinase K digestion at 56°C. Purified DNA was subjected to next generation sequencing of the CDR3 region using immunoSEQ (Adaptive Biotechnology, Seattle, WA) and analyzed with the immunoSEQ Analyzer software (Adaptive Biotechnology).

6.3.7 Autoantibody detection

Detection of autoantibodies was performed on cryosections from lung and liver tissue of a non-reconstituted NSGAb°DR1 mouse. Briefly, sections were warmed to room temperature (RT) for 5' and fixed using pre-cooled acetone for 10' at RT. Samples were washed 3X in PBS and blocked using goat serum for 30' at RT followed by incubation with sera from individual mice reconstituted with either IPEX or control HSCs diluted in PBS containing 0.1% Triton X-100 (PBST) overnight at 4°C. Sections were washed 3X for 5' in PBST followed by an incubated with FITC-conjugated goat anti-human IgG (Santa Cruz Biotechnology, Dallas, Texas) for 60' at RT, washed 3X for 5' in PBST, overlaid with vectashield mounting medium containing DAPI (Vector Laboratories, Burlingame, CA) and detected by indirect immunofluorescence.

6.3.8 Suppression assay

A leukocyte suspension was obtained from peripheral blood using Lympholyte (Cedarlane laboratories, Burlington, NC). The $CD4^+$ T cells were enriched by negative selection (Miltenyi Biotec) following the manufacture's protocol. The flow through was then subjected to CD25 positive selection (Miltenyi Biotec) to isolate putative T_{reg} cells. CD25-depleted allogeneic responder $T_{naïve}$ cells were labeled with 5 mM CFSE and $5x10^4$ cells were co-cultured with or without an equal number of IPEX T_{reg} or control T_{reg} cells in the presence of 1 mg plate-bound aCD3 (clone: OKT3, eBiosceince) and 1 mg soluble aCD28 (clone: CD28.6, eBiosceince) in T cell media. After 4 days of culture, proliferation of $T_{naïve}$ cells was assessed by flow cytometry.

6.3.9 ELISAs

Total immunoglobulins in sera of humanized mice were quantified using standard ELISA kits for human IgM (Bethyl Laboratories, Montgomery, TX) and human IgG (Bethyl Laboratories). Human IgE was measured using a capture antibody (clone: MHE-18, BioLegend) and detected using horseradish peroxidase-conjugated anti-human IgE (cat# A9667, Sigma-Aldrich).

6.3.10 Statistical analysis

Statistical analysis was performed using Prism 6 (GraphPad Software, La Jolla, CA).

6.3.11 Mice

NOD.Cg-*Prkdc*^{scid}*H2-Ab1*^{tm1Doi}.Tg(HLA-DQA1,HLA-DQB1) mice were purchased from The Jackson Laboratory (Bar Harbor, Maine). Since these mice were hemizygous for the HLA transgene, inbreeding of this strain occasionally generated NOD.Cg-*Prkdc*^{scid}*H2-Ab1*^{tm1Doi} mice, which were then crossed with the NOD.Cg-*Prkdc*^{scid}*Il2rg*^{tm1Wjl}.Tg(HLA-DRA*0101,HLA-DRB1*0101) strain, also purchased from The Jackson Laboratory. The resulting F1 offspring were inbred to generate NOD.Cg-*Prkdc*^{scid}*Il2rg*^{tm1Wjl}*H2-Ab1*^{tm1Doi}.Tg(HLA-DRA*0101,HLA-DRB1*0101) (NSGAb^{o}DR1) mice. The *H2-Ab1* mutant allele was maintained homozygous while the mutant *Il2rg* allele was maintained homozygous for females and hemizygous for males since it is X-linked. The HLA-DR1 transgene was maintained in a hemizygous state, allowing for some offspring to be negative for HLA-DR1 giving rise to NSGAb^{o} mice used in our studies. The NSG mice were also purchased from The Jackson Laboratory. All mice were maintained in autoclaved cages with autoclaved food and water *ad libitum* in the specific-pathogen free facility at Boston Children's Hospital. All animal experiments were approved and conducted according to the institutional guidelines at Boston Children's hospital.

6.3.12 Antigen–specific immunoglobulin

Mice were immunized against OVA by injecting a 200 ml emulsion containing 250 mg OVA in 100 ml and 100 ml CFA. After two weeks, mice administered a single immunization booster using a 200 ml emulsion containing 50 mg OVA in 100 ml and 100 ml incomplete Freund's adjuvant. Fourteen days later, mice were bled and serum OVA-specific IgG determined by ELISA.

6.3.13. In vitro T cell stimulation and intracellular staining

Total splenocytes were plated at a concentration of $1x10^6$ cells in RPMI 1640 (Life Technologies, Grand Island, NY) supplemented with 10% FBS, 2 mM L-glutamine (Life Technologies), 1 mM sodium pyruvate (Life Technologies), 100 mM non-essential amino acids (Life Technologies), 10 mM HEPES (Life Technologies), 55 mM 2-mercaptoethanol (Life Technologies), and 100 U/ml Penicillin/Streptomycin (Life Technologies) (hereafter referred to as T cell media). Cells were stimulated with stimulated with 20 $ngml^{-1}$ phorbol myristate acetate (PMA) (Sigma-Aldrich) and 1 $ngml^{-1}$ ionomycin (Sigma-Aldrich) for 4 hours at 37^{o}C in the presence of 10 $ngml^{-1}$ GolgiStop (BD Biosciences, San Jose, CA). Cells were collected and washed 2X with FACS buffer (PBS supplemented with 2% FBS and 0.1% NaN_3). Cell surface staining for human TCRb (clone: IP26, BioLegend) was performed for 30' at RT, washed 2X with FACS buffer, then fixed using BD Cytofix/Cytoperm (BD Biosciences) following the manufacture's protocol. Cells were then stained using a cocktail of anti-human antibodies including IFNg (clone: 4S.B3,

BioLegend), TNF (clone: MAb11, BioLegend), IL17A (clone: BL168, BioLegend), and IL10 (clone: JES3-19F1, Biolegend) for 45' at RT. Cells were washed 2X with FACS buffer and intracellular cytokine production detected using a 3-laser FACSCanto II (BD Biosciences) flow cytometer.

6.3.14 Quantitative real–time PCR

qPCR was performed with SYBR Green (Bio-Rad) using a CFX96 real-time PCR (Bio-Rad, Hercules, CA) machine on cDNA generated with the iScript cDNA kit (Bio-Rad) on 1 mg total RNA isolated from whole tissue homogenized in TRIzol (Life Technologies). 2 mM of each human target primer was used in the reaction and quantified by normalizing the cycle threshold (Ct) of the target gene to the Ct value of HPRT and the fold change was compared to a pooled human RNA control sample using the formula $2^{-(Ct(target) - Ct(HPRT))}$. Sequences for human targets are as follows:

IFNg For: 5'-TCGGTAACTGACTTGAATGTCCA, Rev: 5'-TCGCTTCCCTGTTTTAGCTGC,
TNF For: 5'-GAGGCCAAGCCCTGGTATG Rev: 5'-CGGGCCGATTGATCTCAGC,
IL10 For: 5'-GACTTTAAGGGTTACCTGGGTTG Rev: 5'-TCACATGCGCCTTGATGTCTG,
IL12A For: 5'-ATGGCCCTGTGCCTTAGTAGT Rev: 5'-AGCTTTGCATTCATGGTCTTGA,
IL17A For: 5'-TCCCACGAAATCCAGGATGC Rev: 5'-GGATGTTCAGGTTGACCATCAC,
IL4 For: 5'-CGGCAACTTTGTCCACGGA Rev: 5'-TCTGTTACGGTCAACTCGGTG,
IL2 For: 5'-AACTCCTGTCTTGCATTGCAC Rev: 5'-GCTCCAGTTGTAGCTGTGTTT,
IL13 For: 5'-CCTCATGGCGCTTTTGTTGAC Rev: 5'-TCTGGTTCTGGGTGATGTTGA,
HPRT For: 5'-CCTGGCGTCGTGATTAGTGAT Rev: 5'-AGACGTTCAGTCCTGTCCATAA.

6.3.15 Autoantigen array

Antigens were spotted in triplicates onto SuperEpoxy2 slides (Arrayit, Sunnyvale, CA) with a robotic microarrayer (Genetix, Sunnyvale, CA) fitted with solid spotting pins. Nonspecific binding on microarrays was blocked with 1% bovine serum albumin, followed by incubation with serum samples from Control(DR1), IPEX(NSG) and IPEX(DR1) mice (1:100 dilution in blocking buffer). Microrrays were then washed and incubated with Cy3-conjugated goat antibody to human immunoglobulin G (Jackson ImmunoResearch Labs; 109-166-088) and Cy5-conjugated goat antibody to human immunoglobulin M (Jackson ImmunoResearch Labs; 109-176-129). Antigen reactivity was defined by binding to the replicates of that antigen on the microarray. Significant antibody response to autoantigens was determined by comparing raw reactivity observed in IPEX(NSG) and IPEX(DR1) to the cut-off value (mean + 2*SD) calculated in Control(DR1) group for all antigens; only values greater than the cut-off were considered to be a significant antibody response to autoantigens in IPEX(NSG) and IPEX(DR1). Subsequently, antibody reactivity was normalized in IPEX(NSG) and IPEX(DR1) by taking a ratio of raw reactivity observed in IPEX(NSG) and IPEX(DR1) to maximum reactivity observed in IPEX(NSG) and IPEX(DR1).

6.3.16 Flow cytometry antibodies

mCD45 (clone: 30-F11, BioLegend), mEpCam (clone: G8.8, BioLegend) hCD45 (clone: HI30, BioLegend), CD3 (clone: OKT3, BioLegend), CD4 (clone:OKT4, BioLegend), CD8 (clone: RPA-T8, BioLegend), CD25 (clone: 3G10, Miltenyi Biotec), FOXP3 (clone: PCH101, eBioscience, San Diego, CA), CCR7 (clone: 150503, BD Pharmingen, San Jose, CA), CD45RA (clone: HI100, BD Pharmingen), CD127 (clone: A019D5, BioLegend), CD19 (clone: HIB19, BioLegend), CD38 (clone: HB-7, BioLegend), CD27 (clone: O323, BioLegend), IgD (clone: IA6-2, BioLegend), CD14 (clone: HCD14, BioLegend), HLA-DR (clone: L243, BioLegend), CD16 (clone:3G8, BioLegend), CD56 (clone: CMSSB, eBiosceince), CD11c (clone:3.9, BioLegend), CD123 (clone: 6H6, BioLegend), 7-AAD (BD Pharmingen), and CD107a (clone: H4A3, BD Pharmingen).

6.3.17 NK cell cytotoxicity assay

Effector cells (PBMCs or splenocytes from immune replete NSGAb^{o}DR1 mice) were first stimulated with 6000 U/ml of human IL-2 for 24 hours in DMEM media (Cellgro, Manassas, VA) supplemented with 10% heat-inactivated fetal bovine serum (Life Technologies, Grand Island, NY) and 100 U/ml Penicillin/Streptomycin (Life Technologies). Lymphokine-activated killer cells were then co-cultured with 50,000 K562 target cells (ATCC, CCL-243) previously labeled with 5 mM CellTrace Violet (Life Technologies) at a ratio of 32:1 (E:T) at 37^{o}C 5% CO_2 in a 96-well plate. After 4 hours, cells were washed, stained, and quantified by flow cytometry.

6.4 Results

6.4.1 NSG mice expressing human MHC Class II permit enhanced T cell development

To investigate whether human CD4$^+$ T cells can be positively selected and also promote adaptive immune responses in mice that express human MHCII in the absence of murine MHCII, we generated NOD.*Prkdc*^{scid}*Il2rg*$^{-/-}$*H2-Ab1*$^{-/-}$ (NSGAb^o) mice harboring a human HLA-DR1*0101 transgene (NSGAb^{o}DR1) under the control of the murine MHCII promoter. We first confirmed cell surface expression of HLA-DR1 on murine thymic epithelium and splenic antigen presenting cells (APCs) (**Figure 6.S1**). Next, CD34$^+$ cord blood human HSCs were obtained from a healthy HLA-DR1*0101 positive donor and injected intrahepatically into radiation-conditioned 1-day old NOD.*Prkdc*^{scid}*Il2rg*$^{-/-}$ (NSG), NSGAb^o, and NSGAb^{o}DR1 pups. Human leukocyte chimerism at 20 weeks was found to be equivalent among the three strains in peripheral blood, spleen, and bone marrow (**Figure 6.1A**). Immunophenotyping of human leukocytes isolated from recipient strains revealed a slight but statistically significant increase in the proportion of T cells and monocytes with a concomitant/relative decrease in the proportion of B cells in NSGAb^{o}DR1 mice compared to NSG and NSGAb^o mice (**Figure 6.1B** and **Table 6.S1**). There was a trend towards fewer CD4$^+$ T cells in the thymus of NSGAb^o mice suggesting a requirement for either murine or human class II expression for proper

development of human CD4$^+$ T cells (**Figure 6.1C**). Further evaluation of the specific subtypes of CD4$^+$ and CD8$^+$ splenocytes in NSGAb^{o}DR1 mice demonstrated no significant difference in the proportion of naïve, effector, effector memory or central memory cells compared to the other strains (**Table 6.S1**). Most of the CD4$^+$ and CD8$^+$ T cells from NSGAb^{o}DR1 mice were CD45RA$^-$CD45RO$^+$ (data not shown). Interestingly, the relative frequencies of thymic regulatory T cells (Tregs) was slightly elevated in NSGAb^{o}DR1 mice and may reflect higher avidity between human T cell receptor (TCR) and HLA-DR1 (**Figure 6.1C**). T cells isolated from all reconstituted strains were equally adept in their ability to produce human cytokines in vitro following TCR-independent stimulation (**Figure 6.S2**).

6.4.2 NSGAb^{o}DR1 mice develop delayed–type hypersensitivity

Although there was no obvious difference in thymic selection or the development of the CD4$^+$ T compartment when HLA-DR1 was expressed, we hypothesized that the TCR repertoire might exhibit improved diversity that would improve T cell function. Development of a diverse population of antigen-specific T cells requires rearrangement of germline-encoded TCR gene segments (Alt et al., 1992) and is largely mediated by the complementarity determining region 3 (CDR3) within variable (V) gene segments of the TCRb (*TRB*) genes (Jung and Alt, 2004). To assess if human CD4$^+$ T cell selection on HLA-DR1 modulates T cell clonotype diversity, we profiled the CDR3 region of *TRB* on splenic CD4$^+$ T cells in reconstituted mice using high throughput next-generation sequencing (Freeman et al., 2009). A high degree of combinatorial diversity in V gene segment usage was readily observed in splenic CD4$^+$ T cells isolated from either NSGAb^{o}DR1 or NSG mice with the CDR3 length following standard Gaussian distribution in both strains (**Figure 6.1D-E**). A few T cell clonotypes were overrepresented in each of the reconstituted strains but the NSG mice exhibited a higher degree of clonality compared to NSGAb^{o}DR1 mice (**Figure 6.1F**). This diversity was achieved in the absence of exogenous interleukin-7 (IL7), similar to a previous report in mice reconstituted as neonates (Marodon et al., 2009). Although NSGAb^{o}DR1 CD4$^+$ displayed a greater frequency of unique clonotype sequences in the periphery compared to NSG mice (**Table 6.S2**), the normalized TCR diversity index was similar between NSGAb^{o}DR1 and NSG mice (**Figure 6.1D**). These data indicate that selection of human CD4$^+$ T cells on HLA-DR1 does not alter TCR diversity relative to NSG mice but exhibits reduced T cell oligoclonality. Next, we assessed CD4$^+$-dependent adaptive T cell responses *in vivo*. One of the hallmarks of cell-mediated immunity is the ability to mount a rapid response to a previously encountered antigen. Though delayed-type hypersensitivity (DTH) responses to recall antigens have been reported in BLT mice (Rajesh et al., 2010), recall challenge inflammation-associated swelling is minimal in humanized mouse models where there is

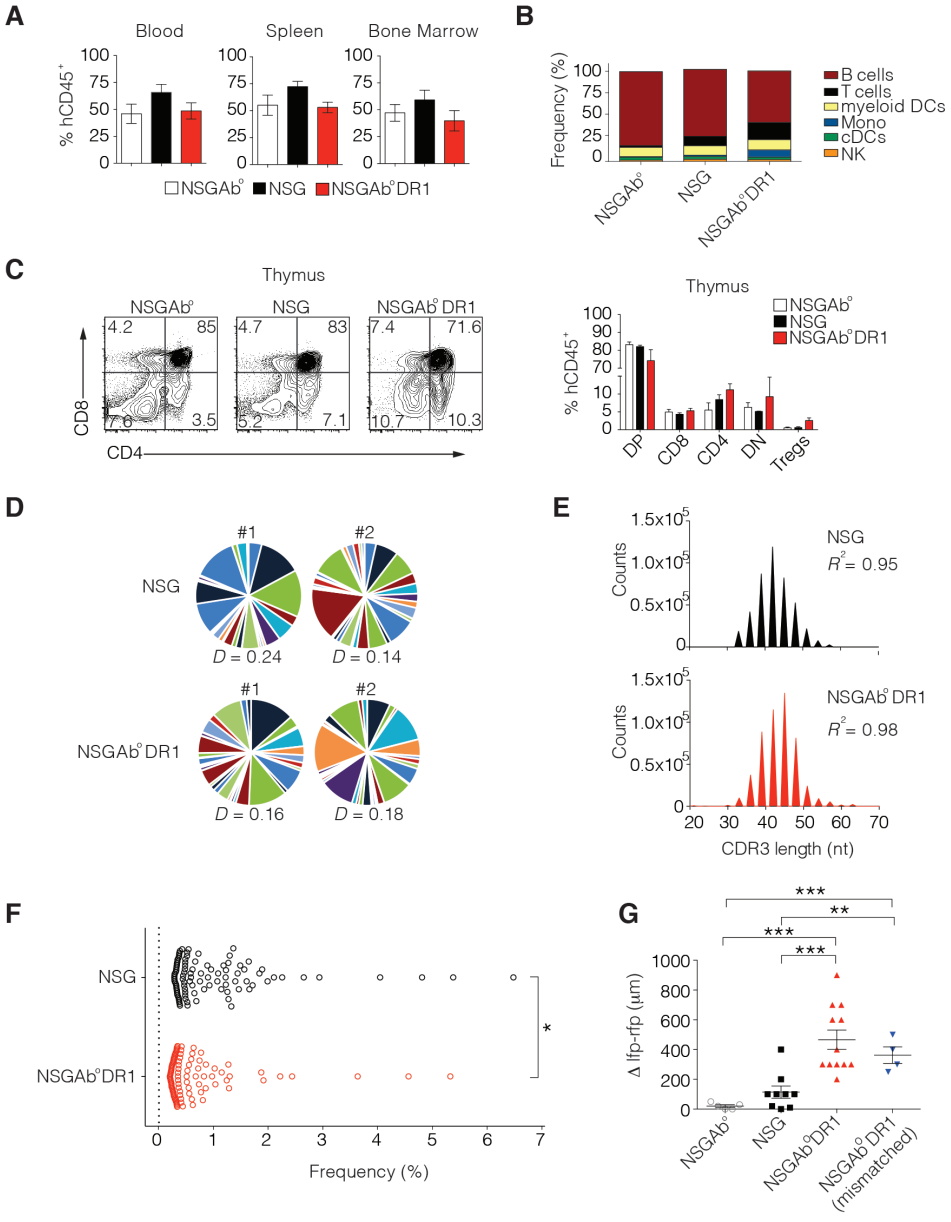


Figure 6.1. Normal T cell development and function in NSGAb°DR1 mice. (**A**) Human chimerism in blood, spleen, and bone marrow determined using human CD45 staining 20 weeks post-reconstitution from a single DR1 allelically matched cord blood donor. n ≥ 6 per group pooled from three independent experiments. (**B**) Human immune cell populations as a percent of human leukocytes in spleens of reconstituted mice. (**C**) Representative flow cytometry dot plots of developing T cells (left) quantified (right) showing double positive (DP), $CD4^+$, $CD8^+$, double negative (DN) and $CD4^+CD25^+FOXP3^+$ Tregs. n ≥ 5 per group from two independent experiments. (**D**) The CDR3 region

of the TCRb locus was profiled using next-generation ultrahigh-throughput sequencing. Pie segments displayed in different colors to highlight unique V-gene segments for NSG and NSGAb^{o}DR1 mice. TCRb repertoire *D*iversity was calculated using normalized Shannon's entropy ($P > 0.05$). (**E**) Histogram of CDR3 nucleotide length in CD4^{+} T cells isolated from spleens of reconstituted NSG or NSGAb^{o}DR1 mice. (**F**) The relative frequency or "evenness" of the CD4^{+} TCRb repertoire was plotted for the top 100 clonotypes in two representative animals in each genotype with each circle depicting an individual clonotype. (**G**) Delayed-type hypersensitivity response in OVA-immunized mice displayed as the difference in swelling between OVA-injected left footpad (lfp) and PBS-injected right footpad (rfp). NSGAb^{o}DR1 (mismatched) refers to reconstitution using HSCs from a donor that was negative for the DRB1*01:01 allele. Data are pooled from two independent experiments. Bars are the mean ± SEM. Statistical analysis performed using one-way ANOVA with Tukey's multiple comparisons test (A), multiple *t* test corrected for multiple comparisons using Holm-Sidak method (C), non-linear regression (E), unpaired *t* test (F, G) $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$

absence of autologous human tissue graft or exogenous recombinant IL7 (Lepus et al., 2009; Unsinger et al., 2009). We compared DTH responses to ovalbumin (OVA) in NSGAb^{o}, NSG, and NSGAb^{o}DR1 mice that were previously immunized subcutaneously against OVA. While we observed low degree of footpad swelling in NSG mice, similar to a previous report (Unsinger et al., 2009), NSGAb^{o}DR1 mice, when compared to NSG or NSGAb^{o} mice, exhibited greater footpad swelling in response to the recall antigen following secondary challenge indicating improved immunological memory responses are present (**Figure 6.1G**). This improved recall response also occurred when NSGAb^{o}DR1 mice were reconstituted using donor HSCs that were not matched for the HLADR1*01:01 allele (**Figure 6.2G**). While these data indicate that CD4^{+} T cells have functional capacity, CD8^{+} T cells and NK cells from NSGAb^{o}DR1 mice also have functional properties at least *in vitro*, as evidenced by IFNg secretion (**Figure 6.S3A**) and target cell lysis respectively (**Figure 6.S3B-C**).

6.4.3 Human B cell maturation and class switch recombination occurs in NSGAb^{o}DR1 mice

T cells also play a critical role in the humoral arm of adaptive immunity as rodents and humans lacking T cells exhibit profound defects in B cell maturation and antibody class switching (Luzzati and Jacobson, 1972; Mond et al., 1982). Previous data in humanized NSG mice show varying stages of human B cell development; however, progression to mature naïve B cells from immature transitional B cells remains poor with low levels of class switched antibodies (Biswas et al., 2011; Chang et al., 2012; Watanabe et al., 2009; Xu et al., 2014b). We analyzed the immature transitional splenic B cell population in reconstituted NSGAb^{o}, NSG, and NSGAb^{o}DR1 mice by staining for CD45, CD19, CD24 and CD38. NSGAb^{o}DR1 mice had a significant decrease in the proportion of CD45^{+}CD19^{+}CD24^{hi}CD38^{hi} immature transitional B cells with a concomitant increase in CD45^{+}CD19^{+}CD24^{int}CD38^{int} mature naïve B cells (**Figure 6.2A**) (Cuss et al., 2006; Palanichamy et al., 2009). Consistent with an increase in mature naïve B cells, serum IgG and IgE levels were also increased, indicating that immunoglobulin class switching was intact in NSGAb^{o}DR1 mice (**Figure 6.2B**), as was previously seen in NRG mice expressing both murine MHCII and human HLA-DR4 (DRAG mice) (Danner et al., 2011). Immunization against OVA led to the development of OVA-

specific IgG in some but not all NSGAb^{o}DR1 mice (**Figure 6.S4**). Collectively, these data indicate that adaptive humoral immune responses are present when human CD4^{+} T cells are restricted by human HLA-DR1 in the absence of murine MHCII.

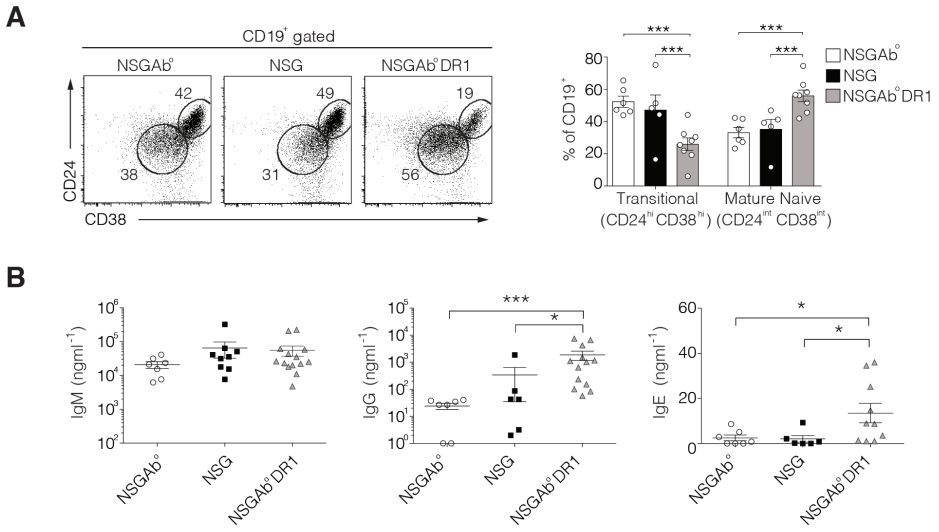


Figure 6.2. Increased B cell maturation and class switched immunoglobulins in reconstituted NSGAb^{o}DR1 mice. (**A**) Representative flow cytometry dot plots of splenic B cells gated on CD45^{+}CD19^{+} (left) and quantified (right) from three independent experiments with n ≥ 5 per group. (**B**) Serum immunoglobulin levels quantified by ELISA. Each data point represents an individual mouse from three independent experiments. Solid bars are the mean ± SEM. Statistical analysis was performed using multiple *t* test corrected for multiple comparisons using Holm-Sidak method (A), Mann-Whitney test (B), $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$

6.4.4 Multi–organ inflammation and mortality in NSGAb^{o}DR1 mice reconstituted with IPEX CD34^{+} HSCs

One inherent limitation of the BLT humanized mouse system is the requirement for an autologous setting comprising human thymus, liver, and HSCs from the same donor source, which prohibits assessment of immune dysfunction associated with patient-specific genetic abnormalities. The improved adaptive immune responses in humanized NSGAb^{o}DR1 mice led us to test whether immune reconstitution using HSCs isolated from a patient with a genetically defined immunological disorder would transfer manifestations of the human disease to recipient mice. Given that CD4^{+} T cell function is relatively intact in NSGAb^{o}DR1 mice, we hypothesized that transplantation of HSCs from a patient in which CD4^{+} T cells, especially Tregs, are dysfunctional and directly involved in disease pathogenesis would permit assessment of the utility of this model system. We identified a 7-month old male

infant patient who presented with secretory watery non-bloody diarrhea, eczema, and enteropathy and found to have a nonsynonymous single-nucleotide polymorphism at position c.A1226C in the forkhead box protein 3 (*FOXP3*) gene (**Figure 6.3A**). This mutation resulted in a p.D409A amino acid change in the C terminal DNA binding domain, the same location previously reported in IPEX syndrome (Rao et al., 2007). IPEX syndrome is a rare, and often fatal, X-linked recessive disorder caused by loss-of-function mutations in FOXP3, the master transcriptional regulator of Tregs that is essential for establishment and maintenance of central tolerance (Bennett et al., 2001; Fontenot et al., 2003; Sakaguchi et al., 1985; Schubert et al., 2001). Patients with IPEX syndrome develop enteropathy, eczema, autoantibodies, and sometimes insulin-dependent diabetes mellitus (Gambineri et al., 2008). Mice harboring a loss-of-function mutation in *Foxp3* (*Foxp3*^{sf}) display a similar phenotype with multi-organ inflammation attributed to autoreactive CD4$^+$ T cells (Blair et al., 1994; Mayer et al., 2014). Although FOXP3 expression was intact in CD4$^+$CD25$^+$ Tregs isolated from this patient (**Figure 6.3B**), which has also been described for other *FOXP3* mutations associated with IPEX syndrome (Kinnunen et al., 2013), suppression of allogeneic naïve T cell proliferation by the patient's Tregs was profoundly impaired (**Figure 6.3C**).

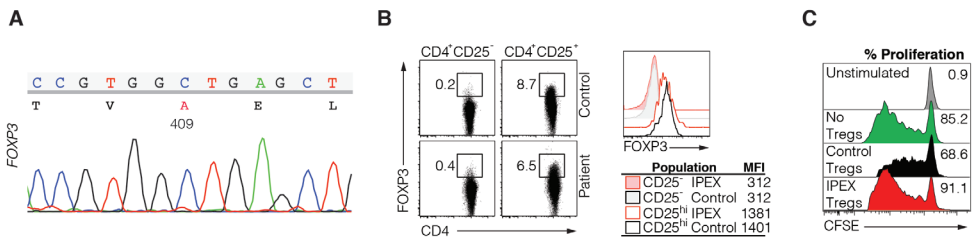


Figure 6.3. Identification of a novel variant in *FOXP3* associated with IPEX syndrome. (**A**) Sequence of the *FOXP3* gene in the IPEX patient. (**B**) FOXP3 expression in T_{regs} (CD4$^+$CD25^{hi}) and $T_{naïve}$ (CD4$^+$CD25$^-$) from the IPEX patient and healthy control shown by flow cytometry (left) and histogram the mean fluorescence intensity (MFI) (right). (**C**) *In vitro* suppression assay using T_{regs} from a healthy control or IPEX patient and carboxyfluorescein succinimidyl ester (CFSE)-labeled allogeneic responder T cells.

To test whether transfer of HSCs from this IPEX patient would cause a spontaneous IPEX-like disease in NSGAb^{o}DR1 and NSG mice, bone marrow-derived CD34$^+$ HSCs were purified and used to engraft 1-day-old NSG and NSGAb^{o}DR1 recipient mice, hereafter referred to as IPEX(NSG) and IPEX(DR1) respectively. In parallel, bone marrow-derived CD34$^+$ HSCs from a healthy pediatric donor were injected into NSGAb^{o}DR1 mice as controls, referred to as Control(DR1) mice. Since the patient was not an allelic match with the DR1 allele expressed in the mice, we also selected mismatched bone marrow as a control to account for any potential pathology due to HLA mismatch. Mice were monitored for the development of clinical

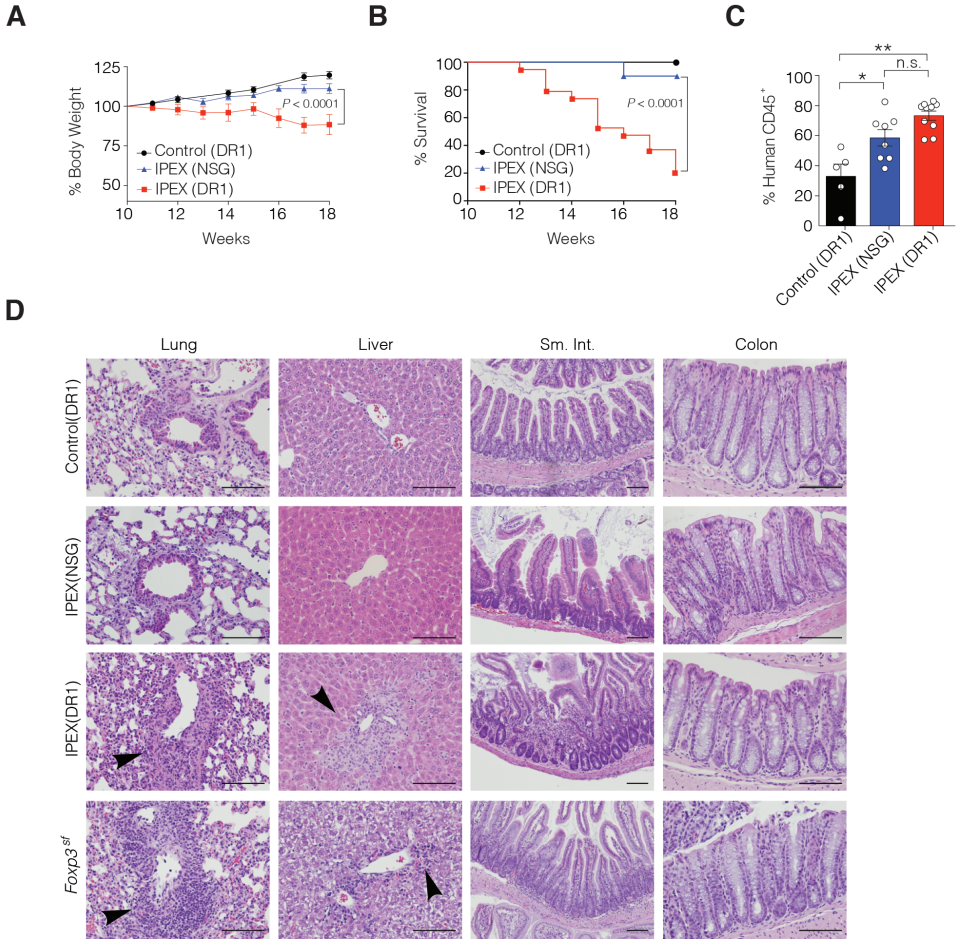


Figure 6.4. NSGAb^{o}DR1 mice reconstituted with IPEX hematopoietic stem cells causes mortality and multi-organ inflammation. (**A**) Longitudinal assessment of body weight change compared to weight at 10 weeks from two independent experiments. (**B**) Kaplan-Meier survival curve comparing reconstituted mice. The 18-week cutoff was selected based on 100% lethality in IPEX(DR1) mice by this time point in the first experimental cohort in order to compare variability across two independent experiments. Control(DR1) n=10, IPEX(NSG) n=10, IPEX(DR1) n=19. (**C**) Human chimerism in spleen of reconstituted mice assessed by flow cytometry pooled from two experiments. Open circles are individual mice. Bars are the mean ± SEM. (**D**) H&E stained sections from the lung, liver, small intestine (Sm. Int.), and colon of NSGAb^{o}DR1 mice reconstituted with control bone marrow CD34^{+} HSCs or IPEX CD34^{+} HSCs, NSG mice reconstituted with IPEX CD34^{+} HSCs, and *Foxp3*^{sf} mice. Black arrowheads denote leukocyte infiltrate. Scale bars are 100 mm. Statistical analysis performed using one-way ANOVA with Tukey's multiple comparisons test (A, C), curve comparison using Log-rank Mantel-Cox test (B). *$P < 0.05$, **$P < 0.01$

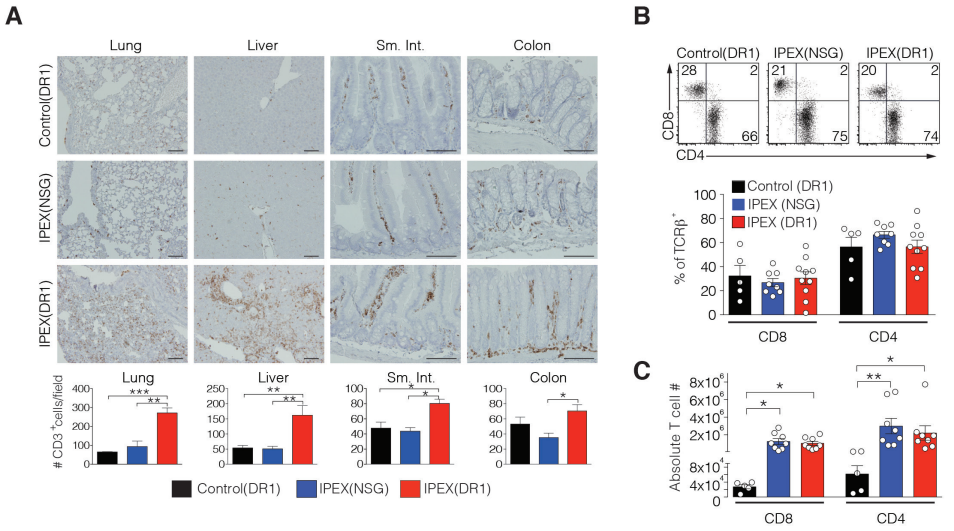


Figure 6.5. Human T cell inflammation in IPEX(DR1) mice. Immunohistochemical analysis detecting human immune cell infiltration using (**A**) anti-hCD3 staining on lung, liver, small intestine, and colon tissue sections (top) quantified by counting the number of $CD3^+$ cells/20X field for 3 mice per group (bottom). (**B**) Representative flow cytometry dot plots of splenic T cells gated on $TCR\beta^+$ (left) with the frequency of $CD4^+/CD8^+$ T cells reported (right). Open circles are individual mice. (**C**) The absolute numbers of splenic T cells were quantified. Open circles are individual mice. All values are mean ± SEM. Statistical analysis performed using one-way ANOVA with Tukey's multiple comparisons test (A), one-way ANOVA using Kruskal-Wallis multiple comparisons test (B, C). $^*P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$

manifestations characteristic of patients with IPEX syndrome and *Foxp3*^{sf} mice. IPEX(DR1) mice exhibited significant weight loss and increased mortality by 18 weeks post-transfer which was rarely observed in IPEX(NSG) mice and never observed in Control(DR1) mice (**Figure 6.4A-B**). Similar to the absence of diabetes in this patient, IPEX(DR1) mice also did not exhibit signs or symptoms of insulitis. At necropsy, IPEX(NSG) and IPEX(DR1) mice exhibited splenomegaly (not shown) with increased human immune cells in the spleen compared to Control(DR1) mice (**Figure 6.4C**). Since IPEX patients, *Foxp3*^{sf} mice, and mice with targeted depletion of FOXP3-expressing cells, develop multi-organ inflammation (Lahl et al., 2007), we analyzed organs known to be affected by Treg-deficiency. Similar to observations in *Foxp3*^{sf} mice (Chen et al., 2005), inflammation was consistently observed by immunohistochemistry in the lung and liver of IPEX(DR1) mice with mild leukocyte infiltration also observed in the small intestine (**Figure 6.4D**). Given the inflammation detected in these organs, we screened whole tissue to quantify human inflammatory cytokines which were broadly elevated in both lung and liver tissue of IPEX(DR1) mice compared with IPEX(NSG) and Control(DR1) mice as anticipated (**Figure 6.S5**). This inflammatory infiltrate consisted of human $CD3^+$ T cells, and

to a lesser extent human CD68$^+$ macrophages, in the lung and liver tissue of IPEX(DR1) mice but not IPEX(NSG) mice or Control(DR1) mice (**Figure 6.5A** and **6.S6**). We assessed the T cell compartment and found similar proportions of CD4$^+$ and CD8$^+$ T cells in the spleens of all three groups (**Figure 6.5B**). The absolute numbers of CD4$^+$ and CD8$^+$ T cells were increased in both IPEX(NSG) and IPEX(DR1) mice, with a normal CD4$^+$/CD8$^+$ ratio, reminiscent of the lymphoproliferation observed in *Foxp3^{sf}* mice (**Figure 6.5C**) (Huter et al., 2008). We tested the function of IPEX human T_{regs} by isolating the CD4$^+$CD25$^+$ fraction from several pooled spleens of IPEX(DR1) mice and found, analogous to the patient's Treg suppression defects, that the ability to suppress autologous CD4$^+$CD25$^-$ T cells was impaired (**Figure 6.S7**).

6.4.5 Engraftment of IPEX CD34$^+$ HSCs leads to autoantibody production

Defective Treg function in both *Foxp3^{sf}* mice and IPEX patients is also associated with hyperimmunoglobulinemia, loss of peripheral B-cell tolerance, and the development of autoantibodies (Aschermann et al., 2013; Bacchetta et al., 2006; Lampasona et al., 2013; López et al., 2011). Similar to observations in patients with IPEX syndrome and in *Foxp3^{sf}* mice (Godfrey et al., 1991; Lampasona et al., 2013; López et al., 2011) serum IgG and IgE levels were elevated in both IPEX(NSG) and IPEX(DR1) mice compared to Control(DR1) mice (**Figure 6.6A**). Patients with IPEX syndrome develop autoantibodies and our patient tested positive for several including antibodies against glutamic acid decarboxylase, thyroperoxidase, and nuclear antigens (Lampasona et al., 2013; Tsuda et al., 2010) We investigated whether human mouse-specific autoantibodies were produced in mice reconstituted with HSCs from the IPEX patient. Indirect immunofluorescence on cryosectioned lung and liver tissue revealed the presence of autoreactive IgG in serum from IPEX(DR1) mice that co-localized with DAPI stained nuclei and was not observed in sera from IPEX(NSG) or Control(DR1) mice (**Figure 6.6B**). Using a more global approach, sera from Control(DR1) mice, IPEX(NSG) mice, and IPEX(DR1) mice were screened using an autoantigen microarray spotted with 500 murine lipid and peptide antigens. Broad IgM and IgG autoreactivity was detected in IPEX(DR1) mice, and to a lesser extent in IPEX(NSG) mice, with several autoantibodies commonly observed in human autoimmune diseases including gliadin, dsDNA, and collagen (**Figure 6.6C** and **6.S8-9**). While there was a clear correlation between autoantibody reactivity and the percentage of human B cells in IPEX(DR1) mice, this was not the case for IPEX(NSG) mice (**Figure 6.6D**). This suggests that the increased autoreactivity is likely attributed to T cell help provided by DR1-restricted CD4$^+$ T cells. It is noteworthy that the higher levels of autoantibodies also did not appear to correspond to early mortality in IPEX(DR1) mice suggesting that additional factors are likely dominant (**Figure 6.6D**).

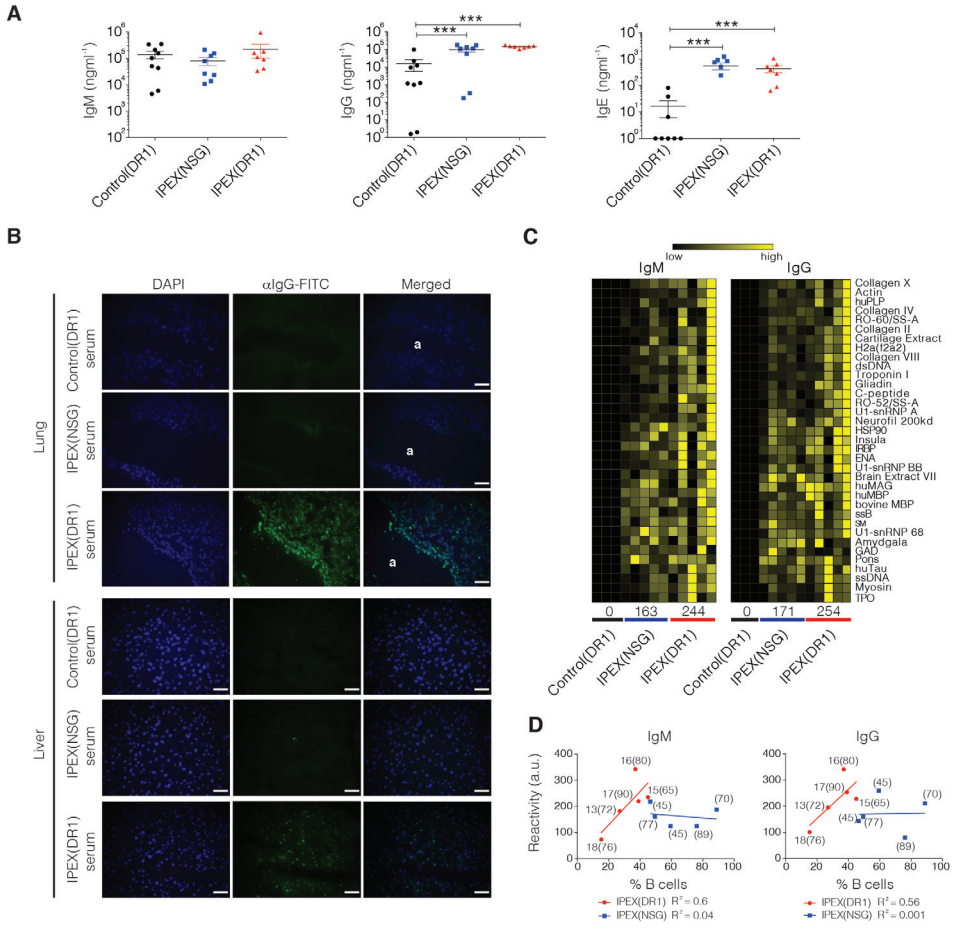


Figure 6.6. Loss of B cell tolerance in IPEX mice. (**A**) Serum immunoglobulin levels quantified by ELISA from two independent experiments. (**B**) Autoantibody detection by indirect immunofluorescence on cryosections of frozen lung and liver tissue from a non-humanized NSGAb°DR1 mouse. Frozen sections were incubated independently with sera from 4 individual reconstituted mice per group. FITC-conjugated anti-human IgG was used as a secondary antibody combined with DAPI to label nuclei. (a) denotes lung airway. Scale bars are 50 mm. (**C**) Heat map of antibody response to select murine antigens determined using an antigen microarray. Each column is the IgM and IgG serum reactivity from an individual mouse in each group with the averaged reactivity score for all antigens per group noted at the bottom. IgM $P = 0.06$, IgG $P = 0.09$ (**D**) The normalized cumulative autoreactive IgM and IgG antibody reactivity values for all 500 mouse antigens in arbitrary units (a.u.) were plotted against the % human B cells in each mouse (n=5). Solid line marks the best-fit linear regression for each cohort with the R^2 value calculated using Pearson correlation coefficients. For each data point the % of human chimerism is shown in parenthesis and for IPEX(DR1) mice, the age in weeks when mice became moribund and were euthanized. Statistical analysis was performed using Mann-Whitney test (A) and nonlinear regression correlation using Pearson correlation coefficients (D). ***$P < 0.001$

6.5 Discussion

The main goal of this study was to develop a humanized mouse strain that would support human adaptive immune function and enable *in vivo* modeling of a monogenic human immune-mediated disease using patient HSCs. Since $CD4^+$ T cells are known to play critical roles in maintaining immune homeostasis, we reasoned that a setting in which human $CD4^+$ T cells were selected on a human HLA class II molecule in the absence of murine MHCII might improve thymic positive selection and, consequently, adaptive immune responses in humanized mice. The NSGAb^{o}DR1 mice described in this study were an attractive strain to test this hypothesis especially given the frequency of the HLA-DR1 allele in the population (Mori et al., 1997).

The improved $CD4^+$ T cell recall response in NSGAb^{o}DR1 mice may result from more effective interactions between DR1-restricted $CD4^+$ T cells and HLA-DR1/peptide complexes presented by $DR1^+$ APCs resulting from co-evolution of the human TCR and human MHCII molecules (Huseby et al., 2005; Kim et al., 2005) Although we observed a slight but significant increase in T cell frequency in NSGAb^{o}DR1 mice compared to NSG mice, we did not observe an increase in the frequency of $CD4^+$ T cells, which is in contrast to the increase in $CD4^+$ T cells reported in DRAG mice that also express a human class II molecule (Danner et al., 2011). This difference may result from expression of both human and murine class II in DRAG mice, which permits interspecies a/b MHCII pairing and enhanced positive selection.

Humoral immunity in humanized mice has generally been poor with B cells exhibiting an immature phenotype and yielding lower levels of serum immunoglobulins compared to humans (Brainard et al., 2009; Gorantla et al., 2007). In our studies, B cell maturation and antibody class switch recombination was increased in reconstituted NSGAb^{o}DR1 mice compared to NSG and NSGAb^o mice. This increase is may be a result of improved cognate interactions between HLA-DR1 restricted $CD4^+$ T cells and HLA-$DR1^+$ B cells triggering cytokine secretion and antibody class switching (Oxenius et al., 1998). The fact that B cells in NSG mice exhibited a more immature phenotype and relatively lower serum IgG and IgE levels suggests that MHCII-restricted $CD4^+$ T cells in NSG mice are less efficient at providing T cell help to B cells.

The most significant finding of our study was that HSCs from a patient with IPEX syndrome caused immunodysregulation in NSGAb^{o}DR1 mice. To date, human HSCs with a causative mutation of a monogenic disease triggering a similar disease phenotype in humanized mice has not been reported. Interestingly the disease phenotype was not readily observed when these HSCs were transferred into the NSG strain despite NSG mice having increased T cell numbers, elevated serum immunoglobulins, and autoantibodies compared with control bone marrow HSCs. This is likely attributed to the enhanced adaptive immune responses seen in reconstituted NSGAb^{o}DR1 mice. Similar to the phenotype in our IPEX(DR1) mice, a recent report also demonstrated a role for human Tregs in immune homeostasis in humanized mice by showing that administration of antibodies blocking CTLA-4, a molecule critical for Treg suppressive function (Jain et al., 2010; Tai et al., 2012; Wing et al., 2008) also

caused weight loss, liver inflammation, and anti-nuclear antibodies (Vudattu et al., 2014). One possibility for the multi-organ inflammation is that the IPEX mice developed allogeneic graft vs. host disease (GVHD) since the IPEX donor was not matched for the DR1*01:01 allele; a previous report using NSGAb^{o} mice expressing human DR4 injected with human PMBCs resulted in allogeneic GVHD (Covassin et al., 2011). However, human stem cell reconstitution in one-day old NSGAb^{o}DR1neonates gives rise to human T cells selected on murine (and likely human) antigens in the mouse thymus where autoreactive T cells are likely deleted. In this regard, using this method, we have never observed characteristic xeno-GVHD symptoms (weight loss or alopecia) following reconstitution even in NSG mice. Moreover, control HSCs that were negative for the DR1*01:01 allele did not result in systemic T cell inflammation following reconstitution in NSGAb^{o}DR1 mice.

In summary, genetic studies in mice have advanced our understanding of gene/protein function in health and disease giving rise to new therapeutic targets. Even so, disparities persist between validated therapeutics using rodent models and improved clinical outcomes necessitating the development of new model systems (McKenzie et al., 1995; Suntharalingam et al., 2006). Humanized mice provide the opportunity to study the function of human immune cells in an *in vivo* setting without risk to human subjects. The model described here exhibits enhanced adaptive immune function which circumvents the requirement of surgically implanted autologous thymic and liver tissue and enables use of HSCs from genetically defined patients (Vudattu et al., 2014; Zheng et al., 2013) These mice will likely be most useful for studies pertaining to T cell responses and immune-mediated diseases resulting from T_{reg} dysfunction and/or aberrant effector CD4^{+} T cell activation. One pre-clinical utility of this model would permit assessment of the efficacy and safety of novel genome editing or vector-based gene therapy strategies aimed at correcting monogenic immunodeficiencies; early trials using retroviral-based vectors had complications due to vector-mediated insertional activation of cellular oncogenes (Cuss et al., 2006; Gaj et al., 2013; Howe et al., 2008). Overall, we propose that NSGAb^{o}DR1 mice, alone or in combination with additional HLA molecules and/or other human cytokines (Rongvaux et al., 2014) will further the utility and usefulness of humanized mice to study human immune responses in health and disease and the development of emerging immunotherapies.

6.6 Acknowledgments

This work was supported by NIH NRSA (F32DK097894) (J.A.G.), NIH Grants HL59561, DK034854, and AI50950, the Helmsley Charitable Trust, and the Wolpow Family Chair in IBD Treatment and Research (S.B.S.), as well as NIH AI075037 (to E.F.). We would also like to thank Emanuele Bosi and Vito Lampasona for their assistance with patient serum and the BIDMC Research Histology core.

6.7 Authorship contributions

J.A.G., S.B, W.S.L, A.Y, S.Y., J.S., W.A.M., D.M, E.F., F.J.Q., B.H.H., A.M., and S.B.S designed the experiments, collected and analyzed data. J.O., D.S.S., K.J.M, L.D.N., L.P., M-G.R., R.B., and S-Y.P assisted with acquisition of patient samples. E.L.M. performed HLA typing. B.P. analyzed autoantigen array data. J.A.G. wrote the manuscript.

6.8 Supplemental data

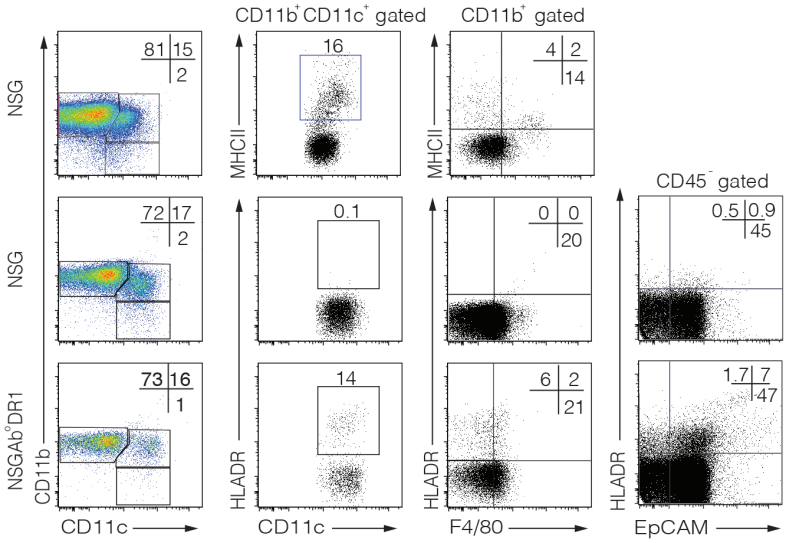


Figure 6.S1. Expression of human HLA-DR1 in NSGAb°DR1 mice. Representative flow cytometry dot plots demonstrating expression of human HLA-DR1 on murine splenic dendritic cells ($CD11b^+CD11c^+$) and macrophages ($CD11b^+F4/80^+$), and thymic epithelium ($CD45^-EpCAM^+$) from NSGAb°DR1 mice compared to murine MHCII expression on splenic dendritic cells and macrophages from NSG control mice.

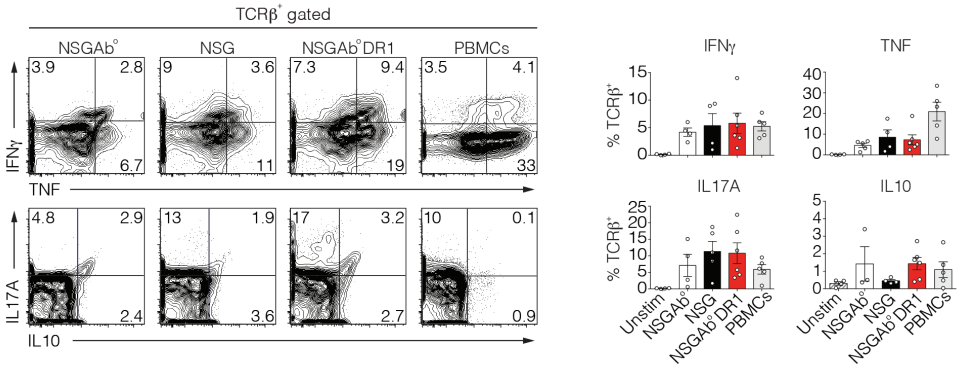


Figure 6.S2. Human T cells in humanized mice are functional. Splenocytes from indicated mice and human peripheral blood mononuclear cells (PBMCs) were stimulated with PMA/Ionomycin for 4 hours. Representative flow cytometry dot plots for intracellular cytokine expression gated on TCRb$^+$ cells (left) and quantified (right). All bars are mean ± SEM.

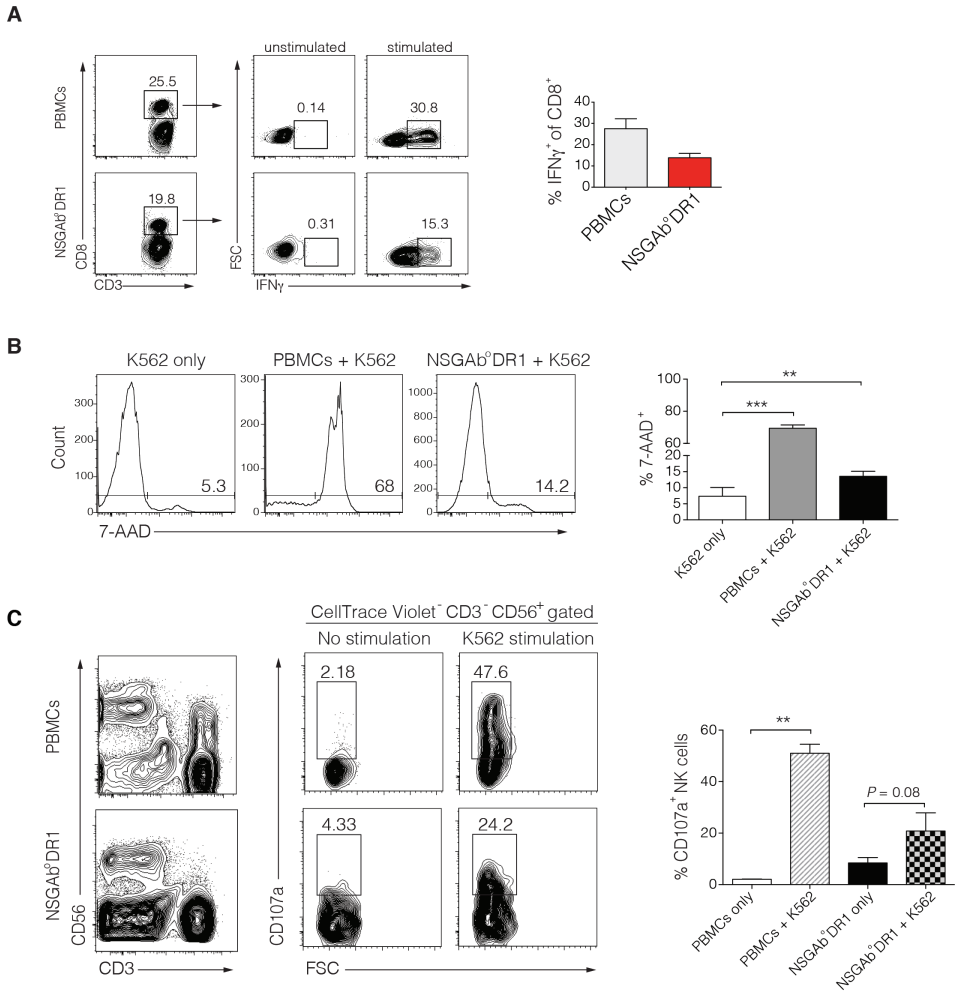


Figure 6.S3. Human CD8⁺ and NK cells isolated from NSGAb°DR1 mice are functional *in vitro*. (**A**) Activation of CD8⁺ T cells in PBMCs and from reconstituted NSGAb°DR1 splenocytes was assessed following stimulation with PMA and ionomycin for 6 hours followed by intracellular staining for IFNg. (**B**) Lymphokine-activated PBMCs or splenocytes from reconstituted NSGAb°DR1 mice were co-cultured with CellTrace Violet-labeled K562 target cells for 4 hours. Cytotoxic killing of K562 cells was determined by 7-AAD incorporation into CellTrace Violet⁺ cells with representative histograms shown (left) and quantified (right) (n=4) from two independent experiments. (**C**) Degranulation of human NK cells (CellTrace Violet⁻ CD3⁻CD56⁺) following stimulation with K562 target cells was measured by cell surface expression of CD107a with a representative flow cytometry dot plot shown (left) and quantified (right). Statistical analysis was performed using one-way ANOVA with multiple comparisons and unpaired *t*-test respectively. $^{**}P < 0.01$, $^{***}P < 0.0001$

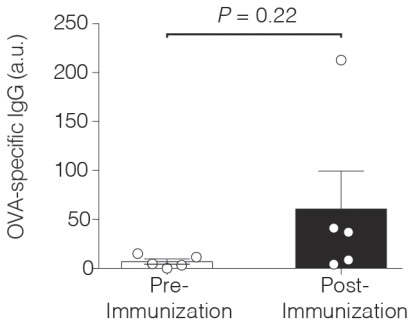


Figure 6.S4. Development of OVA-specific IgG in NSGAb^{o}DR1 mice. Twelve-week-old NSGAb^{o}DR1 mice were bled prior to immunization and serum was stored frozen. Mice were immunized against OVA using an emulsion containing CFA as an adjuvant. A booster immunization containing OVA using incomplete Freund's adjuvant was administered two weeks later with serum isolated after an additional two weeks. Sera was screened for OVA-specific IgG using a standard ELISA and compared to pre-immunization OVA-specific IgG levels. Open circles represent individual animals. Values are the mean ± SEM. Statistical analysis was performed using paired *t*-test.

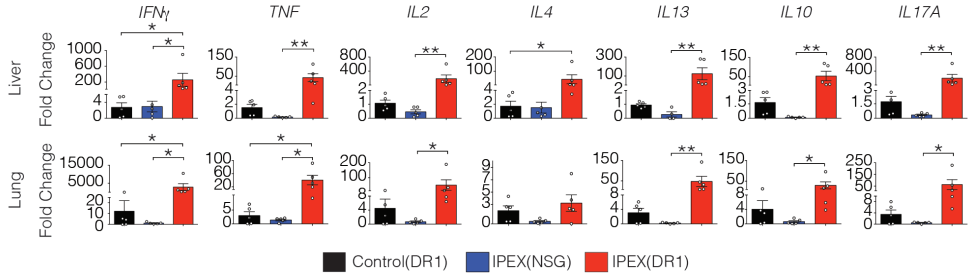


Figure 6.S5. IPEX(DR1) mice have increased pro-inflammatory human cytokines transcripts in lung and liver tissue. mRNA isolated from lung and liver tissue of reconstituted mice was reverse transcribed to make cDNA using oligo(dT) primers. Relative human cytokine transcript levels were determined by quantitative real-time PCR normalized to hypoxanthine phosphoribosyltransferase (*HPRT*) and compared to Control(DR1) mice using the ΔΔCt method to calculate fold change. n ≥ 4 per group shown as the mean ± SEM. Statistical analysis was performed using one-way ANOVA using Kruskal-Wallis multiple comparisons test $^{*}P < 0.05$, $^{**}P < 0.01$

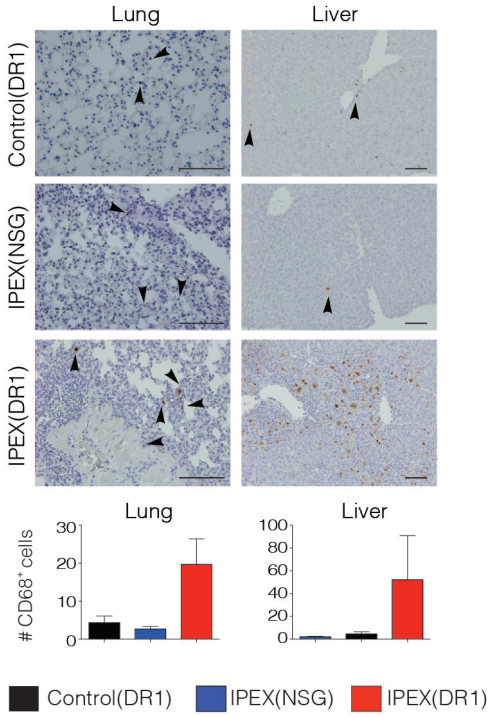


Figure 6.S6. Infiltrating human macrophage in lung and liver of IPEX(DR1) mice. Immunohistochemical analysis detecting human macrophage using anti-hCD68 staining on lung and liver tissue sections counterstained with hematoxylin (top) and quantified by counting the number of $CD68^+$ cells/20X field for 3 mice per group (bottom). Black arrowheads denote human $CD68^+$ cells. Scale bars are 100 mm. Statistical analysis performed using one-way ANOVA with Tukey's multiple comparisons test.

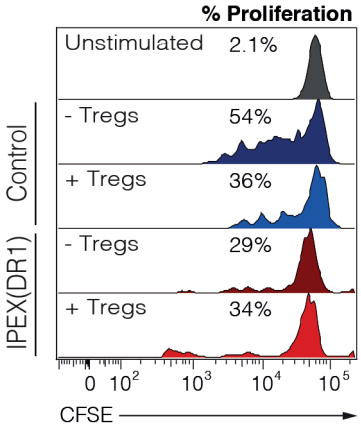


Figure 6.S7. IPEX(DR1) T_{regs} fail to suppress naïve T cell proliferation. $CD4^+CD25^+$ T_{reg} cells were isolated from spleens of Control(DR1) or IPEX(DR1) mice and cultured with 50k CFSE-labeled autologous $CD4^+CD25^-$ responder T cells stimulated with 1 $mgml^{-1}$ plate bound aCD3 and 1 $mgml^{-1}$ soluble aCD28 antibodies for 72 hrs. Percent proliferation was determined by CFSE dilution using flow cytometry.

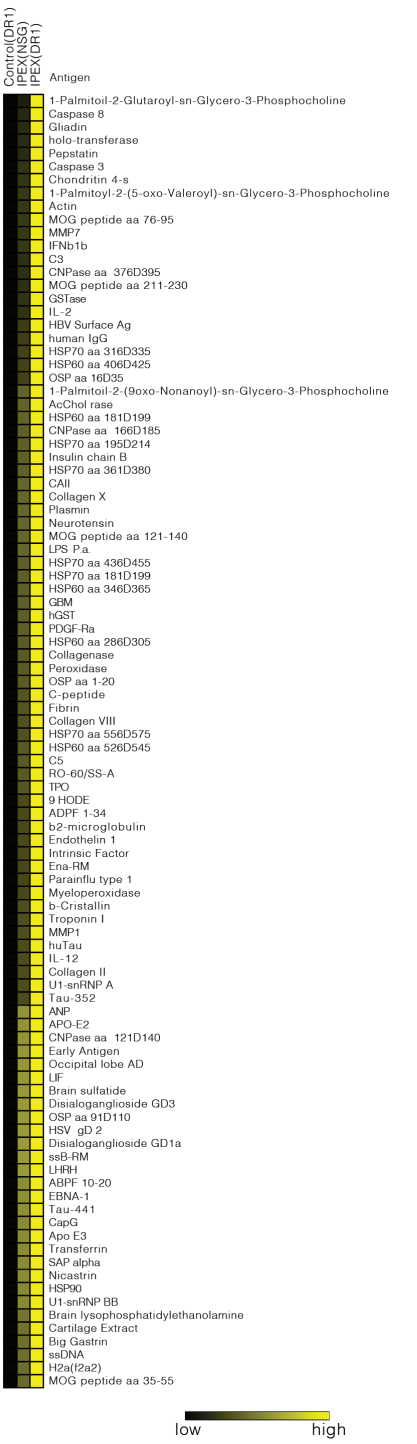


Figure 6.S8. Increased IgM autoantibodies in mice reconstituted with IPEX HSCs. Heat map of antibody response to autoantigens determined using an antigen microarray. Each column represents the mean serum reactivity of IgM for each group. Control(DR1) n=3, IPEX(NSG) n=5, IPEX(DR1) n=5.

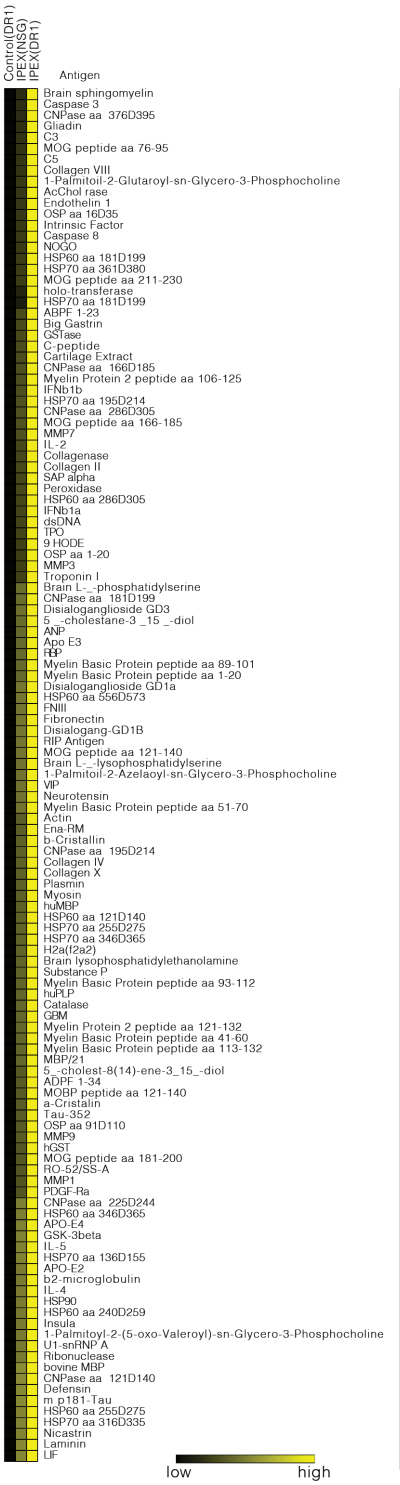


Figure 6.S9. Increased IgG autoantibodies in mice reconstituted with IPEX HSCs. Heat map of antibody response to autoantigens determined using an antigen microarray. Each column represents the m mean serum reactivity of IgG for each group. Control(DR1) n=3, IPEX(NSG) n=5, IPEX(DR1) n=5.

Table S1. Splenic human leukocyte frequencies determined by flow cytometry.

	NSGAb^o	NSG	NSGAb^{o}DR1
T cells (% CD3$^+$ of hCD45$^+$)	2.0 (1.0)	12.6 (7.5)	***23.8 (4.1)***
CD4 (% CD4$^+$ of CD3$^+$)	7.9 (3.4)	63.1 (5.6)	44.9 (6.6)
Naive (CCR7$^+$ CD45RA$^+$)	3.4 (0.88)	10.5 (3.2)	7.2 (1.2)
Effector (CCR7$^-$ CD45RA$^+$)	8.6 (3.2)	15.8 (4.7)	13.9 (2.0)
Effector Memory (CCR7$^-$ CD45RA$^-$)	10.7 (6.0)	32.5 (5.1)	24.7 (3.3)
Central Memory (CCR7$^+$ CD45RA$^-$)	3.5 (1.3)	8.4 (2.4)	4.6 (1.0)
Treg (% CCR4$^+$ CD127$^{lo/-}$of T cells)	0.34 (0.20)	5.5 (2.1)	2.4 (0.43)
CD8 (% CD8$^+$ of CD3$^+$)	42.7 (12.9)	25.4 (4.4)	34.9 (5.8)
Naive (CCR7$^+$ CD45RA$^+$)	18.8 (6.8)	5.22 (2.2)	11.9 (3.5)
Effector (CCR7$^-$ CD45RA$^+$)	12.5 (3.9)	6.4 (1.6)	11.9 (1.3)
Effector Memory (CCR7$^-$ CD45RA$^-$)	7.6 (2.8)	10 (2.2)	7.65 (2.1)
Central Memory (CCR7$^+$ CD45RA$^-$)	3.03 (0.94)	1.06 (0.37)	1.73 (0.47)
B cells (% CD3$^-$ CD19$^+$ of hCD45$^+$)	86.1 (2.8)	78.6 (7.5)	***62.7 (4.5)****
Immature (% CD24^{hi}CD38^{hi} of B cells)	52.3 (3.5)	47.1 (9.3)	***25.9 (3.9)****
Mature Naïve (%CD24^{int}CD38^{int} of B cells)	33.1 (3.2)	35.1 (6.1)	***55.9 (3.6)****
Monocytes (% CD3$^-$ CD19$^-$ CD14$^+$ of hCD45$^+$)	0.80 (0.1)	1.5 (0.50)	***8.6 (2.1)****
cDCs (% CD3$^-$ CD19$^-$ CD14$^-$ HLADR$^+$ CD11c$^+$)	3.2 (0.74)	3.4 (1.2)	2.6 (2.2)
myeloid DCs (% CD3$^-$ CD19$^-$ CD14$^-$ HLADR$^+$ CD123$^+$CD11c$^+$)	10.9 (4.8)	11.6 (5.9)	14.9 (2.9)
NK cells (% CD3$^-$ CD56$^+$ of hCD45$^+$)	1.69 (0.51)	2.72 (0.52)	2.32 (0.38)

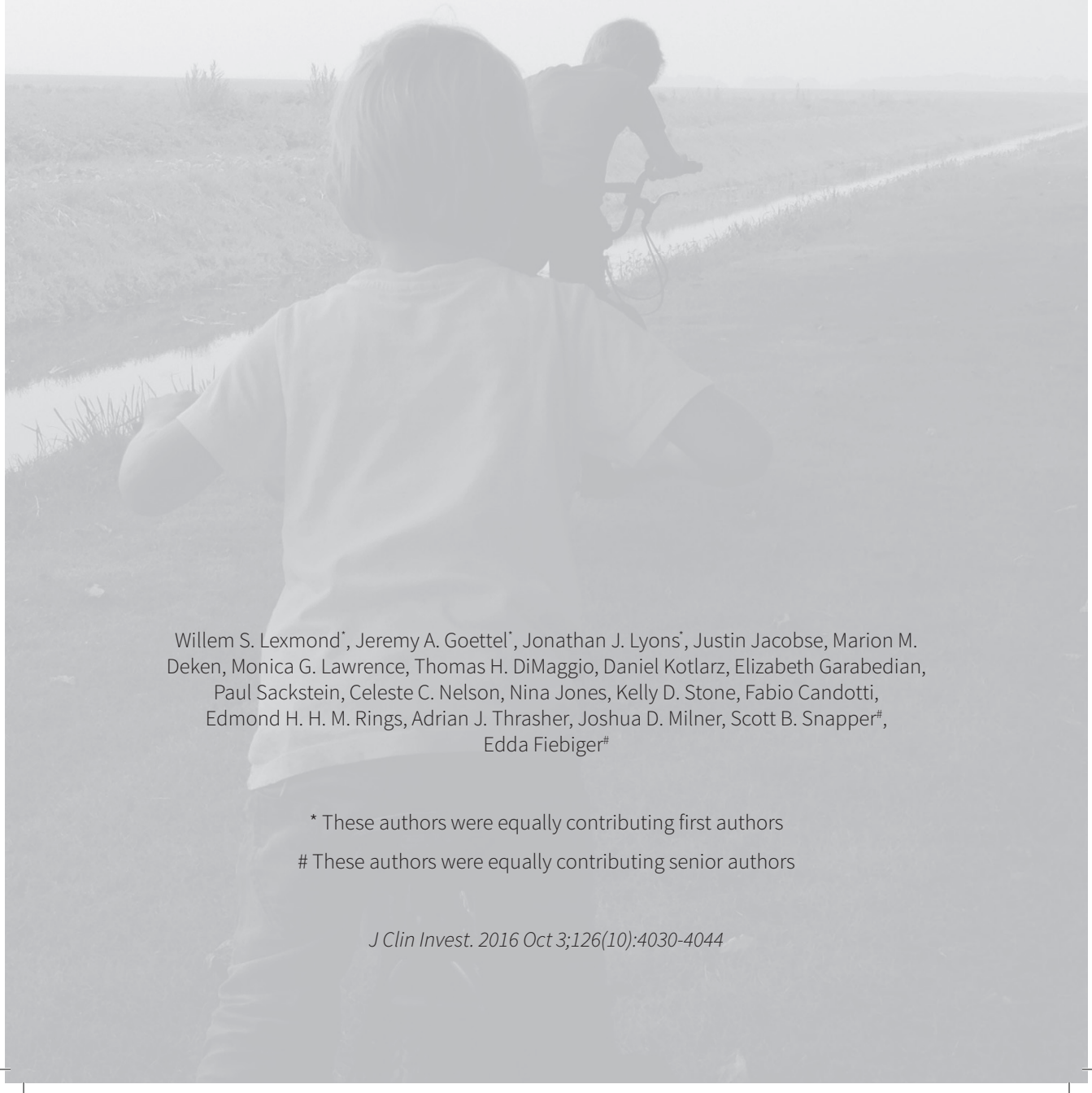
Numbers are the mean (± SEM) * $P < 0.05$

Table S2. TCR repertoire. Sequence analysis of the *TRB* CDR3 region in splenic CD4$^+$ T cells.

Sample	Total sequences	Unique sequences (%)	Diversity index
NSG #1	487,721	846 (0.17)	0.24
NSG #2	280,031	1369 (0.48)	0.14
NSGAb^{o}DR1 #1	411,070	3538 (0.86)	0.18
NSGAb^{o}DR1 #2	597,708	3874 (0.64)	0.16

Chapter 7

FOXP3^{+} Tregs require WASP to restrain Th2–mediated food allergy



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7.1 Abstract

In addition to the infectious consequences of immunodeficiency, patients with Wiskott-Aldrich syndrome (WAS) often suffer from poorly understood, exaggerated immune responses that can result in autoimmunity and elevated levels of serum IgE. Here we show that WAS patients and mice deficient in Wiskott-Aldrich syndrome protein (WASP) frequently develop IgE-mediated reactions to common food allergens. In *Was*$^{-/-}$ animals, this adjuvant-free IgE-sensitization to chow antigens was most pronounced for wheat and soy, and occurred under specific pathogen free as well as germ-free housing conditions. Allergic responses to food allergens in WASP deficiency critically depended upon FOXP3^{+} Tregs, as conditional deletion of WASP in this immune compartment resulted in more severe Th2-type intestinal inflammation than observed in *Was*$^{-/-}$ counterparts. While WASP-deficient Tregs efficiently contained Th1 and Th17-type effector differentiation in vivo, they failed to restrain Th2 effector responses that drive allergic intestinal inflammation. Loss of WASP was phenotypically associated with increased GATA3 expression by effector memory, but not naïve-like, FOXP3^{+} Tregs, which occurred independently of increased IL-4 signaling. Our results reveal a novel, Treg-specific role for WASP that is required for prevention of Th2 effector cell differentiation and allergic sensitization to dietary antigens.

7.2 Introduction

Type 2 immunity is involved in a variety of host-defense functions, ranging from protection against parasites and support of epithelial barrier integrity, to regulation of wound healing and control of metabolic homeostasis (Palm et al., 2012; Pulendran and Artis, 2012; Wynn, 2015). Many of these functions are performed by cells of the innate immune system, including eosinophils, basophils and mast cells, which in turn are orchestrated by Th2 lymphocytes of the adaptive immune system through the production of type 2 cytokines such as IL-4, IL-5 and IL-13. Th2 help can furthermore instruct B cells to produce immunoglobulin E (IgE), which arms mast cells and basophils with antigen-specific effector functions through binding to their high-affinity IgE receptor FcεRI on the cell surface. Despite the presence of multiple inhibitory pathways of type 2 immunity (Wynn, 2015), dysregulation within this system is increasingly common in Westernized societies and can result in the production of allergen-specific IgE, type I hypersensitivity reactions, and allergic tissue inflammation (Palm et al., 2012; Pulendran and Artis, 2012; Wynn, 2015). In order to better understand the recent surge in incidence as well as the pathogenesis of Th2-driven allergic diseases, the study of aberrant activation of Th2 effector responses is of critical importance.

As is true for Th1 responses, Th2-mediated immune reactions critically rely on the function of regulatory T cells (Tregs) for their containment. Functional defects in Foxp3, the lineage-defining transcription factor that identifies the best-characterized population of Tregs, results in the immune dysregulation, polyendocrinopathy, enteropathy, X-linked (IPEX) syndrome in human patients, and in the IPEX-related scurfy phenotype in mice (Ramsdell and Ziegler, 2014). In both species, the severe lymphoproliferation that ensues shows concomitant Th1 and Th2 effector responses, which may counter-regulate each other (Suscovich et al., 2012), and manifests clinically as a loss of tolerance-to-self and autoimmunity in combination with elevated IgE levels and food allergies (Akdis and Akdis, 2009; Lin et al., 2005; Suscovich et al., 2012). Emerging data implicate functionally distinct subsets of $CD4^+FOXP3^+$ Tregs that exert differential control over Th1 and Th2 effector cell proliferation, which may be related to their anatomic location of origin. For instance, thymus-derived Tregs were recently shown to specifically control Th1 immunity (Dhainaut et al., 2015), whereas selective loss of peripherally-induced (i)Tregs resulted in uncontrolled Th2, but not Th1 or Th17-type inflammation (Josefowicz et al., 2012b). These findings fit within the broader paradigm that lineage-committed $FOXP3^+$ Tregs are responsive to environmental cues and can assume tissue-specific phenotypes by co-opting other transcription factors such as GATA3 or T-bet (Josefowicz et al., 2012a; Li and Zheng, 2015; Yu et al., 2015). The mechanisms and consequences of this phenotypic variation and functional plasticity are only beginning to be understood.

In addition to the human IPEX syndrome, dysregulated Th2 responses, atopy and elevated IgE levels occur in a range of primary human immunodeficiencies, some of which

are phenocopied in the corresponding mouse models (Datta and Milner, 2011; Liston et al., 2008; Ozcan et al., 2008; Sassi et al., 2014; Yong et al., 2012; Zhang et al., 2014). The variety of genes that are affected in these disorders - e.g. *STAT3*, *DOCK8, PGM3* and multiple genes involved in TCR signaling such as *LAT, ZAP70* or *RAG* - suggests that hyper IgE phenotypes can result from alterations in a number of distinct immunological pathways. In most of these cases, however, the underlying mechanisms of increased IgE production or the functional consequences of elevated serum IgE have not been studied in detail.

One well-known primary immunodeficiency with elevated serum IgE is Wiskott-Aldrich syndrome (WAS) (Datta and Milner, 2011; Ozcan et al., 2008). WAS is caused by mutations in the Wiskott-Aldrich syndrome (*WAS*) gene on the X-chromosome, which encodes the Wiskott-Aldrich syndrome protein (WASP) with expression restricted to hematopoietic lineages. WASP is the founding member of a family of actin regulators, capable of transducing a variety of signals to mediate changes in the actin cytoskeleton, and has been implicated in a great variety of cellular functions in both lymphocytes and non-lymphocytes (Snapper and Rosen, 1999; Thrasher and Burns, 2010). More than 100 unique loss-of-function mutations in *WAS* have been reported, giving rise to a clinically heterogeneous group of patients (Thrasher and Burns, 2010). The most severely affected males present early in life with thrombocytopenia, eczema, autoimmune sequelae and recurrent infections, which can be fatal in the absence of bone-marrow transplantation or gene therapy. In contrast, milder loss-of-function mutations have been identified in patients who suffer from an attenuated form of the disease termed X-linked thrombocytopenia (XLT), which has excellent long-term survival with medical management alone (Albert et al., 2011). Despite the long-known association between *WAS* mutations and atopy, the antigenic specificity of the expanded IgE pool and the consequences of elevated IgE on the prevalence of allergic disease are only beginning to be investigated in human patients (Tuano et al., 2014) and have not been addressed in WASP-deficient mice.

Here we show that patients with mutations in *WAS* demonstrate an increased frequency of sensitization to food allergens as measured by serum specific IgE and skin prick testing and increased prevalence of clinically relevant food allergy in childhood when compared to the general population. Similarly, *Was*$^{-/-}$ mice spontaneously develop IgE-mediated immune responses and intestinal mast cell expansion. Using conditional WASP-deficient mice, we identified that WASP deficiency limited to FOXP3^{+} Tregs results in a strongly Th2-skewed, allergic inflammation of the small intestine, which was exacerbated compared to complete *Was*$^{-/-}$ counterparts. Phenotypically, WASP-deficient Tregs displayed elevated levels of Th2 transcription factor GATA3 in the effector-like, but not naïve subset of FOXP3^{+} Tregs, which occurred independently of IL-4. These findings demonstrate that WASP is required for FOXP3^{+} Tregs to exert selective control over Th2-type immune responses.

7.3 Methods

7.3.1 Patients

25 consecutive patients with Wiskott-Aldrich syndrome (WAS) or X-linked thrombocytopenia (XLT) followed by NHGRI on an active clinical protocol (NCT00006319), provided informed consent on an NIH IRB-approved research protocol designed to study atopy (NCT01164241). Prior to enrollment, a clinical diagnosis of WAS or XLT was confirmed by mutation analysis of the WAS gene and WASP protein expression as previously described (Wada et al., 2001). Comprehensive allergic histories, review of all available outside records pertaining to prior allergic or immunologic evaluation and therapeutic intervention, as well as physical examinations were performed at the Clinical Center of the National Institutes of Health (NIH). Total and allergen-specific serum IgE levels were quantified by ImmunoCAP (Uppsala, Sweden) from all patients (n = 25). Skin prick testing to a panel of common allergens was performed in patients with sufficient intact / non-inflamed skin (n = 14) and compared to standard clinical positive and negative controls. Because three individuals had previously undergone hematopoietic stem cell transplantation, data from sera and skin prick tests were excluded from analysis. Skin prick testing to morphine (10, 3, 1, 0.3, 0.1 mg/mL) was performed in individuals with WAS mutations (n = 13) and compared to control subjects (n = 15).

7.3.2 Animals

The generation of *Was*$^{-/-}$*, Was*$^{-/-}$*Il4*$^{-/-}$*, and Was*$^{-/-}$*Rag2*$^{-/-}$ mice has been previously described (Nguyen et al., 2007; Snapper et al., 1998) and *Was*$^{-/-}$ animals have since been made commercially available from The Jackson Laboratory (Bar Harbor, ME). All mice used in this study were bred in house and maintained in accordance with institutional guidelines in specific pathogen-free conditions at the Boston Children's Hospital. 129SvEv *Was*$^{-/-}$ animals were re-derived into germ-free conditions at Boston Children's Hospital. The C57BL/6 mice harboring floxed *Was* alleles (*Was*$^{fl/fl}$ females *or Was*^{fl} males*)* were generated and donated by Adrian Thrasher (University College London Institute of Child Health, London UK) as previously described (Recher et al., 2012). We backcrossed these mice for 10 generations onto the 129SvEv background in our facility. *Itgax*-Cre mice and *Foxp3*-Cre mice on the C57BL/6 background were obtained from Jackson laboratory and backcrossed for at least 10 generations onto the 129SvEv background. Serum from *Was*^{fl}*Mb1*-Cre and *Was*^{wt}*Mb1*-Cre animals was kindly provided by Drs. Stefano Volpi and Luigi Notarangelo at the Department of Immunology of Boston Children's Hospital (Boston, USA).

7.3.3 Chow and chow extracts

All mice were kept on irradiated Prolab® Isopro® RMH 3000 (LabDiet, St. Louis, MO) throughout their lives. Crude samples of the five main (%w/w) components (in order of abundance:

ground wheat, soybean meal, wheat middlings, ground yellow corn, and fish meal) were obtained directly from LabDiet. Protein extracts for in vitro studies were generated by soaking approximately 10 gram of homogenized chow pellets or meal component in 40 ml of phosphate buffered saline (PBS) in a 50 ml Falcon® tube, which was placed in a shaking incubator at 37 °C for 4 hours, and then spun at 5000 x g for 10 minutes. The supernatant containing solubilized food antigens was then ultra-centrifuged (100,000 x g) for 1 h and sterile-filtered using a 20-micron syringe filter (Corning, Tewksbury, MA). Protein concentration of extracts was quantified using a colorimetric protein assay (Bio-Rad, Hercules, CA). For allergen elimination experiments, mice were transferred to a clean cage containing a protein-free, amino acid-based formula (Research Diets Inc, New Brunswick, NJ). An elemental chow extract was obtained from mortar-and-pestle-homogenized pellets as described for regular chow.

7.3.4 ELISAs

Total and OVA-specific IgE and IgG1 ELISAs were performed as previously described (Platzer et al., 2015a) using the following reagents: capture antibodies: anti-mouse IgE (southern biotech 1110-01) and anti-mouse IgG1 (southern biotech 1070-01); protein standards: mouse IgE and IgG1 κ isotype controls (BD Pharmingen), and mouse anti-ovalbumin IgE (MCA2259, AbD Serotec); detection reagents: horseradish peroxidase-conjugated anti-mouse IgE (southern biotech 1110-05) and IgG1 (southern biotech 1070-05), biotin-conjugated ovalbumin (US Biologicals), and streptavidin-conjugated horseradish peroxidase (BD Pharmingen).

For food-specific Ig ELISAs, 96-well polystyrene plates (Costar assay plates, Corning) were coated overnight with 100 µl of food extract in PBS (100 $\mu g ml^{-1}$ protein concentration). Plates were subsequently washed 6 times with 300 µl of 0.05% tween in PBS (washing buffer) and blocked with 10% fetal calf serum (FCS) in PBS for a minimum of 2 h at room temperature. Serum dilutions ranging from 1:30 to 1:5000 were prepared in 2% FCS in PBS buffer and transferred to wells in a volume of 100 µl. Plates were incubated at room temperature for 3 h and then washed again 6 times in washing buffer, after which anti-food Ig antibodies were detected with horseradish peroxidase-conjugated antibodies to IgE (1110-05), IgG1 (1070-05), IgG2a (1080-05), IgG2b (1090-05), IgG3 (1100-05), or IgA (1040-05, all from Southern Biotech) in 1:1000 dilutions in 2% FCS in PBS for 1 h at room temperature. Following 6 additional washes, plates were developed in the dark for 3-10 minutes with 100 µl of tetramethylenbenzidine per well (KPL, Gaithersburg, MD). This reaction was stopped by the addition of 50 µl of 2M H_2SO_4 prior to spectrophotometric analysis at 450nm (Perkin Elmer). To allow semi-quantitative comparison of reactivity against different food extracts, all five investigated extracts were coated in equivalent concentrations in adjacent wells on the same 96-well plate. For each sample, a sixth well was included that was coated with PBS alone and the OD of this blank well was subtracted from the OD of the extract-coated wells to correct for any non-specific binding and thus obtain the Ig reactivity against any of the five components. Cumulative

anti-food Ig titers were defined as the sum of the blanked OD values of all five investigated extracts. For some experiments, wells were coated with combined wheat and soy extracts, and 2-fold serial dilutions of a positive serum sample were used to quantify the anti-food IgG1 reactivity as arbitrary units/ml (U/ml).

Serum MCPT1, as well as IL-4, IL-13, IL-2, IL-17a and IFN-g ELISAs were performed using a commercially available kit (eBioscience) according to the manufacturer's instructions.

7.3.5 Flow cytometric analysis

To assess basophil-surface IgE loading, whole blood samples were collected in EDTA. Following lysis of erythrocytes in RBC lysis buffer (eBioscience), cell pellets were blocked with anti-mouse CD16/CD32 (clone 2.4G2, BD Pharmingen) and stained with anti-mouse CD45 (clone 30-F11), anti-mouse CD49b (clone DX5), anti-mouse FcεRIa (clone MAR-1) and anti-mouse IgE (clone RME-1, all from Biolegend). Basophils were identified within peripheral blood mononuclear cells as $CD45^{Mid}CD49b^{+}Fc\varepsilon RIa^{+}IgE^{+}$, and IgE surface loading was determined from the mean fluorescence intensity of the anti-IgE stain. Immune profiling of MLN and PP lymphocytes was performed using the following fluorochrome-conjugated antibodies: antio-CD16/CD32 (clone 2.4G2, BD Pharmingen), anti-CD4 (clone GK1.5), anti-Foxp3 (clone FJK-16s, eBioscience), anti-GATA3 (clone TWAJ, eBioscience), anti-CD44 (clone IM7), anti-CD62L (clone MEL-14), anti-ICOS (clone C398.4A), anti-T-bet (clone 4B10), anti-RORgt (clone B2D, eBioscience) anti-neuropilin-1 (product number FAB556A, R&D systems) and Fixable Viability Dye eFluor® 450 (eBioscience). Transcription factors were stained using the Foxp3/Transcription Factor Staining kit (eBioscience).

PBMCs from patients were isolated and regulatory $CD4^{+}$ T cells were stained as previously described (Milner et al., 2015) with Live/Dead Fixable Blue viability dye (Invitrogen, Carlsbad, CA), anti-CD3 Alexa Fluor (AF)700, anti-CD25 phycoerythrin (PE)-Cy7, anti-CD127 AF647, anti-CD4 brilliant violet 605, anti-GATA3 AF488 (BD Biosciences, San Jose, CA), anti-CD45RO Texas Red-PE (Beckman Coulter, Brea, CA), and anti-FOXP3 efluor 450 (eBioscience, San Diego, CA).

7.3.6 Mast cell degranulation assays

Bone marrow-derived cells from WT BALB/c mice were differentiated to mast cells in vitro by culturing cells in RPMI 1640 medium (Gibco) supplemented with 10% FCS, 100 U/ml penicillin and 100 $\mu g ml^{-1}$ streptomycin, 2 mM L-glutamine, 50 μM β-mercaptoethanol, 10 $ng ml^{-1}$ interleukin-3 and 20 $ng ml^{-1}$ stem cell factor (SCF) (henceforth referred to as mast cell medium). Experiments were performed using cultures that were at least 8 weeks old and had a purity of >90% as assessed by cell surface co-expression of mast cell markers c-kit (clone 2B8) and FceRIa (clone MAR-1, both from Biolegend) by flow cytometry. Prior to degranulation assay, mast cells were resuspended in 10^{6}/ml mast cell medium and loaded overnight with dilutions of mouse serum in concentrations as indicated per experiment. The

next day, cells were washed twice in medium, plated in a 96-well tissue culture plate (10^5 cells in 100 µl per well) and stained with viability dye. Cells were then challenged for 10 minutes with an antigen cocktail containing solubilized chow antigens (protein concentration 5 $\mu g ml^{-1}$) or OVA (500 $ng ml^{-1}$), in combination with anti-mouse LAMP-1 antibody (clone 1D4B, Biolegend). Following two washes with cold flow cytometry buffer (0.01% NaN_3 in PBS), cells were acquired on a FACSCanto II flow cytometer (BD Bioscience) and further analyzed using FlowJo software (Treestar).

7.3.7 Histology and mast cell staining

Following euthanasia, intestinal sections were isolated and flushed with cold PBS. Three jejunal sections per animal were fixed in formalin for 24h and then transferred to 70% ethanol. Tissue samples were paraffin-embedded and sectioned by the Histology Core facility of Beth Israel Deaconess Medical Center (Boston, MA), and mast cell quantification was performed as described (Burton et al., 2012). In brief, slides were deparaffinized in sequential xylene baths and rehydrated in graded alcohol solutions. Chloroacetate esterase staining solution consisted of 0.04% naphtol AS-D chloroacetate (Sigma), 0.04% pararosaniline (Sigma), and 0.04% sodium nitrite (Sigma) in PBS, and was used to stain slides for 30 minutes at room temperature. Slides were counterstained with modified Harris hematoxylin solution (Sigma) and mounted in Permount (Fisher). For statistical analysis, a blinded investigator counted mast cells in 4 randomly selected high-power fields. Images were captured using an Olympus DP70 microscope equipped with DP Controller software (Olympus corporation).

7.3.8 Quantitative RT–PCR

Three jujunal tissue sections were pooled and stored in RNAlater (Qiagen). Following tissue disruption and homogenization, total RNA was extracted using an RNeasy Plus mini kit (Qiagen) and reverse transcribed using iScript Supermix (Bio-Rad). *Mcpt1* gene expression was assessed with iQ SYBR Green Supermix (Bio-Rad) using the following primers: FOR 5'-GAG GAC AGA TGT GGT GGG TTT-3' and REV 5'-AGG AGT CAA CTC AGC TTT CTC TT-3', and normalized against expression of housekeeping gene *Hprt* (FOR 5'-GTT GGA TAC AGG CCA GAC TTT GTT G-3' and REV 5'-GAG GGT AGG CTG GCC TAT AGG CT-3'). Relative expression was determined using the $2^{-\Delta\Delta Ct}$ method, in which the ΔCt of a WT sample was set as reference value.

7.3.9 In vivo allergen challenge

To assess the occurrence of oral anaphylaxis, mice were starved for 3 hours and then challenged with 12.5 mg soy protein in 300 µl PBS by gavage. Body temperature was registered with the use of implantable temperature transponders (IPTT-300, Biomedic Data Systems, Seaford, Del). Baseline temperature was defined as the average of three measurements

prior to challenge, and severity of anaphylaxis was assessed by calculating the ΔT from this average every 10 minutes after challenge.

7.3.10 Ex vivo culture and stimulation of mesenteric lymphocytes

Following isolation of MLNs, single cell suspensions were purified using a MagniSort Mouse $CD4^+$ T cell Enrichment Kit (eBioscience) according to the manufacturer's protocol and resuspended and plated in RPMI 1640 medium (Gibco) supplemented with 10% FCS, 100 U/ml penicillin, 100 μgml^{-1} streptomycin, 2 mM L-glutamine and 50 μM β-mercaptoethanol. Cytokine production was determined by ELISA in supernatants obtained in triplicates from 200,000 cultured cells per well that were stimulated for 18-24h with anti-CD3/CD28 using Dynabeads® (Life Technologies).

7.3.11 In vitro polarization

Naïve T cells were isolated from CD45.1 congenic C57BL/6 mice, stained using antibodies against anti-CD3 clone 145-2C11 (BioLegend), anti-CD4 cloneGK1.5 (Biolegend), anti-CD25 clone PC61 (BioLegend), anti-CD44 cloneIM7 (BioLegend) and anti-CD62L clone MEL-14 and sorted based on $CD3^+CD4^+CD25^-CD44^-CD62L^+$. $2.5x10^5$ cells were plated in a 24-well plate in RPMI 1640 medium (Gibco) supplemented with 10% FCS, 100 U/ml penicillin, 100 μgml^{-1} streptomycin, 2 mM L-glutamine, 50 μM β-mercaptoethanol, and stimulated with plate bound 5 $mgml^{-1}$ anti-CD3 clone 145-2C11 (BD Pharmingen) and 1 $mgml^{-1}$ anti-CD28 clone 16-0281-85 (BD Pharmingen) for 7 days in the presence of 20 $ngml^{-1}$ of IL2 (Peprotech), 10 $ngml^{-1}$ IL-12 (R&D systems), and 10 $mgml^{-1}$ anti-IL-4 clone 11B11 (BD Pharmingen) for Th1 polarization; 20 $ngml^{-1}$ of IL-2, 100 $ngml^{-1}$ IL-4, 10 $mgml^{-1}$ anti-IFNg clone XMG1.2 (BD Pharmingen), and 10 $mgml^{-1}$ anti-IL-12 clone C17.8 (BD Pharmingen) for Th2 polarization; 20 $ngml^{-1}$ of IL-6 (BioLegend), 2 $ngml^{-1}$ of human TGFb (BioLegend), 10 $mgml^{-1}$ anti-IFNg and 10 $mgml^{-1}$ anti-IL-4 for Th17 polarization.

7.3.12 Treg suppression assay

Following in vitro Th polarization, responder cells were labeled with CFSE and $5x10^4$ cells were co-cultured in a 1:1 ratio with sorted $CD3^+CD4^+GFP^+$ Tregs isolated from *Was*$^{+/+}$*Foxp3*$^{Cre\text{-}EGFP}$ or *Was*$^{fl/fl}$*Foxp3*$^{Cre\text{-}EGFP}$ mice plated in a 96-well round bottom plate in RPMI 1640 (Gibco) supplemented with 10% FCS, 100 U/ml penicillin, 100 μgml^{-1} streptomycin, 2 mM L-glutamine, 50 μM β-mercaptoethanol and stimulated with aCD3/aCD28 Dynabeads® (Life Technologies) for 3 days. Responder cell proliferation was analyzed by staining with anti-CD45.1 clone A20 (BioLegend) and assessing CFSE dilution using a 3-laser BD FACSCanto II (BD Biosciences) flow cytometer.

7.3.13 In vitro Treg generation assay

$CD3^+CD4^+CD25^-GFP^-$ naïve T cells were sorted from *Was*$^{+/+}$*Foxp3*-Cre-EGFP and *Was*$^{fl/fl}$*Foxp3*-Cre-EGFP mice. $5x10^4$ sorted cells were plated in a 96-well plate in RPMI 1640 medium (Gibco) supplemented with 10% FCS, 100 U/ml penicillin, 100 $\mu g ml^{-1}$ streptomycin, 2 mM L-glutamine, 50 µM β-mercaptoethanol, and stimulated with anti-CD3/anti-CD28 Dynabeads (Life Technologies) in the presence of 2 $ng ml^{-1}$ TFGβ (BioLegend) for 5 days. WT and *Was*$^{-/-}$ GFP^+ iTregs were assessed for GATA3 expression using a 3-laser FACSCanto II (BD Biosciences).

7.3.14 Nanostring assay and hierarchical cluster analysis

Digital gene expression profiling was performed on isolated whole tissue jejunal RNA as previously described using a customized Nanostring® codeset consisting of 86 inflammatory targets and five housekeeping genes (Platzer et al., 2015a). 57 out of 86 investigated genes that were differentially expressed in any pair-wise 2-sided t-test comparison ($p<0.05$) were subjected to hierarchical cluster analysis based on absolute Pearson correlation value with pairwise average linkage using the GenePattern algorithm (http://genepattern.broadinstitute.org).

7.3.15 Statistical analysis

All results were analyzed and visualized using GraphPad Prism version 6.0c for Mac (GraphPad Software). Details pertaining the use of specific statistical tests are provided in the figure legends. A p-value of less than 0.05 was considered significant.

7.3.16 Study approval

All patients provided informed consent prior to inclusion in the study. Clinical studies were performed under an NIH IRB-approved research protocol (NCT01164241). All animal experiments were performed in accordance with Institutional Animal Care and Use Committee-approved protocols number 13-06-2415R and 14-04-2677R (IACUC, Boston Children's Hospital), and adhered to the National Research Council's 'Guide to the care and Use of Laboratory Animals'.

7.4 Results

7.4.1 Sensitization to food antigens and food allergy are enriched among patients with WAS mutations

We assessed the overall burden of clinical food allergy within a cohort of 25 patients with mutations in the *WAS* gene. Results from three patients were excluded from the analysis as they could not be tested prior to hematopoietic stem cell transplantation. A variety of *WAS* mutations were observed in this cohort (**Figure 7.1A**), which consisted of both patients with WAS (n=15) as well as XLT (n=10). Elevated IgE (normal range 0-90 IU/ml) was observed in 59%

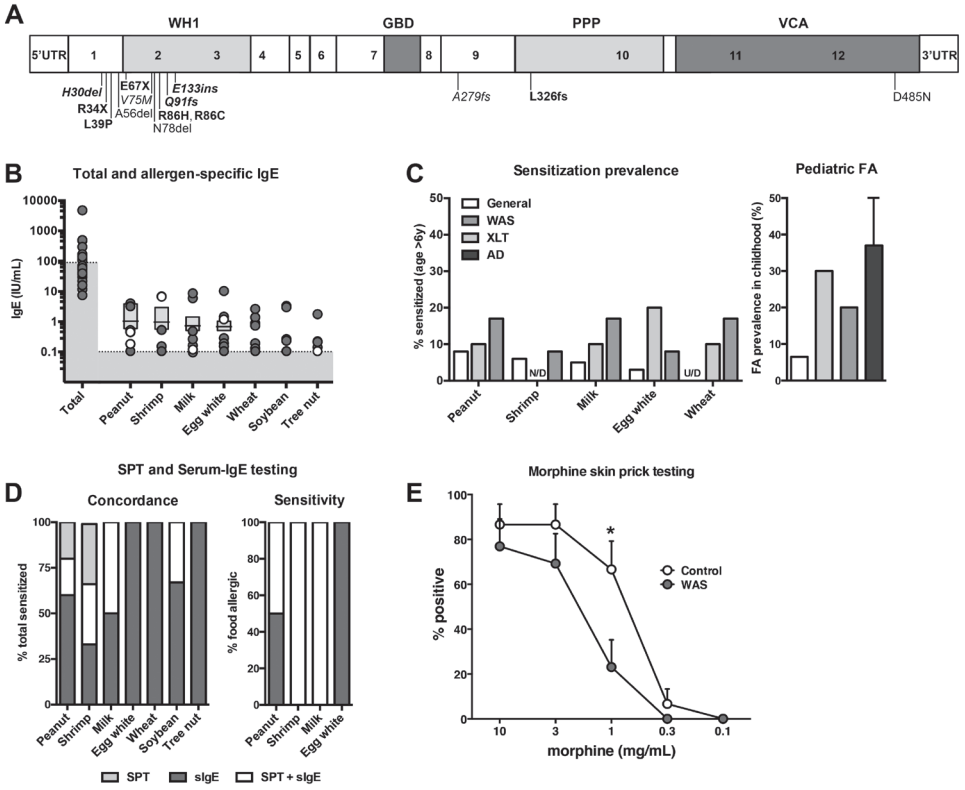

Figure 7.1. Increased sensitization and prevalence of food allergy among patients with *WAS* mutations. (**A**) Schematic of *WAS* gene with mutations identified among cohort (n = 25). Bold indicates Wiskott-Aldrich syndrome (WAS) diagnosis; unbolded indicates X-linked thrombocytopenia (XLT); italicized indicates mutations associated with food allergy in childhood. (**B**) Total serum IgE levels and food allergen specific IgE (sIgE) levels among cohort; limit of detection for sIgE was 0.1 IU/ml. Light grey fill indicates the normal range; boxes indicate median and interquartile ranges for four foods reported in NHANES (Ref 23). White circles indicate food allergic individuals. (**C**) Among patients with sera obtained prior to transplantation (n = 22), percent of WAS (n = 12) or XLT (n = 10) patients with positive serum IgE (sIgE) to foods (minimum cutoff 0.35 IU/mL) compared to the general population as reported in NHANES analysis (left panel). Prevalence of food allergy during childhood among all WAS (n = 15) and XLT (n = 10) patients compared to those reported in the general population (NHANES) and in patients with moderate to severe atopic dermatitis (AD) (right panel) (Refs 24 and 25). (**D**) Concordance of sIgE measurement to skin prick testing to foods (SPT) for individuals who underwent both modalities (n = 14) (left panel). Results of SPT and sIgE testing among patients with persistent clinical food allergy (n = 4); two individuals with food allergy in childhood without current evidence of sensitization were excluded (right panel). (**E**) Percent positive wheal responses to SPT with morphine titration among patients with *WAS* mutations (n = 13) compared to sex-matched controls (n = 15), *p=0.03 by Fisher's exact test. U/D – undefined; N/D – none detected; WH1 – WASP Homology domain 1; GBD – GTP-ase binding domain; PPP – Polyproline domain; VCA – verprolin homology, cofilin homology and acidic region domain.

of patients (13/22), and food allergen-specific IgE was detected in 33% (4/12) of WAS and 20% (2/10) of XLT patients (**Figure 7.1B**) (Salo et al., 2014). Compared to the general population, individuals with *WAS* mutations were more likely to demonstrate serum sensitization to peanut, milk, and egg (**Figure 7.1C**) (Salo et al., 2014). The prevalence of physician-diagnosed food allergy among WAS and XLT patients in childhood (20% and 30%, respectively) was also increased compared to rates reported among children in NHANES (6.5%) (McGowan and Keet, 2013) and approached levels reported among children with moderate-severe atopic dermatitis (37±13%) (**Figure 7.1C**) (Eigenmann et al., 1998), although none of the patients reported a history of anaphylaxis. Food sensitization was generally detected with greater sensitivity using serum IgE (sIgE) testing than by skin prick test (SPT, **Figure 7.1D**, left panel): peanut and egg white allergens failed to elicit positive SPT responses in two physician-diagnosed food allergic patients who were found positive for sIgE against these two allergens (**Figure 7.1D**, right panel). One patient with a clinical history consistent with food allergy was negative both by SPT and by serum-specific IgE to all tested food antigens. Five additional patients who underwent sIgE testing could not have SPT performed and were not included in evaluating the sIgE/SPT concordance rates. Patients with mutations in WAS demonstrated dampened wheal responses to skin-challenge with morphine (**Figure 7.1E**), suggesting that WASP deficiency in mast cells may contribute to the high discordance of sIgE levels and SPT responses. Taken together, our data demonstrate a marked enrichment of clinically relevant food-antigen-specific IgE in patients with mutations in *WAS*.

7.4.2 *Was*$^{-/-}$ mice spontaneously develop IgE–mediated immune responses to food allergens

Following the observation that WAS patients frequently develop IgE antibodies against food antigens, we investigated the occurrence of IgE-mediated responses to allergens in chow in WASP-deficient mice. *Was*$^{-/-}$ females or hemizygous *Was*$^{-}$ males (henceforth both referred to as *Was*$^{-/-}$ mice) on BALB/c, C57BL/6, and 129SvEv backgrounds developed elevated levels of total serum IgE and IgG1 in comparison to background-matched wild-type (WT) controls (**Figure 7.2A** and **7.S1A,C**). Serum IgE increased with age and correlated positively with the density of surface-bound IgE on circulating basophils, indicating that binding of IgE to its high-affinity receptor FceRI was unperturbed in *Was*$^{-/-}$ animals (**Figure 7.S1A,B**). We next developed ELISA-based assays to screen for antibodies against the five main components of mouse chow. Food-specific antibodies were not detected in WT mice while soy and wheat-specific IgE and IgG1 were readily detected in the serum of *Was*$^{-/-}$ mice (**Figure 7.2B**). For all investigated food extracts, a strong positive correlation between ingredient-specific titers of IgE and IgG1 was observed (**Figure 7.S1D**). The observation that food-specific IgE could be detected in *Was*$^{-/-}$ mice on three different genetic backgrounds indicated that sensitization to ingested antigens did not result from colonic inflammation since, in contrast to *Was*$^{-/-}$ mice

on the colitis-prone 129SvEv background (Nguyen et al., 2007; Snapper et al., 1998), animals on the BALB/c and C57BL6 background are resistant to colitis (**Figure 7.S1E,F**).

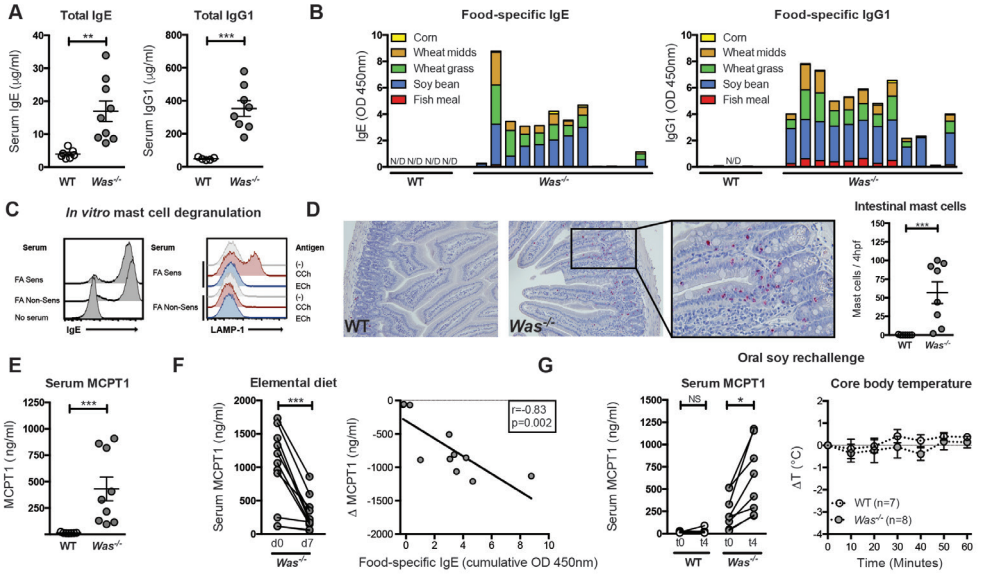

Figure 7.2. Spontaneous sensitization to food antigens and food allergy in *Was*$^{-/-}$ mice. (**A**) Comparative analysis of total serum IgE and IgG1 levels in 3-month old WT BALB/c (open circles, n=7) and *Was*$^{-/-}$ mice (gray circles, n=9) of mixed genders. (**B**) IgE and IgG1 reactivity against the five main (%w/w) chow components as determined by ELISA in 1:30 (IgE) or 1:1000 (IgG1) diluted serum samples. (**C**) Loading of WT bone-marrow-derived mast cells with serum of food allergic (FA Sens) or non-food allergic (FA Non-Sens) *Was*$^{-/-}$ mice compared to no-serum control (left panel). Appearance of surface LAMP-1 as a marker of mast cell degranulation after stimulation with antigen extracts from conventional chow (CCh), elemental chow (ECh) or PBS (-). (**D**) Intestinal mast cell expansion as determined by chloroacetate esterase staining of jejunal cross-sections (20X, with digital magnification to 50X shown in window) and quantification in WT (n=7) and *Was*$^{-/-}$ mice (n=8). (**E**) Serum levels of mast cell protease 1 (MCPT1) determined by ELISA. (**F**) Effect of 7-day treatment with elemental diet on serum MCPT1 in *Was*$^{-/-}$ mice (n=11). Spearman's rank correlation between cumulative anti-food IgE titers of mice and response to allergen elimination defined as ΔMCPT1. (**G**) Effect of oral rechallenge with 12.5 mg soy protein extract on body temperature and serum MCPT1 after 4h. Symbols represent individual mice and error bars depict SEM. N/D: not detectable; ** p<0.01; ***p<0.001; NS: not significant as determined by two-tailed Student's t-test. Results in **C** are representative of 2 independent experiments. Equivalent results were obtained in a cohort of WT and *Was*$^{-/-}$ mice on the 129sv background.

To assess whether food-specific serum IgE from *Was*$^{-/-}$ mice was functional in mediating type I hypersensitivity reactions, bone marrow-derived WT mast cells were loaded with serum from *Was*$^{-/-}$ mice previously identified by ELISA to be sensitized to food antigens (FA Sens) or with serum from *Was*$^{-/-}$ mice of comparable IgE titer that had been found to not be sensitized to food (FA Non-Sens, **Figure 7.2C**). The appearance of lysosomal-associated

membrane protein 1 (LAMP-1) at the cell surface of mast cells is an established surrogate marker for antigen/IgE-mediated histamine release (Burton et al., 2014a; Föger et al., 2011), and we found that WT mast cells loaded with serum from food-sensitized *Was*$^{-/-}$ mice degranulated in response to stimulation with an antigen extract from conventional chow (CCh), but not in response to an extract from a protein-free, amino-acid-based elemental diet (ECh, **Figure 7.2C**). In contrast, mast cells loaded with serum from non-food-sensitized animals were not activated by either food extract, which further demonstrated that IgE-dependent degranulation was antigen-specific and established that screening of antibody reactivity against the five main chow components is a reliable marker for the overall anti-food IgE reactivity.

Expansion of intestinal mast cells is a common symptom in food allergic patients (Caffarelli et al., 1998; Hogan et al., 2012) and has been demonstrated to correlate with disease severity in experimental mouse models of food allergy (Brandt et al., 2003; Burton et al., 2012; Platzer et al., 2015a). In line with these findings, we observed expansion of the small intestinal mast cell pool in *Was*$^{-/-}$ mice as detected by chloroacetate staining on jejunal tissue sections (**Figure 7.2D**). In addition, mRNA expression levels of the mucosal mast cell marker *Mcpt1* was elevated in *Was*$^{-/-}$ animals and positively correlated with the cumulative anti-food IgE titer (**Figure 7.S1G**). Since *Was*$^{-/-}$ mice cannot eliminate food antigens from their diet, we hypothesized that persistent oral allergen exposure would result in continuous IgE/FceRI-mediated degranulation of intestinal mast cells. Indeed, *Was*$^{-/-}$ animals had elevated serum levels of the mast cell protease MCPT1 (**Figure 7.2E**), which is released from mucosal mast cells in response to IgE-mediated detection of food antigens (Brandt et al., 2003; Khodoun et al., 2011). Moreover, anti-food IgE titers positively correlated with serum MCPT1 levels (**Figure 7.S1H**). Consistent with a critical role for food antigens in mast cell activation, elimination of the dietary allergens via switching *Was*$^{-/-}$ mice from conventional chow to an elemental amino acids-based chow resulted in a decline in serum MCPT1 after 7 days, with the most pronounced therapeutic effect (defined as ΔMCPT1) observed in mice with the highest cumulative anti-food IgE titers (**Figure 7.2F**, right panel). Oral rechallenge with soy extract after more than a week of allergen elimination led to rapid activation of mucosal mast cells indicated by increased levels of serum MCPT1, but did not result in signs of anaphylactic shock as measured by a reduction in core body temperature (**Figure 7.2G**). Combined, these data demonstrate that *Was*$^{-/-}$ mice develop food-specific IgE, which effectively mediates mast-cell degranulation in vitro and in vivo.

7.4.3 The microbial flora is dispensable for spontaneous oral sensitization to chow, but shapes the humoral anti–food response in *Was*$^{-/-}$ mice

Since commensal microbes play a dominant role in regulating IgE-mediated responses to food antigens (Cahenzli et al., 2013; Noval Rivas et al., 2013; Stefka et al., 2014), we assessed the contribution of the microbiome to the induction of food-specific IgE responses in *Was*$^{-}$

$^{-/-}$ animals. When compared to specific pathogen free-housed (SPF) WT mice, both SPF and germ-free (GF) *Was*$^{-/-}$ mice showed elevated total IgE and IgG1 levels. Although total IgE was lower in GF *Was*$^{-/-}$ than in SPF *Was*$^{-/-}$ animals (**Figure 7.3A**), detailed food-specificity profiling of IgE and IgG1 revealed that sensitization to food occurred efficiently in mice housed under either condition (**Figure 7.3B**) with MCPT1 titers comparable between SPF and GF *Was*$^{-/-}$ mice (**Figure 7.3C**). Further comparative analysis of the isotype composition of the humoral anti-food response in GF *Was*$^{-/-}$ and SPF *Was*$^{-/-}$ mice showed that anti-food IgG1 and IgG2b titers were elevated in the absence of microbes, whereas food-specific IgG3 and IgA levels were diminished (**Figure 7.3D** and **Figure 7.S2**). Since the chow used in SPF and GF setting differed only by one additional cycle of high-dose irradiation in the latter, it is reasonable to assume that all *Was*$^{-/-}$ animals were exposed to food of nearly identical antigenicity. Consequently, food-specific IgE responses in *Was*$^{-/-}$ mice do not require microbial modifications or co-signals that could potentially confer allergenic properties to food antigens. Moreover, these results demonstrate that food allergy in *Was*$^{-/-}$ animals does not require alterations in the commensal flora that may possibly occur as a consequence of WASP deficiency.

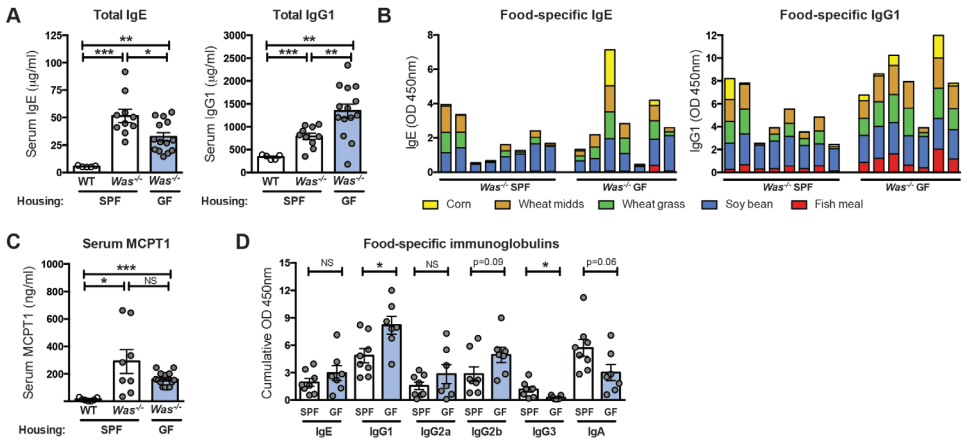

Figure 7.3. Commensals are dispensable for spontaneous sensitization to food in *Was*$^{-/-}$ mice but shape the isotype composition of the humoral anti-food response. (**A**) Comparison of total IgE and total IgG1 serum levels in 4-6-month old WT (open circles, n=5) or *Was*$^{-/-}$ (gray circles) on the 129SvEv background that were housed under either specific-pathogen-free (SPF, n=10) or germ-free (GF, n=14) conditions. (**B**) Food-specific IgE and IgG1 for the five main chow constituents in IgE (serum dilution 1:100) or IgG1 (1:5000) from SPF (n=8) and GF (n=7) *Was*$^{-/-}$ mice. (**C**) Comparison of serum MCPT1 levels. (**D**) Comparison of cumulative anti-food titers of IgE, IgG1, IgG2a (1:1000), IgG2b (1:1000), IgG3 (1:200), and IgA (1:5000) in SPF (n=8) and GF (n=7) *Was*$^{-/-}$ animals. Symbols represent individual mice and error bars depict SEM. *p<0.05; ** p<0.01; ***p<0.001; NS: not significant as determined by two-tailed Student's t-test. Results are shown from sera obtained from mice from ≥3 independent cohorts.

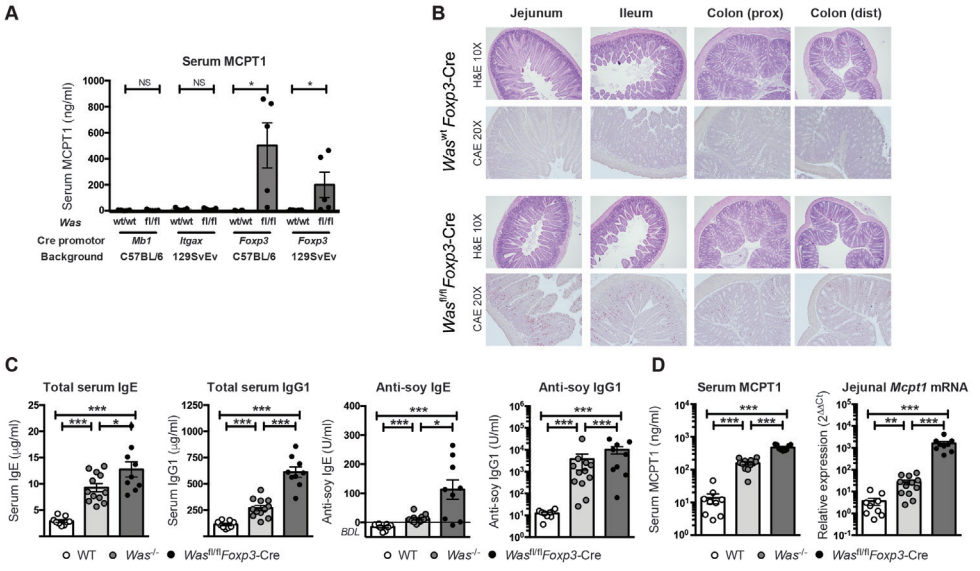

Figure 7.4. WASP deficiency in Tregs is sufficient for the development of spontaneous food allergy and results in more severe disease. (**A**) Comparison of MCPT1 levels in mice with cell type-specific WASP deletions. Mice with conditional deletion of *Was*$^{fl/fl}$ alleles in B cells (*Was*$^{fl/fl}$*Mb1*-Cre), CD11c^{+} dendritic cells (*Was*$^{fl/fl}$*Itgax*-Cre) or Tregs (*Was*$^{fl/fl}$*Foxp3*-Cre) of ≥2 months of age, n≥5 per group. (**B**) Representative H&E (10X) and chloroacetate staining (20X) of intestinal cross-sections in *Was*$^{fl/fl}$*Foxp3*-Cre or *Was*^{wt}*Foxp3*-Cre littermates on C57BL/6 background. (**C**) Comparison of total and soy-specific IgE and IgG1 at 2 months in co-housed WT (open circles, n=9), *Was*$^{-/-}$ (gray circles, n=12), and *Was*$^{fl/fl}$*Foxp3*-Cre (black circles, n=9) mice of mixed genders on the C57Bl6 background. (**D**) Comparison of serum protein and jejunal mRNA expression levels of mucosal mast cell marker MCPT1. Symbols represent individual mice and error bars depict SEM. *p<0.05; ** p<0.01; ***p<0.001; NS: not significant as determined by two-tailed Student's t-test (panel A) or one-way ANOVA with Tukey's multiple comparisons test (panels C, D). *BDL* below detection limit. Data in panels C and D are representative of 2 independent cohorts.

7.4.4 Conditional deletion of WASP in FOXP3^{+} regulatory T cells results in exacerbated Th2–type intestinal inflammation

WASP is expressed in all hematopoietic lineages, and its deficiency in dendritic cells, B cells, effector T cells or regulatory T cells (Tregs) has been described to have a variety of consequences for immune responses (Maillard et al., 2007; Nguyen et al., 2007; Thrasher and Burns, 2010; Westerberg et al., 2005). Unlike *Was*$^{-/-}$ mice, *Was*$^{-/-}$*Rag2*$^{-/-}$ double knockout mice presented with serum MCPT1 levels comparable to WT or *Rag2*$^{-/-}$ animals (**Figure 7.S3A**), indicating that intestinal mast cell expansion and activation in WASP deficiency does not occur in the absence of an adaptive immune system. We next analyzed strains with conditional deletion of WASP in B cells (*Was*$^{fl/fl}$*Mb1*-Cre), CD11c^{+} dendritic cells (*Was*$^{fl/fl}$*Itgax*-Cre) or FOXP3^{+} Tregs (*Was*$^{fl/fl}$*Foxp3*-Cre). Elevated serum MCPT1 was not found in animals harboring conditional deletions of WASP in B cells or dendritic cells, but was present in *Was*$^{fl/fl}$*Foxp3*-

Cre females or *Was*^{fl}*Foxp3*-Cre males (henceforth also referred to as *Was*$^{fl/fl}$*Foxp3*-Cre mice) on both the C57BL/6 and 129SvEv backgrounds (**Figure 7.4A**). Histological analysis confirmed a profound expansion of mucosal mast cells in the small intestine of *Was*$^{fl/fl}$*Foxp3*-Cre mice but not in their *Was*^{wt}*Foxp3*-Cre littermates (**Figure 7.4B**). Mucosal mast cell infiltration occurred in the absence of gross histological changes and we observed no evidence of colitis in *Was*$^{fl/fl}$*Foxp3*-Cre mice on the C57BL/6 background (**Figure 7.4B**).

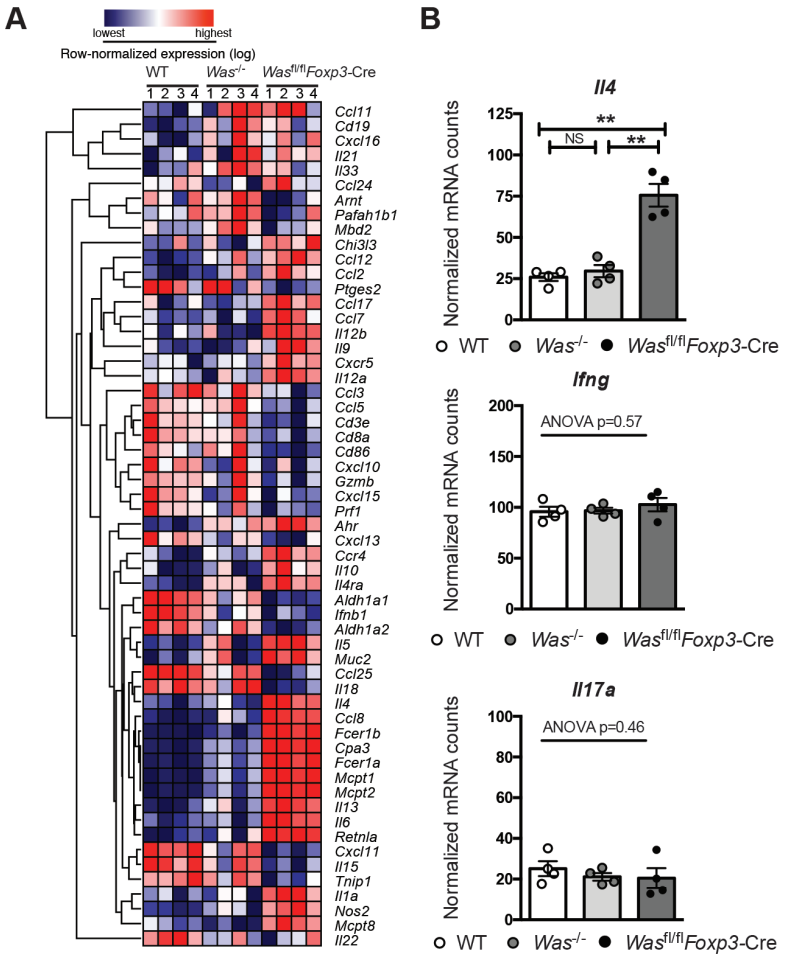

Figure 7.5. WASP deficiency in Tregs results in Th2-type small intestinal inflammation. (**A**) Hierarchical cluster analysis of differentially expressed genes within a panel of 86 inflammatory targets in tissue sections obtained from jejunum of cohoused female mice. Row-normalized, log-transformed mRNA counts are shown from 4 animals per group. (**B**) Bar graph representation and statistical analysis of mRNA expression of *Il4*, *Ifng*, and *Il17a* in jejunal sections as shown in panel A. Symbols represent individual mice and error bars depict SEM. ** p<0.01; NS: not significant as determined by one-way ANOVA with Tukey's multiple comparisons test.

Following the observation that Treg-specific WASP deletion was sufficient for expansion of intestinal mast cells, we next asked whether WASP deficiency in other immune compartments contributed further to the pathogenesis of food allergic sensitization. Comparison of co-housed, age and sex-matched *Was*$^{fl/fl}$*Foxp3*-Cre, *Was*$^{-/-}$ and WT animals revealed that *Was*$^{fl/fl}$*Foxp3*-Cre mice developed higher levels of total and soy-specific IgE and IgG1 (**Figure 7.4C**), as well as higher serum MCPT1 levels and increased intestinal *Mcpt1* mRNA expression (**Figure 7.4D**) than mice with complete deletion of WASP. More severe allergic intestinal inflammation was confirmed by digital mRNA expression profiling on jejunal tissue, which revealed a type 2 immune cluster that contained multiple mast-cell markers in addition to *Mcpt1*, including *Mcpt2, Fcer1a, Fcer1b,* and *Cpa3,* together with Th2-type cytokines *Il4, Il13* and *Il5* that were more prominently expressed in *Was*$^{fl/fl}$*Foxp3*-Cre than *Was*$^{-/-}$ mice (**Figure 7.5A** and **Figure 7.S3B**). This analysis indicated that jejunal inflammation was Th2-specific since mRNA levels of *Ifng*, *Il17a* and *Tnfa* in the small intestine were equivalent to WT levels in both *Was*$^{-/-}$ and *Was*$^{fl/fl}$*Foxp3*-Cre mice (**Figure 7.5B** and **Figure 7.S3B**). Taken together, these results demonstrate that conditional deletion of WASP in FOXP3^{+} Tregs resulted in increased development of IgE-mediated immune responses to food antigens and Th2-specific small intestinal tissue inflammation.

7.4.5 WASP–deficient Tregs fail to selectively suppress Th2 effector responses in vivo

Was$^{-/-}$ animals are known to have reduced FOXP3^{+} Treg numbers, and WASP-deficient Tregs exhibit aberrant proliferation and suppression in response to TCR stimulation in vitro (Humblet-Baron et al., 2007; Maillard et al., 2007; Marangoni et al., 2007). These abnormalities, which can be partly attributed to reduced levels of IL-2, have previously been associated with the occurrence of autoimmunity and colitis in *Was*$^{-/-}$ mice (Humblet-Baron et al., 2007; Maillard et al., 2007). The sequelae of Treg-conditional WASP deficiency on intestinal immune homeostasis, however, have thus far remained undefined.

In sharp contrast to *Was*$^{-/-}$ mice, both relative and absolute FOXP3^{+} Treg numbers in the MLNs and Peyer's patches (PPs) of *Was*$^{fl/fl}$*Foxp3*-Cre were equal to or higher than those of WT counterparts (**Figure 7.6A** and **Figure 7.S4A**). This increase in Treg numbers compared to *Was*$^{-/-}$ mice could have been due to higher levels of IL-2, since CD4^{+} T cells from the MLNs of *Was*$^{fl/fl}$*Foxp3*-Cre mice were found to produce WT levels of IL-2 upon anti-CD3/CD28 stimulation (**Figure 7.6B**). We reasoned that a relative underrepresentation of peripherally-induced FOXP3^{+} Tregs (iTregs), which particularly control tolerance to foreign antigens (Josefowicz et al., 2012b), could potentially underlie the occurrence of IgE-mediated immune responses to food antigens. However, cell surface staining of neuropilin-1, the absence of which specifically defines iTregs (Weiss et al., 2012; Yadav et al., 2012), revealed that iTregs made up equivalent fractions of total FOXP3^{+} Tregs in WT, *Was*$^{-/-}$ and *Was*$^{fl/fl}$*Foxp3*-Cre mice (**Figure 7.S4B**). Similarly, we found no differences in the activation-status of Tregs as *Was*$^{fl/fl}$*Foxp3*-Cre and WT mice showed equal fractions of CD44^{hi}CD62L^{lo} effector-memory Tregs (**Figure 7.S4C**).

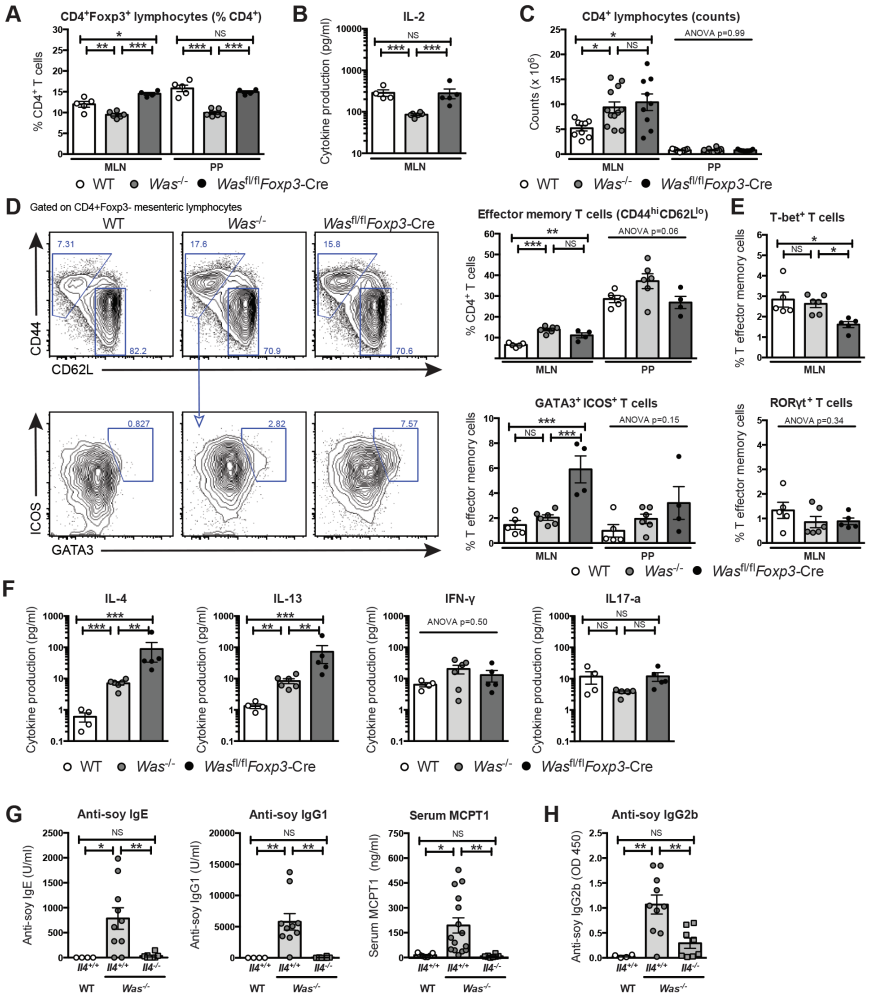

Figure 7.6. WASP-deficient Foxp3⁺ Tregs fail to suppress Th2 type lymphoproliferation in vivo. (**A**) Quantification by flow cytometry of Foxp3⁺ Tregs amongst CD4⁺ T cells obtained from mesenteric lymph nodes (MLNs) or Peyer's patches (PP) of WT (open circles, n=5), *Was*$^{-/-}$ (gray circles, n=6) and *Was*$^{fl/fl}$*Foxp3*-Cre (black circles, n=4) mice. (**B**) Production of IL2 by CD4⁺ mesenteric T lymphocytes stimulated with anti-CD3/CD28 ex vivo. Each dot represents the average cytokine production from triplicate cell suspensions from a single mouse. (**C**) Total CD4⁺ T cell numbers obtained from MLN and PP. (**D**) Gating strategy of GATA3⁺ICOS⁺ Th2-type effector cells within the parent gate of effector memory T cells from MLNs of representative samples, with quantification and statistical testing in the right panels. (**E**) Fraction of T-bet⁺ and RORgt⁺ effector memory T cells. (**F**) Production of IL-4, IL-13, IFN-g and IL-17a by CD4⁺ mesenteric T lymphocytes stimulated with anti-CD3/CD28 ex vivo. Each dot represents the average cytokine production from triplicate cell suspensions from a single mouse. (**G**) Serum levels of anti-soy specific IgE and IgG1, and MCPT1 in *Was*$^{-/-}$ mice on the 129SvEv background with either *Il4*$^{+/+}$ (gray circles, n=10 or 15) or *Il4*$^{-/-}$ alleles (gray squares, n=8). (**H**) Anti-soy IgG2b titer as determined by ELISA in 1:1000 serum dilution. Symbols represent individual mice and error bars depict SEM. *p<0.05; ** p<0.01; ***p<0.001; NS: not significant as determined by two-tailed Student's t-test or one-way ANOVA with Tukey's multiple comparisons test. In B and F, data were log-transformed prior to statistical testing. Data from 2 pooled experiments (panels C, G and H) or representative results from ≥2 independent experiments (panels A, B, D, E and F) are shown.

Despite these normal numerical and phenotypical characteristics of WASP-deficient FOXP3$^+$ Tregs, we observed that both *Was*$^{-/-}$ and *Was*$^{fl/fl}$*Foxp3*-Cre mice developed a mild CD4$^+$ lymphoproliferation in MLNs (**Figure 7.6C**). Within this expanded CD4$^+$ T cell pool in MLNs of *Was*$^{-/-}$ and *Was*$^{fl/fl}$*Foxp3*-Cre mice, an increased fraction of cells displayed the CD44^{hi}CD62L^{lo} effector-memory T cell phenotype. Within this latter fraction, we observed an increase in Th2-skewed effector T cells as determined by their co-expression of ICOS and the Th2 transcription factor GATA3 (Nurieva et al., 2003; Tindemans et al., 2014) (**Figure 7.6D** and **Figure 7.S4D**). In sharp contrast, the fraction of Th1-skewed, T-bet$^+$ effector T cells was decreased in *Was*$^{fl/fl}$*Foxp3*-Cre mice, whereas the abundance of Th17-skewed, RORgt$^+$ T cells was equivalent amongst all three genotypes (**Figure 7.6E**). This selective Th2 skew in the CD4 effector compartment was corroborated further by analysis of cytokine production from CD4$^+$ mesenteric lymphocytes cultured *ex vivo*, which showed significantly increased production of IL-4 and IL-13, whereas IFN-g and IL-17 production was similar to WT mice in both *Was*$^{-/-}$ and *Was*$^{fl/fl}$*Foxp3*-Cre animals (**Figure 7.6F**). These results demonstrated that WASP-deficient Tregs are capable of normally regulating Th1 or Th17 differentiation, but fail to specifically contain Th2 effector responses in vivo.

We then aimed to investigate whether WASP-deficient FOXP3$^+$ Tregs were selective in their capacity to suppress Th1, Th2, or Th17 polarized effector cells in vitro. To do this, we first isolated congenically marked CD45.1$^+$ naïve CD4$^+$ T cells and polarized them into Th1, Th2, or Th17 effector subsets respectively (**Figure 7.S5A**). We confirmed the identity of the polarized cells by staining for Th-specific transcription factors T-bet, GATA3, and RORgt (**Figure 7.S5B**). We then CFSE-labeled these responder cells and co-cultured them with sorted EGFP$^+$ Tregs from *Was*$^{+/+}$*Foxp3*-Cre or *Was*$^{fl/fl}$*Foxp3*-Cre mice (**Figure 7.S5C**) in the presence of aCD3/aCD28 and examined CD45.1$^+$ responder cell proliferation 72 hours later (**Figure 7.S5D**). Both WT and WASP-deficient FOXP3$^+$ cells proved equally poor in their ability to suppress in vitro polarized effector cells (**Figure 7.S5D**) even though the WT Tregs were competent to suppress naïve T cells proliferation (**Figure 7.S5E**). With this set of experiments, we were therefore unable to demonstrate that the selective loss of Th2 suppression by WASP-deficient Tregs in vivo also occurs with co-cultured polarized cells in vitro.

We next assessed the pertinence of the Th2 cytokine IL-4 on the pathogenesis of food allergy in *Was*$^{-/-}$ mice, by analyzing the extent to which food allergic sensitization occurred in *Was*$^{-/-}$*Il4*$^{-/-}$ animals. Anti-soy, as well as total IgE and IgG1 responses were completely abrogated in the absence of IL-4, and mucosal mast cell expansion and activation did not occur as assessed by serum MCPT1 levels (**Figure 7.6G** and **Figure 7.S4E**). Furthermore, anti-soy titers of the IL-4-independent IgG2b isotype were also significantly lower in *Was*$^{-/-}$*Il4*$^{-/-}$ mice (**Figure 7.6H**), signifying a critical role for Th2-mediated inflammation in the induction of anti-food humoral immune responses in WASP-deficient mice.

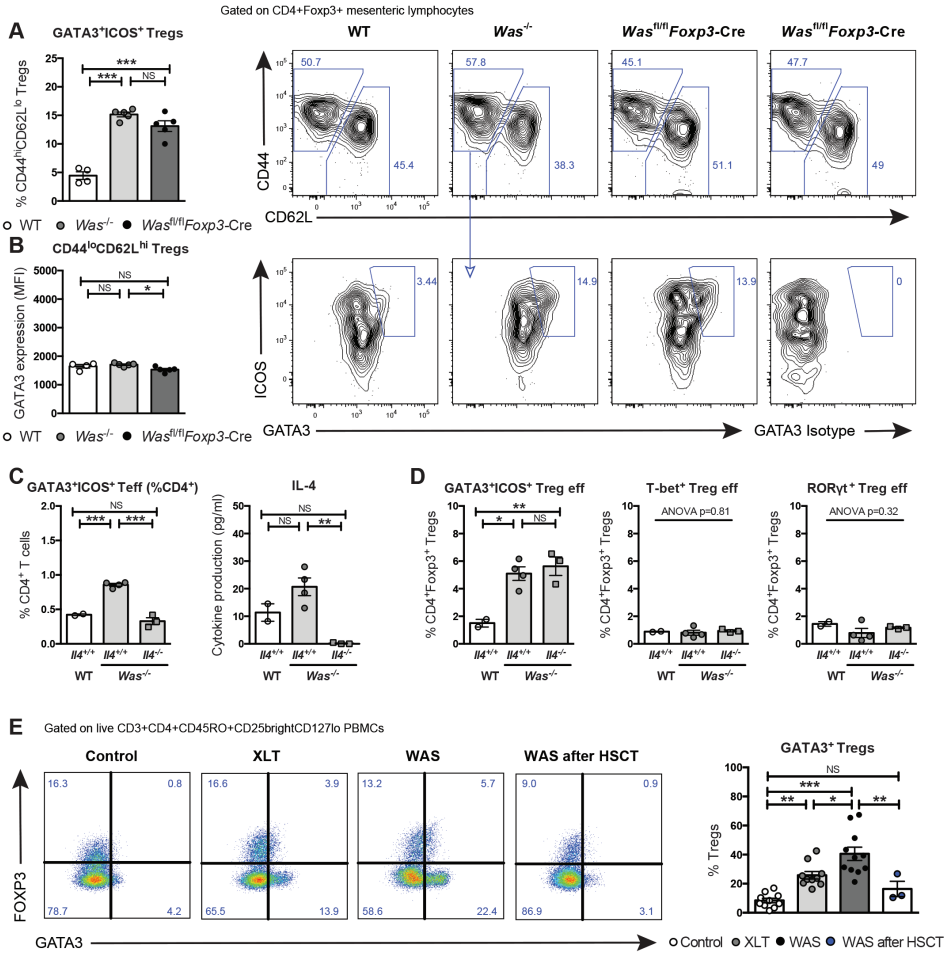

Figure 7.7. WASP-deficient effector Tregs assume a Th2-like phenotype in mice and human patients. (**A**) Fraction of CD44^{hi}CD62L^{lo}CD4^{+}Foxp3^{+} Tregs that co-express GATA3 and ICOS in WT (open circles, n=4), *Was*$^{-/-}$ (gray circles, n=6) and *Was*$^{fl/fl}$*Foxp3*-Cre (black circles, n=5) mice, with gating strategy in representative samples depicted on the right. (**B**) Intracellular GATA3 levels in CD44^{lo}CD62L^{hi}CD4^{+}Foxp3^{+} Tregs determined by flow cytometry. (**C**) GATA3^{+}ICOS^{+} effector T cells in WT or *Was*$^{-/-}$ mice with either *Il4*$^{+/+}$ (gray circles, n=4) or *Il4*$^{-/-}$ alleles (gray squares, n=3) and IL-4 production by CD4^{+} mesenteric lymphocytes stimulated with anti-CD3/CD28 ex vivo. Each dot represents the average cytokine production from triplicate cell suspensions from a single mouse. (**D**) Percentage of Foxp3^{+} Tregs co-expressing GATA3 and ICOS, T-bet, or RORgt in in WT or *Was*$^{-/-}$ mice with either *Il4*$^{+/+}$ or *Il4*$^{-/-}$ alleles. **(E)** Gating strategy and cumulative data of percent of total effector (CD45RO^{+}) Tregs co-expressing GATA3 in isolated peripheral blood mononuclear cells (PBMCs) from XLT (n=10) and WAS (n=11) patients compared to age and sex matched controls (n=10) or WAS patients following hematopoietic stem cell transplantation (HSCT, n=3). Symbols represent individual mice or patients and error bars depict SEM. *p<0.05; ** p<0.01; ***p<0.001; NS: not significant as determined by one-way ANOVA with Tukey's multiple comparisons test. Mouse experiments representative of 2 independent experiments. Results in panel E are cumulative data from 1 experiment

7.4.6 WASP–deficient effector Tregs assume a Th2–like phenotype

Recent data indicate a role for Th2-type-programmed Tregs in an experimental model of murine food allergy as well as human children (Noval Rivas et al., 2015). Similarly, a Th2-promoting fraction of Tregs that depends on TCR signaling was identified in asthmatic mice and patients (Ulges et al., 2015). Since aberrant responses to TCR activation are a feature of WASP-deficient T cells (Matalon et al., 2013), we analyzed the extent to which WASP-deficient Tregs display a Th2-like phenotype. In both *Was*$^{-/-}$ and *Was*$^{fl/fl}$*Foxp3*-Cre mice, we observed increased fractions of GATA3^{+}ICOS^{+} Tregs in MLNs compared to WT counterparts (**Figure 7.7A** and **Figure 7.S6A**). These cells were confined to the CD44^{hi}CD62L^{lo} effector-like Treg subset as GATA3 expression in the naïve-like fraction of Tregs was equivalent to WT CD4^{+}FOXP3^{+}CD44^{lo}CD62L^{hi} Tregs (**Figure 7.7B**). Th2-type reprogrammed Tregs in the context of food allergy had previously been observed in a genetic model that relies on increased signaling through the IL-4 receptor (Noval Rivas et al., 2015). We therefore asked whether WASP-deficient CD44^{lo}CD62L^{hi} Tregs exhibit an increase GATA3 expression pattern in response to increased availability of IL-4 in a Th2-skewed inflammatory setting. However, analysis of *Was*$^{-/-}$*/Il4*$^{-/-}$ animals revealed that while Th2 skewing of effector T cells and IL-4 production from CD4^{+} mesenteric lymphocytes was abrogated (**Figure 7.7C**), an equivalent increase in the fractions of GATA3^{+}ICOS^{+} memory-effector Tregs was found when compared to *Was*$^{-/-}$ IL-4 competent counterparts, while relative Treg numbers were similar between groups (**Figure 7.7D** and **Figure 7.S6B**). Additional analysis revealed that increased expression of transcription factors did not extend to T-bet or RORgt, as memory-effector Tregs co-expressing these markers were found in similar frequencies amongst all genotypes (**Figure 7.7D**). When naïve T cells were stimulated with anti-CD3/CD28 and TGFβ in vitro, we observed no difference in the induction of GATA3^{+} Tregs from WT or *Was*$^{fl/fl}$*Foxp3*-Cre donor-derived cells (**Figure 7.S6C**), indicating that differentiation into the Treg lineage in the absence of WASP by itself is insufficient to cause increased GATA3^{+} expression. This suggests that the appearance of GATA3^{+}ICOS^{+} Tregs depends on additional external signals in vivo. Combined, these data demonstrate that WASP-deficient effector memory Tregs, but not naïve-like Tregs, assume a Th2-like phenotype independently from increased IL-4 signaling.

Lastly, we looked whether GATA3^{+} Tregs are increased in patients with mutations in *WAS.* Individuals with either XLT or WAS displayed increased fractions of GATA3^{+} Tregs among the effector Tregs isolated from peripheral blood mononuclear cells (PBMCs, **Figure 7.7E**). Similarly, mean fluorescence intensity of GATA3 was higher in effector Tregs from WAS and XLT patients than in age-matched, male controls (**Figure 7.S6D**). Interestingly, we observed a correlation between severity of *WAS* mutation and GATA3-expression in Tregs, as the fraction of GATA3^{+} Tregs was significantly higher in WAS patients than in individuals with XLT. In agreement with our observations in mice, WAS patients also had a higher fraction of FOXP3^{-} GATA3^{+} effector T cells (Th2 cells, **Figure 7.S6E)**. Three WAS patients who had previously undergone hematopoietic stem cell transplantation (HSCT) showed a normal

GATA3^{+} effector Treg compartment, as well as Th2 effector cells among PBMCs (**Figure 7.7E** and **Figure 7.S6E**). These results indicate that loss of suppression of Th2 effector responses and assumption of a Th2-type phenotype of Tregs also occur in human WAS patients.

7.5 Discussion

The Wiskott-Aldrich syndrome is an illustrative example of how investigating a monogenetic human disease can advance our understanding of immune pathways in health and disease (Liston et al., 2008; Thrasher and Burns, 2010). Data from *Was*$^{-/-}$ mice (Snapper et al., 1998) revealed that WASP-dependent functions are highly conserved between mouse and man and that many aspects of the human disease are faithfully mimicked in these animals. WASP is best characterized for its role as stimulator of ARP2/3-mediated actin polymerization, which enables a variety of downstream effector functions including tissue migration and filopodia stability. A vast body of literature has accumulated that addresses the molecular and immunological consequences of deficiency of WASP in not just T and B lymphocytes, but also iNKT cells, platelets, NK cells, mast cells, dendritic cells, monocytes and neutrophils (Massaad et al., 2013). Because many of these WASP-dependent functions have been defined in either patients and mice lacking WASP in all hematopoietic lineages, or in isolated cell systems *ex vivo* or in vitro, the systemic sequelae of cell-specific perturbations in WASP-mediated signals has remained largely unknown (Massaad et al., 2013).

Here we report an increased prevalence of allergic responses against common food antigens as a feature of WAS in humans. Although elevated levels of serum IgE and eczema are well-described characteristics of the disease, the burden of IgE-mediated allergic disorders among patients with WAS or XLT has remained largely uncharacterized. Our studies demonstrate that the elevated serum IgE pool in these patients is functional and has the capacity to mediate allergic reactions to common food antigens, which should prompt treating physicians to be vigilant for food allergy among these patients and to obtain a careful history of adverse events after food ingestion.

The connection between WASP deficiency and the occurrence of IgE-mediated reactions against foods was further corroborated by studies in *Was*$^{-/-}$ animals. WASP-deficient mice produce IgE and IgG1 antibodies with specificity for components of chow, most prominently soy and wheat, which are also two allergens commonly implicated in IgE-mediated food allergy in human patients (Sicherer and Sampson, 2010; Wang and Sampson, 2011). These responses were observed in *Was*$^{-/-}$ mice on three different genetic backgrounds, independent of colonic inflammation, and occurred similarly in mice that were housed under germ-free settings when compared to those colonized with an SPF-flora. As such, our findings suggest that mutations in *Was* predispose to allergic disease independently from microbe-derived signals or differences in strain-specific immune constitution.

Using a loxP/Cre-based system permitting cell-specific deletion of WASP (Recher et al., 2012), we demonstrated that FOXP3$^+$ Tregs are critically dependent on WASP-mediated signals for their ability to maintain tolerance to ingested food antigens. These results indicate that WASP deficiency in other immune compartments such as antigen presenting cells, B cells, or effector CD4$^+$ T cells is dispensable for the pathogenesis of food allergic responses in *Was*$^{-/-}$ mice and, potentially, WAS patients. In fact, the absence of WASP in non-FOXP3$^+$ immune cells is likely to have a net dampening effect on IgE sensitization to food antigens since both food-specific IgE titers and allergic intestinal tissue inflammation were significantly lower in *Was*$^{-/-}$ mice when compared with *Was*$^{fl/fl}$*Foxp3*-Cre mice. In support of this conclusion, work by Morales-Tirado et al. previously identified a Th2-specific dysfunction of WASP-deficient CD4$^+$ effector T cells, manifesting as a reduced production of IL-4 and compromised expulsion of *N. Brasiliensis* (Morales-Tirado et al., 2010). Indeed, following ex vivo TCR stimulation, we confirmed reduced production of IL-4 and IL-13 by CD4$^+$ mesenteric lymphocytes obtained from *Was*$^{-/-}$ mice when compared to cells from *Was*$^{fl/fl}$*Foxp3*-Cre mice. In human WAS patients, which are more likely to resemble *Was*$^{-/-}$ mice than *Was*$^{fl/fl}$*Foxp3*-Cre animals, reduced effector function in Th2 lymphocytes may account for the observation that only 20-30% of WAS and XLT patients in our cohort suffer from clinically relevant food allergy. Notably, none of the food allergic patients suffered from anaphylaxis upon allergen exposure, which may be related to reduced mast-cell histamine secretion in response to IgE-mediated activation, as has been previously described for WASP-deficient mice (Pivniouk et al., 2003). Using morphine as an antigen/IgE-independent mast cell stimulus, we observed reduced wheal responses in WAS patients when compared to controls. Together with the high discordance between sIgE levels and SPT in patients with mutations in *WAS*, with several false-negative SPT results, these data suggest that mast cell dysfunction may contribute further to the moderate allergic phenotype in WAS patients, and in *Was*$^{-/-}$ mice when compared to *Was*$^{fl/fl}$*Foxp3*-Cre animals.

Previous studies on Tregs in *Was*$^{-/-}$ mice have hypothesized that the immunopathology in these animals results in part from reduced numbers of FOXP3$^+$ CD4$^+$ lymphocytes in secondary lymphoid organs (Humblet-Baron et al., 2007; Maillard et al., 2007; Marangoni et al., 2007). However, we found that conditional deletion of WASP in *Was*$^{fl/fl}$*Foxp3*-Cre mice did not lead to lower Treg numbers in PP's or MLNs when compared to WT animals. These findings implicate functional abnormalities in WASP-deficient Tregs as the principal cause of immune dysregulation in *Was*$^{-/-}$ mice. Although these Treg abnormalities manifest in vivo as an inability to control Th2 effector cells, in vitro suppression assays showed that WASP-deficient Tregs were unable to suppress other effector cell subsets as well. The basis for this difference is not yet understood. In further support of a Treg-intrinsic role for WASP in controlling aberrant immune responses, we observed increased expression of the Th2 transcription factor GATA3 in activated WASP-deficient Tregs from either *Was*$^{fl/fl}$*Foxp3*-Cre or *Was*$^{-/-}$ hosts when compared to WT animals, and similarly in human patients with WAS in comparison with age and sex-matched controls.

Although the molecular mechanism of Treg-specific dysfunction in the absence of WASP remains to be elucidated, emerging data suggest aberrant signaling downstream of TCR ligation as a possible candidate. Increased Th2 responses and elevated IgE also result from loss of function of the TCR-associated scaffold protein LAT (Aguado et al., 2002; Sommers et al., 2002). WASP is rapidly recruited to the TCR upon ligation (Barda-Saad et al., 2005), and is required for internalization of the TCR complex (Zhang et al., 1999). Since Tregs have been shown to require continuous TCR-dependent signaling for optimal suppressive function (Levine et al., 2014; Vahl et al., 2014), Alterations in downstream events following TCR-mediated activation in the absence of WASP may be related to the loss of Th2-suppressive capacity. Moreover, deficiency of the TCR-associated phosphatase SHP-1 results in an increased population of GATA3^{+} Tregs and food allergy similar to our observations in patients and mice with WASP-deficiency (Noval Rivas et al., 2015). Together, these findings support a link between aberrant TCR signaling in WASP-deficient Tregs and unrestrained Th2 pathology in vivo. However, our studies also indicate that disruption of TCR-mediated signals in WASP-deficient effector T cells is not required for Th2-skewed immune responses to occur, as *Was*$^{fl/fl}$*Foxp3*-Cre mice, which are sufficient for WASP in effector cells, showed increased numbers of GATA3^{+} effector T cells compared to *Was*$^{-/-}$ animals. Molecular profiling and immune phenotyping of WASP-deficient Tregs following TCR-mediated activation may further elucidate the mechanism that results in a Th2-skewed regulatory phenotype.

In summary, we identified an increased prevalence of IgE-mediated food allergy amongst a cohort of patients with mutations in *WAS*. Using *Was*$^{-/-}$ mice, we showed that IgE sensitization occurs both in the presence and absence of an intestinal flora and that the deficiency of WASP in FOXP3^{+} Tregs alone is sufficient to drive allergic intestinal inflammation. While capable of fully containing Th1 and Th17 effector responses in vivo, WASP-deficient Tregs exhibit a Th2-like phenotype and fail to selectively restrain Th2 effector differentiation resulting in intestinal mast cell expansion and activation. Our findings demonstrate that in WAS, defective FOXP3^{+} Tregs promote Th2-type immune hyperactivation and allergic disease.

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7.7 Author contributions

Designed and conducted the clinical patient study: JJL, MGL, EG, TD, PS, CCN, NJ, KDS, FC, JDM; Conceived and designed the mouse experiments: WSL, JAG, SBS, EF; Performed mouse experiments: WSL, JAG, JJ, MD, DK; Acquired and analyzed data: WSL, JJL; Obtained funding: WSL, EHHMR, JDM, SBS, EF; Provided reagents: AJT; Wrote the manuscript: WSL; Edited the paper: JJL, JAG, JDM, SBS, EF. All authors approved the final version of the manuscript.

7.8 Supplemental data

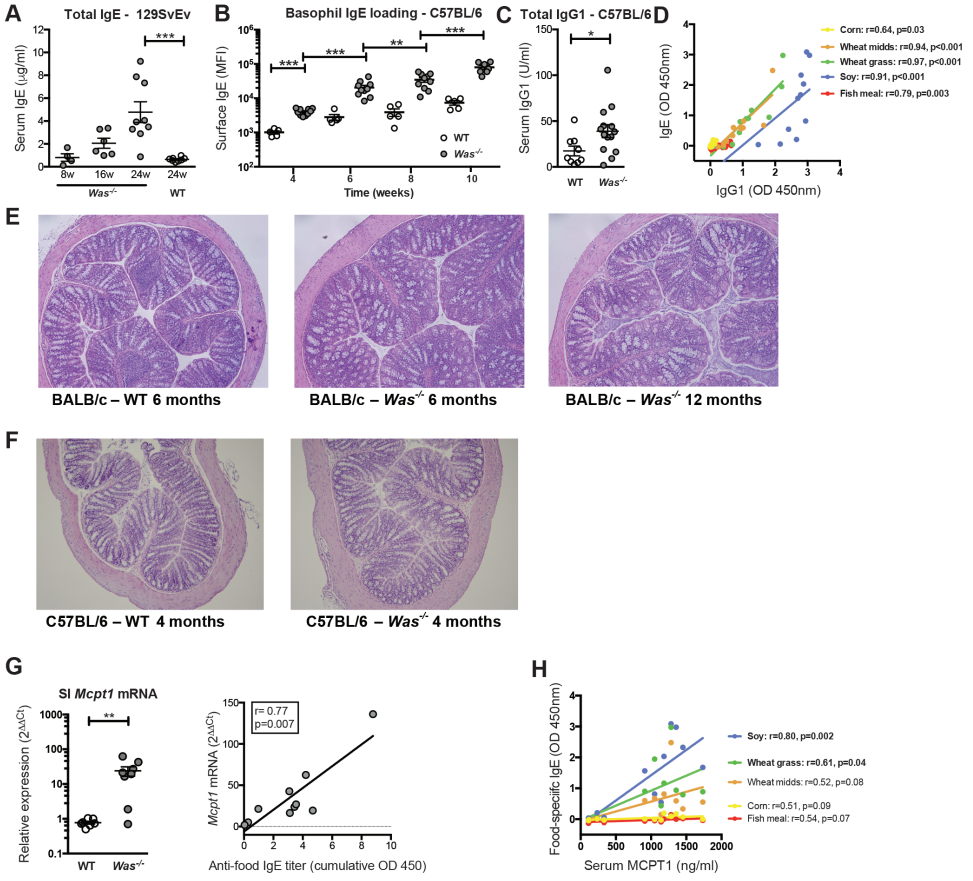


Figure 7.S1. Age-dependency of IgE levels in *Was*$^{-/-}$ mice and absence of colonic inflammation in C57BL/6 and BALB/c *Was*$^{-/-}$ animals. (**A**) Serum IgE levels in 129SvEv *Was*$^{-/-}$ animals between 8 and 24 weeks. (**B**) Bi-weekly assessment of surface IgE loading on blood basophils (CD45^{Mid}CD49b^{+}) from WT (open circles, n=5) and *Was*$^{-/-}$ (gray circles, n=10) on the C57BL/6 background. (**C**) Total levels of serum IgG1 in C57BL/6 *Was*$^{-/-}$ mice. (**D**) Pearson correlation coefficients between anti-food IgE and IgG1 titers for the five main chow components in Balb/c *Was*$^{-/-}$ mice. (**E**) H&E staining of colonic cross-sections from 6-12 month old WT and BALB/c *Was*$^{-/-}$ mice (20X) and (**F**) 4-month old *Was*$^{-/-}$ mice on the C57BL/6 background (20X). (**G**) Relative gene expression of *Mcpt1* in small intestinal (SI) tissue samples and correlation of *Mcpt1* mRNA expression with anti-food IgE titer. (**H**) Pearson correlation coefficients (r) between food-specific IgE titers and baseline serum MCPT1. Symbols represent individual mice and error bars depict SEM. *p<0.05; ** p<0.01; ***p<0.001; NS: not significant as determined by two-tailed Student's t-test or paired t-test for intraindividual analyses.

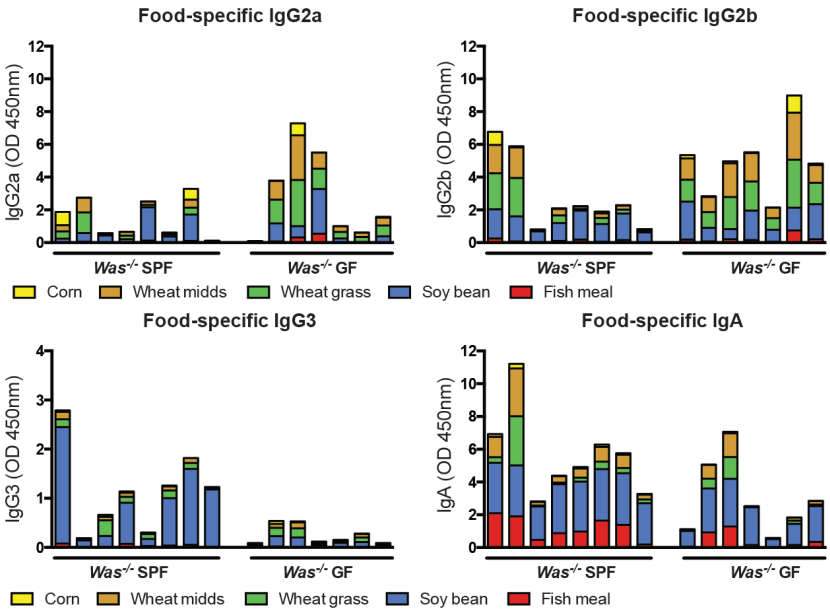


Figure 7.S2. Detailed analysis of food-specific titers for the five main chow constituents. Contribution of individual component-specific immunoglobulins to the cumulative anti-food isotype titers of IgG2a, IgG2b, IgG3, and IgA in SPF (n=8) and GF-housed (n=7) *Was*$^{-/-}$ mice. Bars represent results from individual mice.

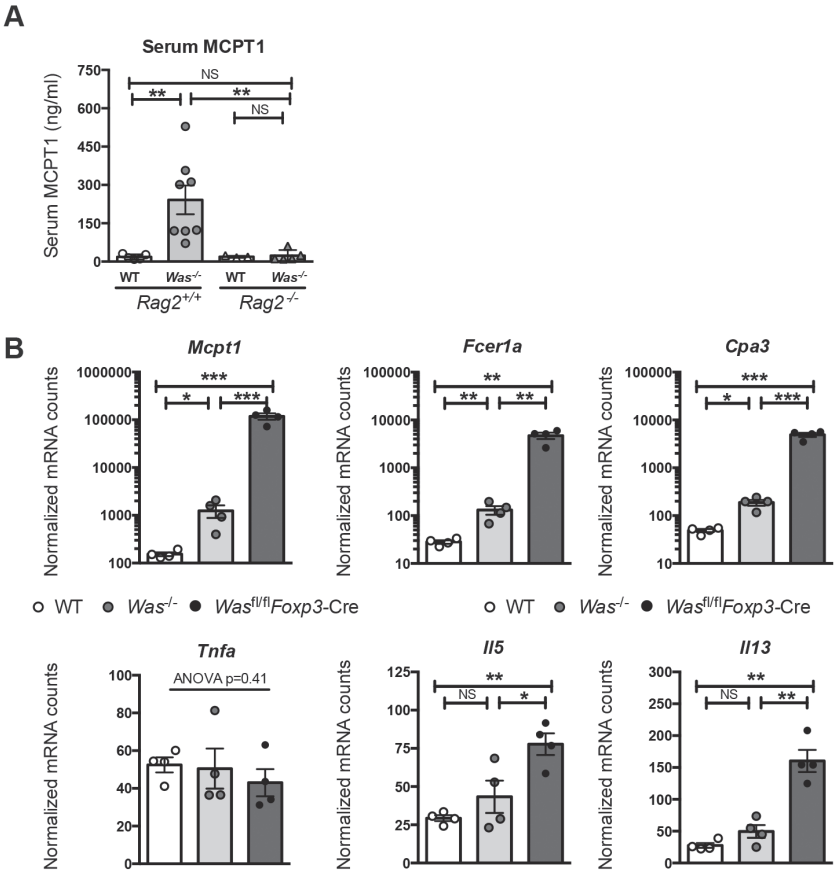


Figure 7.S3. Intestinal mast cell expansion in *Was*$^{-/-}$ mice requires adaptive immunity and *Was*$^{fl/fl}$*Foxp3*-Cre mice develop Th2-type intestinal inflammation. (**A**) Comparison of serum MCPT1 in WT (open circles, n=5), *Was*$^{-/-}$ (gray circles, n=8), *Rag2*$^{-/-}$ (open triangles, n=3) and *Was*$^{-/-}$*Rag2*$^{-/-}$ (gray triangles, n=5) mice ≥ 2 months old on the 129SvEv background. (**B**) Normalized absolute mRNA counts obtained from digital mRNA profiling of jejunal tissue for indicated genes. Symbols represent individual mice and error bars depict SEM. *p<0.05; ** p<0.01; ***p<0.001; NS: not significant as determined by two-tailed Student's t-test (panel A) or one-way ANOVA with Tukey's multiple comparisons test (panel B).

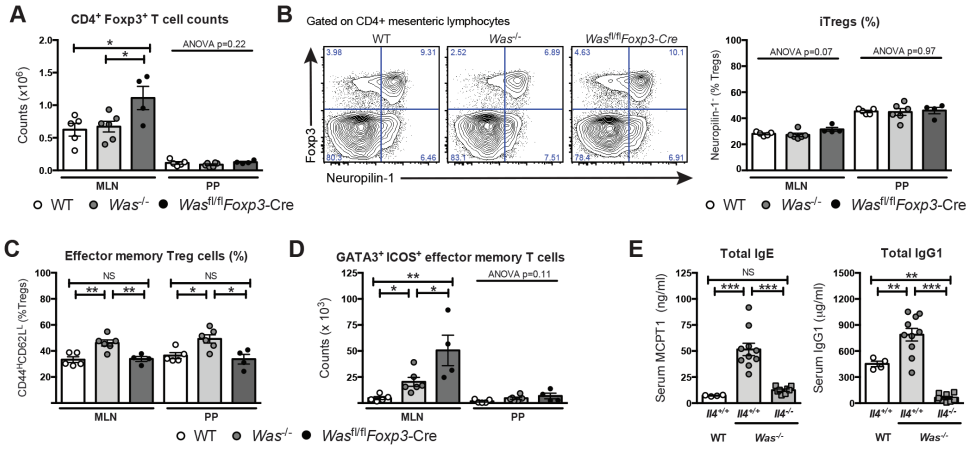


Figure 7.S4. WASP-deficient Foxp3⁺ Tregs fail to suppress Th2 type lymphoproliferation in vivo. (**A**) Quantification of Foxp3⁺ Tregs counts in MLNs and PPs of WT (n=5), *Was*$^{-/-}$ (n=6), and *Was*$^{fl/fl}$*Foxp3*-Cre (n=4) animals. (**B**) Gating strategy and summary statistic of the fraction of iTregs, defined as Foxp3⁺ Neuropilin1^{-}, within the total population of Tregs obtained from MLNs. (**C**) Summary statistic of the subpopulation of CD44^{Hi}CD62L^{Lo} effector memory Tregs within the total population of CD4⁺Foxp3⁺ lymphocytes. (**D**) Absolute numbers of Th2-type, GATA3⁺ICOS⁺ effector memory cells in MLNs and PPs. (**E**) Serum levels of total IgE and IgG1 in *Was*$^{-/-}$ mice on the 129SvEv background with either *Il4*$^{+/+}$ (n=10) or *Il4*$^{-/-}$ (n=8) alleles. Symbols represent individual mice and error bars depict SEM. *p<0.05; ** p<0.01; ***p<0.001; NS: not significant as determined by two-tailed Student's t-test or one-way ANOVA with Tukey's multiple comparisons test. Representative results of 2 independent experiments.

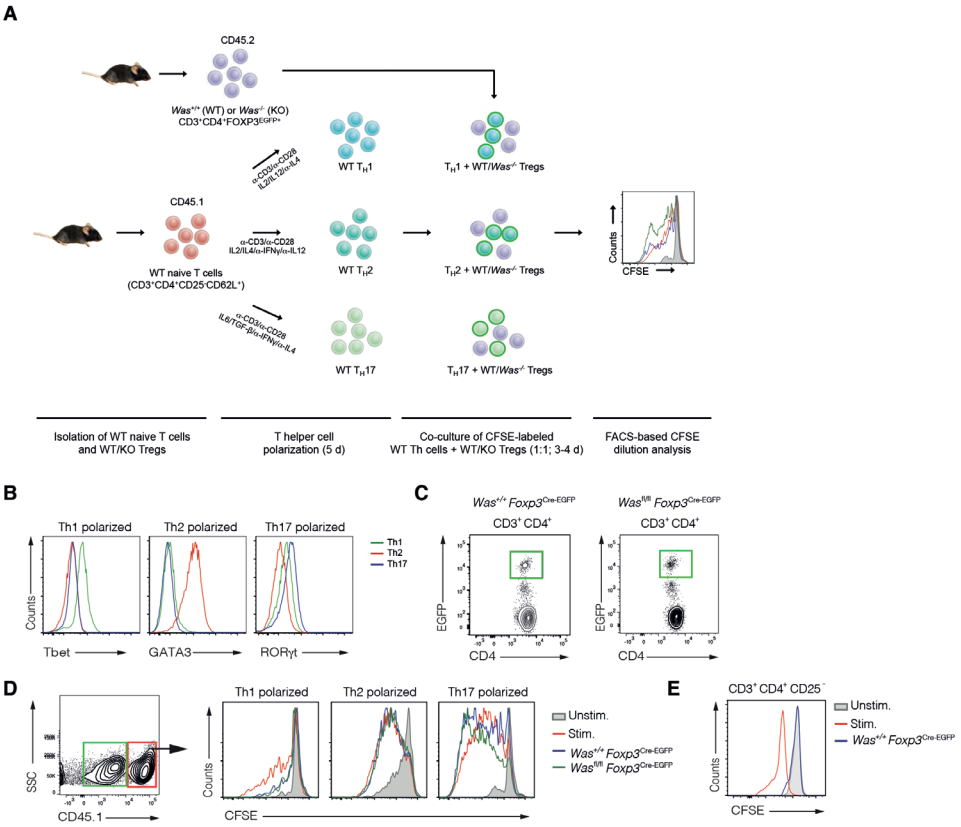


Figure 7.S5. In vitro *suppression of polarized Th cells*. (**A**) Schematic of in vitro Th1, Th2, and Th17 polarization of WT naïve T cells and subsequent suppression assay using *Was*$^{+/+}$*Foxp3*$^{Cre\text{-}EGFP}$ or *Was*$^{-/-}$ *Foxp3*$^{Cre\text{-}EGFP}$. (**B**) Expression of Th-specific transcription factors following Th1/Th2/Th17 polarizing conditions. (**C**) Sorting of FOXP3-GFP^{+} cells from *Was*$^{+/+}$*Foxp3*$^{Cre\text{-}EGFP}$ or *Was*$^{fl/fl}$*Foxp3*$^{Cre\text{-}EGFP}$ mice. (**D**) Gating of CD45.1^{+} responder Th cells labeled with CFSE (left) with Th cell proliferation indicated by CFSE dilution in overlaid histograms (right). (**E**) Suppression of freshly isolated naïve CD4^{+} T cells by GFP^{+} WT Tregs isolated from *Was*$^{+/+}$*Foxp3*$^{Cre\text{-}EGFP}$ mice. Data are representative of three independent experiments.

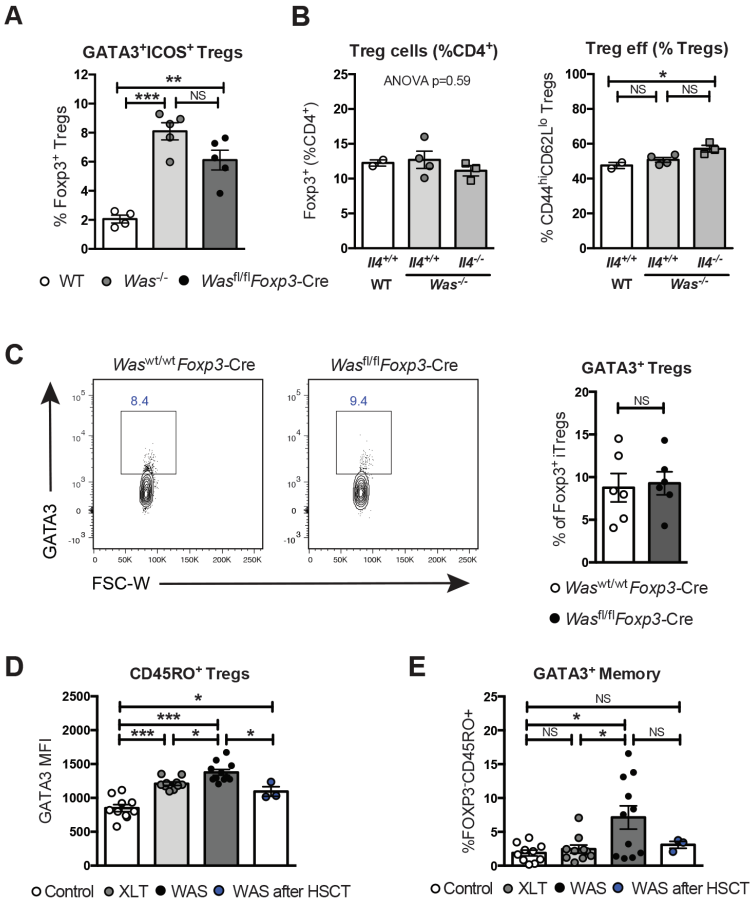
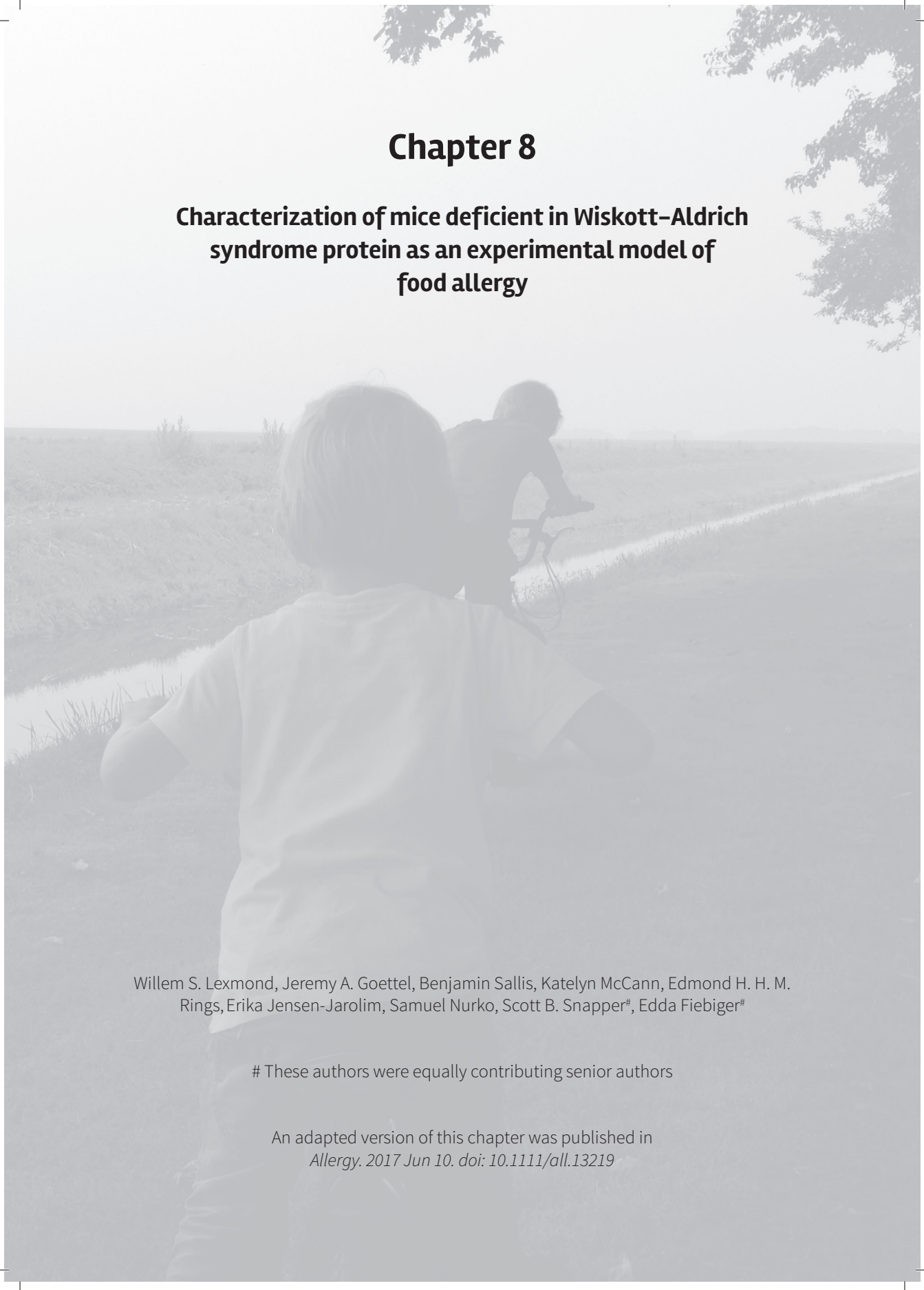


Figure 7.S6. WASP-deficient effector Tregs assume a Th2-like phenotype. (**A**) Fraction of total CD4$^+$Foxp3$^+$ Tregs that co-express GATA3 and ICOS in WT, *Was*$^{-/-}$ and *Was*$^{fl/fl}$*Foxp3*-Cre mice. (**B**) Fraction of Foxp3$^+$ Tregs amongst CD4$^+$ mesenteric lymphocytes and effector memory Tregs amongst total Foxp3$^+$ Tregs in WT or *Was*$^{-/-}$ mice with either *Il4*$^{+/+}$ or *Il4*$^{-/-}$ alleles on the 129sv background. (**C**) Comparison of TGFβ-mediated induction of GATA3$^+$ Tregs from naïve CD3$^+$CD4$^+$CD25$^-$GFP$^-$ T cells from WT or *Was*$^{fl/fl}$*Foxp3*-Cre mice in vitro. Representative dot plots are shown with the percentage of GATA3$^+$ iTregs depicted gated on CD3$^+$CD4$^+$GFP$^+$ cells. (**D**) Mean fluorescent intensity (MFI) of GATA3 expression among total effector (CD45RO$^+$) Tregs and (**E**) percent of GATA3$^+$ effector T cells among XLT (n=10) and WAS (n=11) patients compared to age and sex matched controls (n=10) or WAS patients after hematopoietic stem cell transplantation (HSCT) (n=3). Dots represent cells from individual mice or patients and error bars depict SEM. *p<0.05; ** p<0.01; ***p<0.001; NS: not significant as determined by one-way ANOVA with Tukey's multiple comparisons test. Panels A-C are representative of 2 independent experiments. Results in panels D and E are cumulative data from 1 experiment.

Chapter 8

Characterization of mice deficient in Wiskott–Aldrich syndrome protein as an experimental model of food allergy



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8.1 Abstract

Background: Food allergies are a rapidly growing health problem worldwide. Mechanisms underlying the natural development of this disease are poorly understood, in part, because spontaneous disease models have not been reported.

Objective: We sought to determine if sensitization to chow as spontaneously occurring in mice deficient for Wiskott-Aldrich syndrome (WAS) protein results in IgE-mediated food allergy.

Methods: ImmunoCAP ISAC microarray was performed in serum of food allergic and WAS patients. Adjuvant-free sensitization to ovalbumin (OVA) was assessed following repetitive OVA gavage in WASP deficient (*Was*$^{-/-}$) mice or in animals with a Treg-specific *Was* deletion (*Was*$^{fl/fl}$*Foxp3*-Cre mice) and compared with an experimental model induced in wild-type mice by gastric gavage after intraperitoneal sensitization with OVA/alum.

Results: Polysensitization to multiple food antigens was found in food allergic and WAS patients. As seen in food allergic patients, low titers of OVA-specific IgE were found in the spontaneous *Was*$^{-/-}$ model. Anaphylaxis and allergic diarrhea occurred to a similar degree in both models. Adjuvant-free intestinal sensitization in *Was*$^{-/-}$ mice occurred independently of FcεRI but expansion of jejunal mast cells was less pronounced. Significant spontaneous sensitization and pronounced intestinal allergy was also observed in *Was*$^{fl/fl}$*Foxp3*-Cre animals.

Conclusion: *Was*$^{-/-}$ and *Was*$^{fl/fl}$*Foxp3*-Cre animals provide models for food allergy that address the current experimental gap in analyzing aberrant immune responses at the onset of food allergy. *Was*$^{fl/fl}$*Foxp3*-Cre mice are a genetically parsimonious alternative to this model that allows the study of allergy without confounding influence of WASP deficiency in non-Treg immune compartments.

8.2 Introduction

Immunoglobulin E (IgE)-mediated food allergies are a rapidly growing health problem in industrialized societies. Although the determination of the exact prevalence is hampered by non-overlapping diagnostic definitions, estimates for US adults and children currently lie around 5% and 8% respectively (Sicherer and Sampson, 2014). In these patients, exposure to minute quantities of food allergens can trigger anaphylactic reactions, which range in severity from oral tingling and nausea to profuse diarrhea and anaphylactic shock (Longo et al., 2013). Allergic reactions to food can be fatal, and form a common reason for emergency room visits and hospitalization (Bock et al., 2007; Henson and Burks, 2012). Considerable research effort is therefore dedicated to understanding the origin of IgE-mediated food allergy (Sampson et al., 2014).

The pathogenesis of food allergic reactions consists of a sensitization phase, during which food-specific IgE antibodies are first produced, and an effector phase, in which IgE antibodies mediate activation of immune effector cells upon repeated exposure to food antigens. Allergic sensitization results from perturbations in the pathways that normally maintain a state of immune tolerance to innocuous food antigens. While the physiological outcome of food antigen presentation by dendritic cells is anergy of T cells and induction of FOXP3^{+} regulatory T cells (Tregs), sampling of intestinal allergens in food allergic patients instead results in Th2 effector responses. Allergen-specific Th2 cells respond to subsequent allergen encounters with production of Th2 type cytokines such as IL-4 and IL-13, and thereby can instruct allergen-specific B cells to undergo class-switching to IgE. Food-specific IgE circulates freely, until it binds to the high-affinity IgE receptor FcεRI that is expressed on the cell surface of mast cells and basophils. During the allergic effector phase, these IgE effector cells respond to antigen-mediated crosslinking of FcεRI by rapid secretion of pre-formed inflammatory mediators such as histamine and proteases, which are responsible for the local and systemic symptoms associated with allergic reactions.

Epidemiologic studies have identified numerous associations between genetic, microbial, environmental and nutritional factors and the development of food allergy, but experimental and mechanistic evidence to prove causality remains scarce. This gap in our understanding is, at least in part, attributable to the lack of animal models that mimic spontaneous sensitization to food as it occurs in humans (Van Gramberg et al., 2013). Wild-type mice have proven to be highly resistant to the dysregulation of IgE-mediated immune responses to food allergens, testifying to the robustness of the cellular regulatory mechanisms that normally mediate tolerance to ingested foreign antigens (Oyoshi et al., 2014). Consequently, murine models of food allergy commonly rely on adjuvant-based strategies that either induce systemic production of antigen-specific IgE prior to first intestinal exposure, or, alternatively, disrupt the induction of oral tolerance by intestinal co-exposure to adjuvants such as cholera toxin or staphylococcal enterotoxin B (Berin and Mayer, 2009).

These models, which recapitulate the symptoms and treatment responses of human disease but not the underlying cause of disease, are considered 'isomorphic' (Oyoshi et al., 2014). A 'homologous' animal model of food allergy, in contrast, would not only capture the IgE effector phase of disease, but also faithfully mimic oral, spontaneous sensitization to food antigens (Oyoshi et al., 2014). Unfortunately, true homologous food allergy models do not exist at this time (Oyoshi et al., 2014), and while conventional adjuvant-based, isomorphic models have proven to be highly valuable tools to decipher the mechanisms of the symptoms of the mast cell-dependent IgE-effector phase, their use for understanding spontaneous disease onset remains contentious.

Recently, we documented an increased prevalence of food allergy in human patients suffering from the primary immunodeficiency Wiskott-Aldrich syndrome (WAS). Similarly, mice that are deficient in the homologous murine *Was* gene (*Was*$^{-/-}$ females or hemizygous *Was*$^{-}$ males) were found to develop IgE antibodies against common food allergens such as wheat and soy that are ubiquitously present in mouse chow. Given this shared phenotype between human patients and the corresponding mouse model, we hypothesized that *Was*$^{-/-}$ animals could potentially be developed as a novel experimental model to study allergic sensitization to intestinal allergens in a more homologous fashion. Here we show that *Was*$^{-/-}$ mice can be sensitized without a requirement for oral or systemic adjuvant against the model antigen OVA and that IgE-mediated effector responses are equivalent to those observed in a conventional food allergy model of disease. These data identify WASP-deficient mice as a novel model of experimental food allergy with a direct corollary to human disease.

8.3 Materials and Methods

8.3.1 Patients

Serum from pediatric WAS patients was obtained from a bio repository maintained under IRB-approved protocol number P00000529 at Boston Children's Hospital. Samples from age-matched food allergic and non-allergic control patients was available from a previously described longitudinal cohort study at Boston Children's Hospital (Lexmond et al., 2013) that was approved by the Investigational Review Board of Boston Children's Hospital (Harvard Medical School, Boston, MA, approval number: 07-11-0460). All patients or their legal guardians provided informed consent prior to donation of blood.

8.3.2 Animals

The generation of *Was*$^{-/-}$ and *Was*$^{fl/fl}$*Foxp3*-Cre mice has been previously described (Snapper et al., 1998) and **Chapter 7**. *Was*$^{-/-}$ and *Was*$^{+/-}$ or WT animals on both BALB/c and C57BL/6 backgrounds were bred in house and kept under SPF conditions in compliance with institutional guidelines. Experiments were performed in littermate setting where possible

and comprised both sexes. Where additional mice were required to increase animal numbers, mice were cohoused prior to the start of the experiment. C57BL/6 *Fcer1a*$^{-/-}$ mice were crossed with *Was*$^{-/-}$ mice and then bred to give rise to cohorts of *Was*$^{-/-}$*Fcer1a*$^{-/-}$ and *Was*$^{-/-}$*Fcer1a*$^{+/-}$ littermates. All described experiments were approved by the Institutional Animal Care and Use Committee under protocol number 13-06-2415R (IACUC, Boston Children's Hospital).

8.3.3 ELISAs

IgE and IgG levels in human serum were detected by ELISA using anti-human IgE (clone: MHE-18, BioLegend) and anti-human IgG (Southern Biotech 2014-01) capture antibodies and horseradish peroxidase-conjugated anti-human IgE (Sigma-Aldrich A9667) or biotin-conjugated anti-human IgG (eBioscience 13-4998-83) detection antibodies and quantified against serial dilutions of human IgE (NBS-C BioScience 0911-0-050) or human IgG (Southern Biotech 0150-01). Quantification of murine total, food antigen-specific and OVA-specific IgE and IgG1 has been previously described (**Chapter 7** and (Platzer et al., 2015a). MCPT1, IL-13, IL-6 and TNF-α protein levels were quantified using Ready-Set-Go! ELISA sets (eBioscience) according to manufacturer's instructions.

8.3.4 ImmunoCAP ISAC microarray

Allergen-specific IgE and IgG4 in serum samples was detected using the ImmunoCAP® ISAC 112 solid phase assay (Thermo Scientific, 81-1012-01) according to manufacturer's instructions. For detection of IgG4, serum samples were diluted 1:50 with ImmunoCAP®-specific IgA/IgG Sample Diluent and incubated for 2h at room temperature to ISAC 112 ImmunoCAP® microchips in a humidified chamber. After washing and drying, bound IgG4 was detected with fluorescently labeled anti-IgG4 as described by the manufacturer, and quantified as relative ISAC standard units between 0.1-12 ISU-G4 with Phadia Microarray Image Analysis (MIA®) and Xplain® software by ThermoFisher.

8.3.5 In vivo food allergy models and allergen challenges

The adjuvant-based model of OVA food allergy has been previously described (Brandt et al., 2003). In brief, WT Balb/C mice were immunized with 50 µg of OVA (Sigma) in 100 µl aluminum hydroxide (Thermo Scientific) via a total of 3 intraperitoneal injections every 7 days. Adjuvant-free sensitization to OVA in *Was*$^{-/-}$ mice was obtained by gavaging 5 mg of OVA in 200 µl PBS every 5th day for a total of 35 days as described in the main text. Following sensitization, mice were challenged by gavage with 50 mg OVA in 250 µl PBS. To assess anaphylaxis, body temperature was registered with the use of implantable temperature transponders (IPTT-300, Biomedic Data Systems, Seaford, Del). Baseline temperature was defined as the average of three measurements prior to challenge, and severity of anaphylaxis was assessed by calculating the ΔT from this average every 10 minutes after challenge.

8.3.6 In vitro mast cell degranulation assays

Bone marrow-derived cells from WT or *Was*$^{-/-}$ mice on either the Balb/C or C57BL/6 background were differentiated *in vitro* under influence of 10 ng/ml interleukin-3 and 20 ng/ml stem cell factor (Peprotech) for 8-15 weeks in RPMI 1640 medium (Gibco) supplemented with 10% FCS, 100 U/ml penicillin and 100 µg/ml streptomycin, 2mM L-glutamine and 55 µM β-mercaptoethanol (mast cell medium). Purity of mast cell cultures was established using mast cell markers c-kit (clone 2B8) and FcεRI (clone MAR-1, both from Biolegend). For degranulation assays, cells were loaded for 16-24h with IgE using either diluted serum or 100 ng/ml TNP-specific IgE, and then challenged with 500 ng/ml OVA or TNP-specific OVA in indicated doses. Degranulation was assessed by staining with anti-mouse LAMP-1 antibody (clone 1D4B, Biolegend) and viability dye (eBioscience). Cells were acquired on a FACSCanto II flow cytometer (BD Bioscience) and further analyzed using FlowJo software (Treestar). For some experiments, IgE-loaded mast cells were stimulated for 6h with 100 ng/ml TNP-specific OVA prior to collection of the supernatant for the assessment of cytokine production.

8.3.7 Flow cytometric analysis of basophils

Whole blood samples were lytreated with RBC lysis buffer (eBioscience) and peripheral blood mononuclear cells were stained with an antibody cocktail containing: anti-mouse CD16/CD32 (clone 2.4G2, BD Pharmingen), anti-mouse CD45 (clone 30-F11), anti-mouse CD49b (clone DX5), anti-mouse FceRIa (clone MAR-1) and anti-mouse IgE (clone RME-1, all from Biolegend). Basophils were identified as $CD45^{Mid}CD49b^{+}$ and analyzed for IgE surface loading using the mean fluorescence intensity of the anti-IgE stain.

8.3.8 pERK WB and quantification

Bone-marrow derived mast cells were lysed in RIPA buffer containing protease inhibitor cocktail, 2mM PMSF, 1 mM sodium orthovanadate and phosphotase inhibitor cocktail B (Santa Cruz Biotechnologies, Dallas, TX). Samples were run on 12% SDS-PAGE gels, transferred to PVDF membranes (Pierce, Rockford, IL) and probed with following antibodies: anti-ERK1/2 and anti-phosphoERK1/2 (both reagents from Cell Signaling Technology, Danvers, MA) followed by peroxidase-conjugated goat anti-rabbit IgG. Peroxidase activity was detected with SuperSignal chemiluminescent substrate reagents (Pierce). Western blots were quantified with ImageJ (NIH).

8.3.9 mRNA expression analysis

Intestinal *Mcpt1* mRNA expression was determined as previously described (**Chapter 7**) using iQ SYBR Green Supermix (Bio-Rad) and the following primers: FOR 5'-GAG GAC AGA TGT GGT GGG TTT-3' and REV 5'-AGG AGT CAA CTC AGC TTT CTC TT-3', and normalized against expression of housekeeping gene *Hprt* (FOR 5'-GTT GGA TAC AGG CCA GAC TTT GTT G-3' and REV 5'-GAG GGT AGG CTG GCC TAT AGG CT-3'). For mast cell assays, mRNA expression

was assessed by Nanostring® Mouse Immunology codeset (nCounter) according to the manufacturer's protocol.

8.3.10 Histology and mast cell staining

Mast cells were quantified using by chloroacetate esterase staining solution as previously described (Burton et al., 2012) and **Chapter 7**, and representative images were captured using an Olympus DP70 microscope equipped with DP Controller software (Olympus corporation).

8.3.11 Statistical analysis

All results were analyzed and visualized using GraphPad Prism version 6.0c for Mac (GraphPad Software). Details pertaining the use of statistical tests are provided in the figure legends.

8.4 Results

8.4.1 IgE and IgG4 antibodies against multiple foods are common in both food allergy and Wiskott–Aldrich syndrome

We have previously documented an increased prevalence of physician-diagnosed food allergy among patients suffering from WAS (**Chapter 7**). To further investigate the extent to which WAS-associated food allergy corresponds to common food allergic disease, we compared total IgE levels in serum samples from five pediatric WAS patients (aged 14 months to 7 years) with serum from age-matched patients who either suffered from food allergy (FA) or had no history of allergy (control). Pediatric FA and WAS patients demonstrated equivalent increases in total serum IgE levels when compared to control patients without allergic disease, whereas no differences were observed in serum total IgG levels between any of the groups (**Figure 8.1A**).

Patients with food allergy are at increased risk of sensitization to other, unrelated food antigens (de Jong et al., 2011; Liu et al., 2010). To assess whether polysensitization is also a feature of WAS-associated food allergy, we performed an immunochip-based assay that allowed for high-throughput screening for IgE reactivity against 112 common allergens, 49 of which represented food-derived epitopes from 19 unique foods. In three out of five patients with WAS, we observed IgE sensitization against food antigens, and two of these patients were found to be sensitized to at least two of the investigated foods. Similarly, four out of six FA patients were polysensitized to food antigens (**Figure 8.1B**). To further map the occurrence of anti-food antibody responses in FA and WAS patients, we also screened for the presence of allergen-specific IgG4, another Th2-dependent immunoglobulin that is thought to have a protective effect on the occurrence of food allergic reactions. IgG4 reactivity against

food allergens was common amongst all three diagnostic groups and across all investigated foods. Combined, these results demonstrate a high degree of overlap in total and food-specific IgE and IgG4 sensitization profiles between FA and WAS patients.

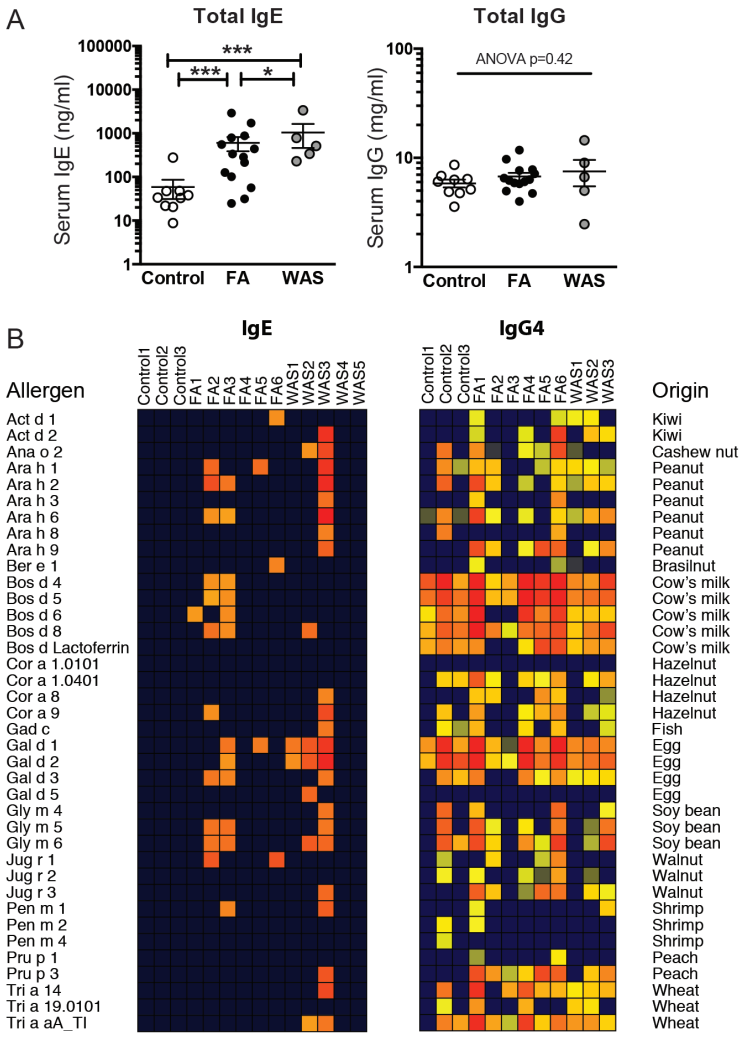

Figure 8.1. IgE and IgG4 antibodies against multiple foods are common in both food allergy and Wiskott-Aldrich syndrome. (**A**) ELISAs for total serum immunoglobulin levels in pediatric WAS patients and age-matched controls without (control) or with food allergy (FA). (**B**) Antigen specificity profiling by ImmunoCAP® demonstrates the presence of IgE and IgG4 with specificity for common food allergens in WAS patients. Serum from 3 control, 6 FA, 5 WAS patients was analyzed (3 WAS patients for IgG4). **p<0.01; ***p<0.001; NS: not significant as determined by ANOVA with Tukey's multiple comparisons test on log-transformed data.

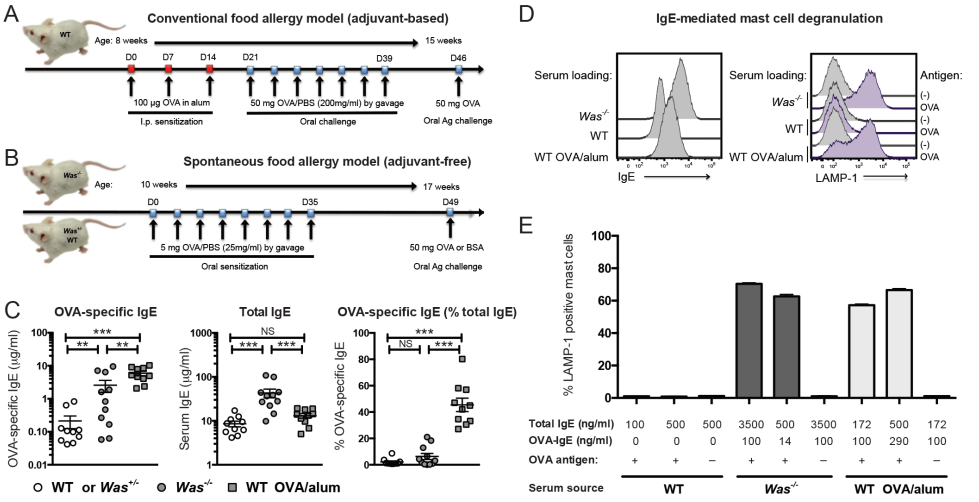

Figure 8.2. *Was*$^{-/-}$ mice develop adjuvant-free IgE sensitization to orally administered OVA. (**A-B**) Experimental time course of adjuvant-based and adjuvant-free sensitization protocols. (**C**) Total IgE, OVA-specific IgE, and % OVA-specific IgE of total IgE before final challenge in OVA/alum sensitized (gray squares) WT mice and OVA-gavaged *Was*$^{-/-}$ (gray circles) or WT/ *Was*$^{+/-}$ littermates (open circles). (**D**) Mast cell degranulation following OVA stimulation. Wild-type cells were loaded with serum (diluted to contain 100 ng/ml OVA-specific IgE) from *Was*$^{-/-}$ or OVA/alum-sensitized WT. Representative of 2 experiments. (**E**) Degranulation assays in response to OVA after loading of WT mast cells with serum of similar total IgE levels from spontaneously sensitized *Was*$^{-/-}$ mice (dark gray bars) or OVA/alum-sensitized WT animals (light gray bars). The mean +SEM of biological triplicates is shown. Symbols represent individual mice. Error bars depict SEM. *p<0.05; ** p<0.01; ***p<0.001; NS: not significant as determined by ANOVA with Tukey's multiple comparison test. Where data are displayed on a log-axis a log-transformation was performed prior to statistical analysis to meet normality assumptions.

8.4.2 *Was*$^{-/-}$ mice develop OVA–specific IgE following oral antigen exposure without adjuvant

Based on the presence of food antigen-specific IgE in the serum of *Was*$^{-/-}$ mice (hemizygous *Was*$^{-}$ males are also referred to as *Was*$^{-/-}$ throughout the manuscript), we hypothesized that allergic sensitization would occur in *Was*$^{-/-}$ animals following exposure to orally administered antigens in the absence of adjuvant. To be able to quantify and compare the efficiency of oral sensitization, we chose to study ovalbumin (OVA) as a model allergen, which is used in an established, isomorphic model of food allergy that relies on systemic sensitization by injection of OVA in aluminum hydroxide (Brandt et al., 2003) (**Figure 8.2A**). As predicted, after a sensitization phase of 8 oral gavages of 5 mg OVA in PBS (**Figure 8.2B**), OVA-specific IgE was detected in the serum of *Was*$^{-/-}$ mice, but not their *Was*$^{+/-}$ or WT littermates (**Figure 8.2C**). However, absolute titers of OVA-specific IgE were significantly lower than those obtained following adjuvant-mediated sensitization. In addition, because *Was*$^{-/-}$ mice have an expanded IgE pool at baseline, an even greater difference existed in the fraction of OVA-specific to total IgE between spontaneously sensitized *Was*$^{-/-}$ and OVA/alum-treated WT

animals (**Figure 8.2C**). To assess whether these disparate ratios of antigen-specific to total IgE levels affected the efficiency of antigen-mediated activation of the IgE-effector axis, we loaded bone marrow-derived mast cells with IgE in serum of mice from either model. When mast cells were loaded with equivalent concentrations of OVA-specific IgE, we observed denser surface IgE coating on cells loaded with serum from *Was*$^{-/-}$ mice (**Figure 8.2D**), which was expected given the greater concentration of serum IgE in *Was*$^{-/-}$ mice. However, the extent of OVA-induced degranulation was comparable between mast cells that were loaded with serum derived from either *Was*$^{-/-}$ mice or adjuvant-induced allergic WT mice (70% vs. 57% LAMP-1 positive cells respectively in a representative experiment, **Figure 8.2D**). When loading was alternatively corrected for the differences in total IgE concentrations, we also observed similar degrees of degranulation despite the approximately 20-fold lower concentration of OVA-specific IgE in *Was*$^{-/-}$ serum (**Figure 8.2E**). Very low titers of antigen-specific IgE are thus sufficient to mediate efficient activation of mast cells *in vitro*. Combined, our results demonstrate that *Was*$^{-/-}$ animals produce physiologically relevant levels of antigen-specific IgE after oral gavage of a foreign antigen in the absence of an adjuvant.

8.4.3 IgE effector responses in *Was*$^{-/-}$ mice are intact

A homologous model of food allergy needs to not only mimic spontaneous allergic sensitization, but should also faithfully recapitulate the IgE effector phase. Since anaphylaxis is a critical phenotype in food allergic individuals, we sought to determine if OVA-specific IgE antibodies in *Was*$^{-/-}$ mice would be associated with anaphylaxis *in vivo*. In line with results from other institutions (Berin and Mayer, 2009; Brandt et al., 2003), OVA/alum-sensitized WT mice in our facility were found to be resistant to oral anaphylaxis as assessed by a drop in body temperature upon repeated allergen challenge. Similarly, body temperature stayed constant in OVA-sensitized *Was*$^{-/-}$ mice after high-dose oral OVA challenge (**Figure 8.3A**). In contrast, allergic diarrhea, which has also been termed intestinal anaphylaxis (Brandt et al., 2003) was readily observed in 79% of *Was*$^{-/-}$ and 60% of OVA/alum-sensitized animals 60 min after gavage, but not in *Was*$^{+/-}$ or WT controls, or in *Was*$^{-/-}$ mice challenged with an irrelevant antigen (**Figure 8.3B**). Oral OVA challenge in *Was*$^{-/-}$ mice and OVA/alum-sensitized WT animals furthermore resulted in equivalent increases in serum levels of MCPT1, a mast cell protease that is released from mucosal mast cells upon allergen-dependent crosslinking of surface-bound IgE and that can therefore be used as a marker for the severity of experimental food allergy (Brandt et al., 2003; Burton et al., 2012; Khodoun et al., 2011) (**Figure 8.3C**). In line with these results, expansion of mucosal mast cells in the small intestine as assessed by *Mcpt1* mRNA levels was found to be similar between both spontaneously sensitized *Was*$^{-/-}$ and OVA/alum WT mice (**Figure 8.3D**). These data thus corroborate our *in vitro* findings by demonstrating that oral allergen-dependent IgE effector responses in *Was*$^{-/-}$ mice occur with equivalent vigor as observed in a conventional adjuvant-based model of food allergy.

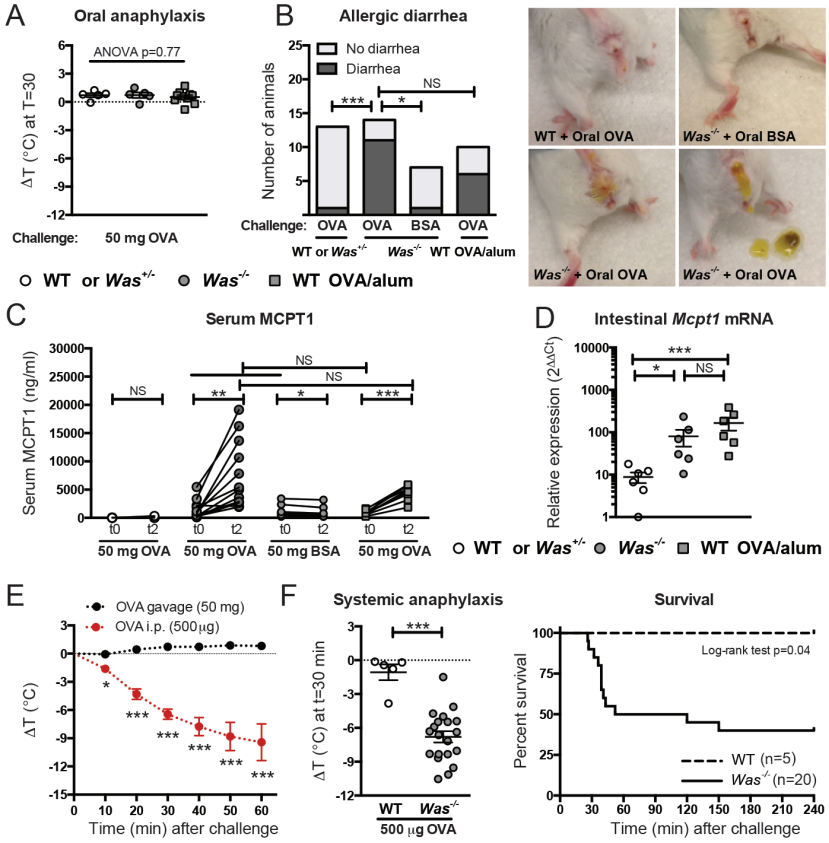

Figure 8.3. ***In vivo*** **IgE effector responses are intact in** ***Was*$^{-/-}$** **mice.** (**A**) Oral anaphylaxis in OVA/alum sensitized (gray squares) WT mice and OVA-gavaged *Was*$^{-/-}$ (gray circles) or WT/ *Was*$^{+/-}$ controls (open circles). (**B**) Occurrence of allergic diarrhea after oral challenge with OVA or Bovine Serum Albumin (BSA) as an irrelevant antigen control. Significance assessed by Fisher's exact test. Upper panels show WT or BSA-gavaged controls. (**C**) Levels of serum MCPT1 after oral challenge with OVA or BSA. (**D**) Comparison of small intestinal *Mcpt1* mRNA expression levels. (**E**) Occurrence of anaphylaxis following systemic, but not oral OVA challenge in *Was*$^{-/-}$ mice. (**F**) Comparison of decrease in body temperature between OVA-gavaged *Was*$^{-/-}$ or WT/ *Was*$^{+/-}$ littermates and Kaplan-Meier survival curves. Symbols represent individual mice. Error bars depict SEM. *p<0.05; ** p<0.01; ***p<0.001; NS: not significant as determined by t-test or Mann-Whitney U test (Panels C, E and F), paired t-test or Wilcoxon matched-pair rank test for intra-individual analyses (Panels C), or ANOVA on log-transformed data with Tukey's multiple comparison test (Panel D).

Despite their resistance to oral anaphylaxis, anaphylactic shock to food allergens in BALB/c mice can be studied using systemic allergen exposure. Whereas body temperature had remained stable in *Was*$^{-/-}$ mice following high-dose oral OVA challenge, we consistently observed a rapid temperature decrease characteristic of anaphylactic reactions in the same mice in response to systemic challenge with 500 μg OVA (**Figure 8.3E**). While *Was*$^{+/-}$ or WT controls subjected to the adjuvant-free sensitization protocol were protected from a drop in

body temperature upon systemic challenge, we observed systemic anaphylaxis in 19 out of 20 *Was*$^{-/-}$ littermates, which was fatal in >50% of mice (**Figure 8.3F**).

8.4.4 Absence of WASP in mast cells permits IgE–mediated degranulation but abrogates inflammatory cytokine production

The susceptibility of OVA-sensitized *Was*$^{-/-}$ mice to anaphylaxis was surprising because we had not observed any anaphylactic reactions among a cohort of 25 patients with WAS. Furthermore, a previous study had found decreased histamine release from mast cells in the absence of WASP and resistance of *Was*$^{-/-}$ mice to passive anaphylaxis (Pivniouk et al., 2003). To further investigate the consequences of WASP deficiency in mast cells, we generated bone marrow-derived mast cells from either WT or *Was*$^{-/-}$ donors on both the BALB/c and C57BL/6 background. All cultures differentiated normally under the influence of IL-3 and SCF and showed equal surface expression levels of cKit and the high-affinity IgE receptor FcεRI (**Figure 8.4A**). In contrast to previously published findings (Pivniouk et al., 2003), we found only a modest, non-significant decrease in the fraction of IgE-loaded mast cells that displayed degranulation upon challenge with a range of antigen concentrations in cells from mice of either genetic background (**Figure 8.4B**). Analysis of the downstream IgE/FcεRI-signaling cascade, however, revealed a significant reduction in ERK1/2 phosphorylation in WASP-deficient mast cells (**Figure 8.4C**). Reduced ERK-signaling was associated with a striking decrease in the concentrations of pro-inflammatory cytokines that were found in the supernatant of mast cells 4h after IgE-mediated activation (**Figure 8.4D**). Because mRNA transcript levels of these cytokines were found to be comparable between WT and *Was*$^{-/-}$-derived mast cells, reduced cytokine production was likely the result of an inability to secrete pre-formed cytokines (**Figure 8.4E**), which has been previously shown to be true for WASP-deficient T effector cells (Morales-Tirado et al., 2010). These findings explain why *Was*$^{-/-}$ mice are susceptible to the sequelae of acute, IgE-mediated immune activation, but also demonstrate that WASP deficiency in mast cells may affect levels of mast-cell derived inflammatory cytokines in the intestine of *Was*$^{-/-}$ mice.

8.4.5 Spontaneous allergic sensitization occurs in the absence of IgE–mediated signaling through FcεRI

Recent studies in two different murine models of food allergy have demonstrated a role for IgE:FcεRI-mediated signals in expansion of mucosal mast cells and the pathogenesis of food allergy (Burton et al., 2014b; Chen et al., 2015). Following our observation that WASP deficiency in mast cells affects their ability to produce inflammatory cytokines in response to IgE-mediated activation, we asked whether IgE:FcεRI-mediated signaling in mast cells contributed to spontaneous oral sensitization to ingested allergens in *Was*$^{-/-}$ mice. To address this question, we generated *Was*$^{-/-}$*Fcer1a*$^{-/-}$ and their *Was*$^{-/-}$*Fcer1a*$^{+/-}$ littermates to study the induction of IgE responses against OVA. Genetic ablation of FcεRI was confirmed by measuring

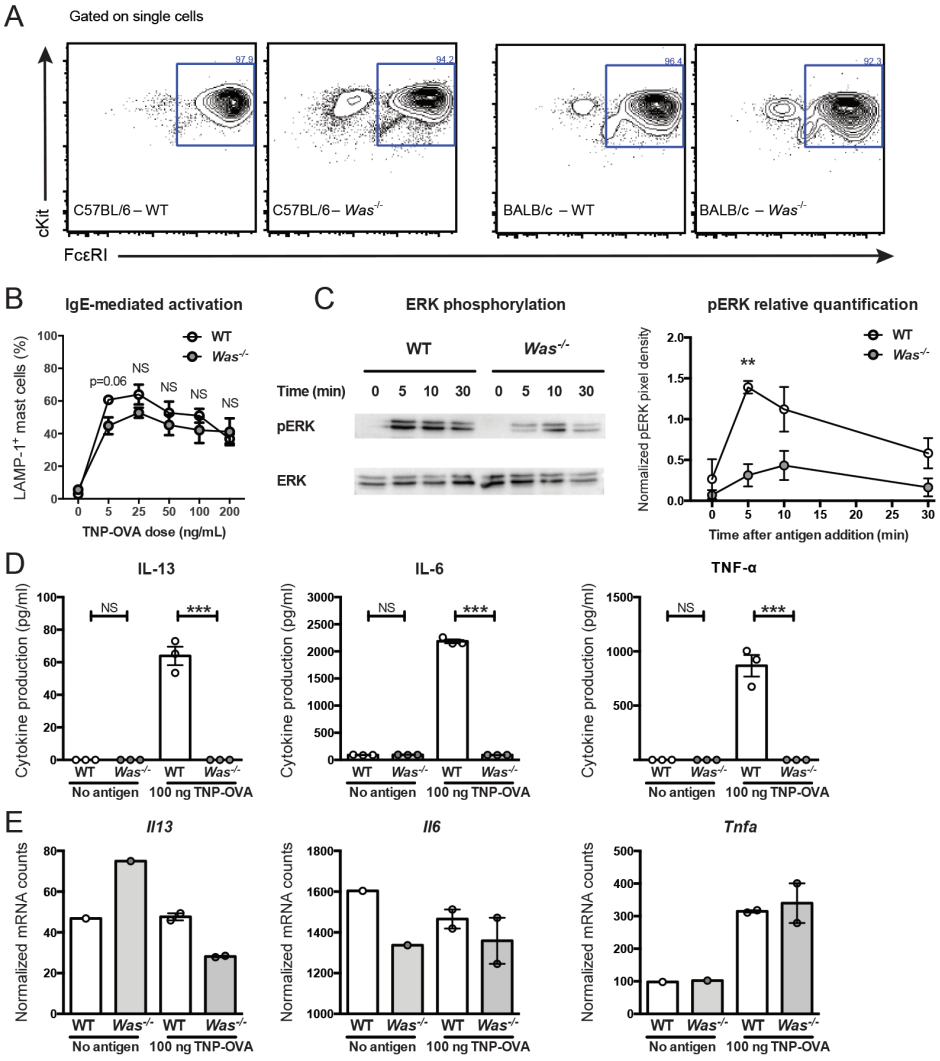

Figure 8.4. WASP is dispensable for IgE-mediated mast cell degranulation but is required for cytokine production. (**A**) Flow cytometric analysis of in parallel-cultured bone marrow-derived mast cells from WT or *Was*$^{-/-}$ donors from either the C57BL/6 or BALB/c background, with cKit^{+}FcεRI^{+} cells representing differentiated mast cells. (**B**) LAMP-1 degranulation assay measuring the response to different doses of antigen in TNP-specific IgE-loaded mast cells derived from bone marrow of either WT (open circles) or *Was*$^{-/-}$ mice (gray circles). (**C**) Time course of the appearance of phosphorylated ERK (pERK) in response to antigen-mediated IgE crosslinking in WT and *Was*$^{-/-}$ mast cells, with total ERK shown as loading control and analysis of pixel intensity on the right. (**D**) Inflammatory cytokine concentration in supernatants of WT and *Was*$^{-/-}$ mast cells 6h after IgE-mediated antigen stimulus. (**E**) Normalized mRNA counts for *Fcer1a*, *Il13, Il6* and *Tnfa* in IgE-loaded WT and *Was*$^{-/-}$ mouse-derived mast cells with or without stimulation with 100 ng TNP-OVA *in vitro*. Error bars depict SEM. ** p<0.01; ***p<0.001; NS: not significant as determined by student t-test.

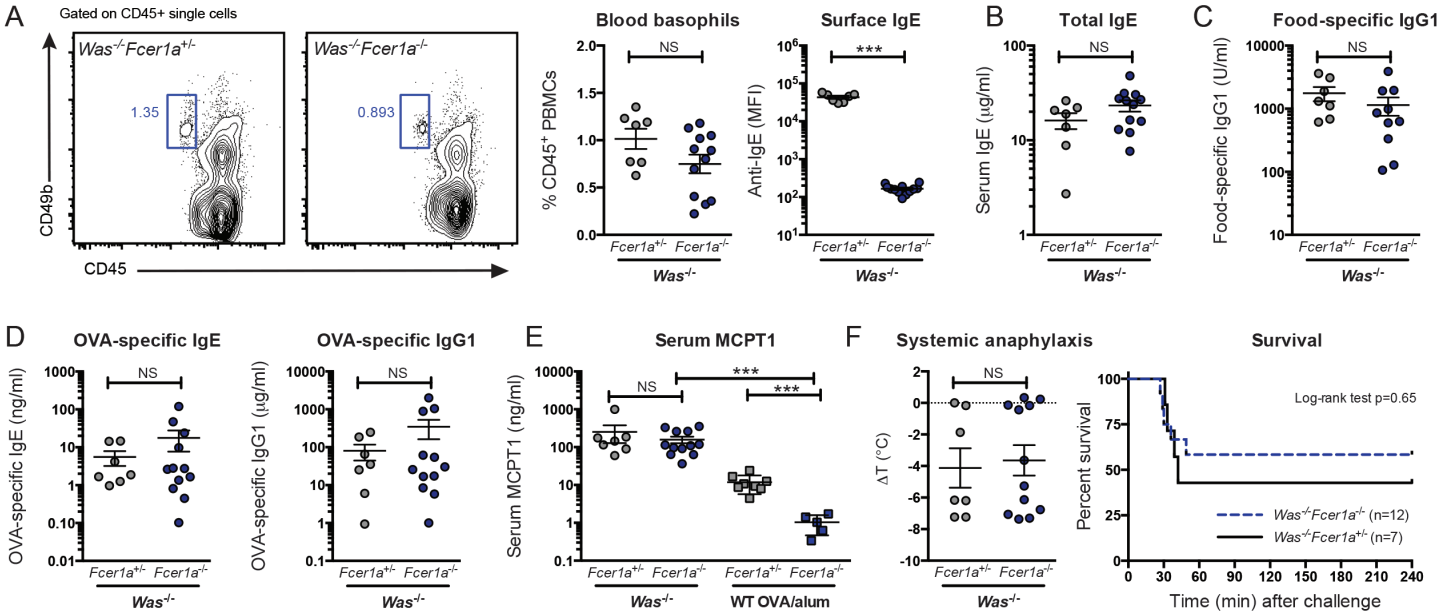

Figure 8.5. Adjuvant-free OVA sensitization occurs in the absence of FcεRI. (**A**) Basophils in peripheral blood of *Was*$^{-/-}$*Fcer1a*$^{+/-}$ (gray circles, n=7) or *Was*$^{-/-}$*Fcer1a*$^{-/-}$ littermates (blue circles, n=12) expressed as percent of total CD45^{+} peripheral blood mononuclear cells (PBMCs), with absence of IgE binding on cell surface of basophils in *Was*$^{-/-}$*Fcer1a*$^{-/-}$ mice. (**B**) Total IgE and (**C**) food-specific IgG1 levels in serum from *Was*$^{-/-}$*Fcer1a*$^{+/-}$ and *Was*$^{-/-}$*Fcer1a*$^{-/-}$ littermates. (**D**) Induction of OVA-specific IgE and IgG1 following adjuvant-free oral sensitization. (**E**) Baseline serum MCPT1 levels in sensitized and challenged *Was*$^{-/-}$*Fcer1a*$^{+/-}$ and *Was*$^{-/-}$*Fcer1a*$^{-/-}$ littermates compared to OVA/alum-sensitized WT mice with or without *Fcer1a*. (**F**) Body temperature and survival curves following systemic challenge with 500 μg OVA. Symbols represent individual mice. Error bars depict SEM. ***p<0.001; NS: not significant as determined by student t-test or ANOVA with Tukey's multiple comparison test (panel E).

surface IgE loading on basophils and was associated with only a slight and non-statistically significant reduction in the fraction of basophils amongst peripheral blood mononuclear cells (**Figure 8.5A**). Serum levels of total IgE (**Figure 8.5B**) and IgG1 with reactivity to soy and wheat antigens (**Figure 8.5C**) were equivalent between FcεRI-deficient and FcεRI-competent *Was*$^{-/-}$ mice. Similarly, oral sensitization with OVA following our established protocol of oral sensitization resulted in similar levels of OVA-specific IgE and IgG1 between groups (**Figure 8.5D**). Surprisingly, we did not observe lower baseline levels of serum MCPT1 before or after sensitization in *Was*$^{-/-}$*Fcer1a*$^{-/-}$ mice (**Figure 8.5E**), indicating that mucosal mast cell expansion in *Was*$^{-/-}$ animals occurs independently of IgE:FcεRI-mediated signals. This was in stark contrast with OVA/alum-sensitized WT mice, in which we confirmed the recently published result that mast cell expansion occurs to a lesser extent in challenged animals that are deficient in *Fcer1a* (Chen et al., 2015) (**Figure 8.5E**).

Early studies in *Fcer1a*$^{-/-}$ animals have clearly demonstrated that anaphylactic responses can occur in the absence of the high-affinity IgE receptor, which are then mediated primarily through IgG:FcγRIII activation on macrophages (Burton and Oettgen, 2011; Dombrowicz et al., 1997; Khodoun et al., 2011). Since we had observed efficient induction of IgG1 responses against OVA, we aimed to assess the susceptibility of *Was*$^{-/-}$*Fcer1a*$^{-/-}$ mice to IgG-mediated anaphylaxis. Systemic OVA challenge of *Was*$^{-/-}$*Fcer1a*$^{-/-}$ and *Was*$^{-/-}$*Fcer1a*$^{+/-}$ littermates resulted in anaphylactic shock in mice of both groups, with similar survival curves in mice with and without expression of FceRI (**Figure 8.5F**). These results demonstrate that *Was*$^{-/-}$ mice are susceptible to IgG-mediated anaphylaxis upon systemic allergen challenge.

8.4.6 Spontaneous oral sensitization to OVA in *Was*$^{fl/fl}$*Foxp3*–Cre mediates more pronounced IgE effector responses compared to adjuvant–sensitized mice

Using loxP-Cre-mediated conditional deletion of WASP in different lineages, we recently identified deficiency of WASP in FOXP3^{+} Tregs to be sufficient for WAS-associated food allergic responses to occur (**Chapter 7**). This genetic system allows the study of spontaneous oral sensitization without any confounding influences of WASP deficiency in other hematopoietic lineages such as mast cells. Similar to our findings in *Was*$^{-/-}$ mice on the BALB/c genetic background, we detected OVA-specific IgE in the serum of C57BL/6 *Was*$^{fl/fl}$*Foxp3*-Cre mice following our protocol outlined in Figure 8.2A-B (**Figure 8.6A**). OVA-IgE titers were again found to be significantly lower than those observed in co-housed C57BL/6 WT mice subjected to repeated OVA challenge following OVA/alum-mediated systemic sensitization (**Figure 8.6B**). However, these low IgE titers effectively mediated mast cell degranulation in all 12 *Was*$^{fl/fl}$*Foxp3*-Cre mice in this cohort, as evidenced by significant increases in levels of serum MCPT1 after high dose OVA challenge. Post-challenge MCPT1 levels were significantly higher than observed in OVA/alum-treated mice, which correlated to a much greater expansion of the small intestinal mast cell pool in *Was*$^{fl/fl}$*Foxp3*-Cre mice that was apparent from elevated pre-challenge serum levels of MCPT1 (**Figure 8.6B**), increased mRNA expression of *Mcpt1* in the

jejunum (**Figure 8.6C**) and higher numbers of small intestinal mast cells upon chloroacetate esterase staining of histological sections (**Figure 8.6D**). Combined, these results indicate that *Was*$^{fl/fl}$*Foxp3*-Cre mice can be used to study adjuvant-free induction of IgE against lumenal antigens and intestinal mast cell effector responses in the normally Th2-resistant C57BL/6 genetic background.

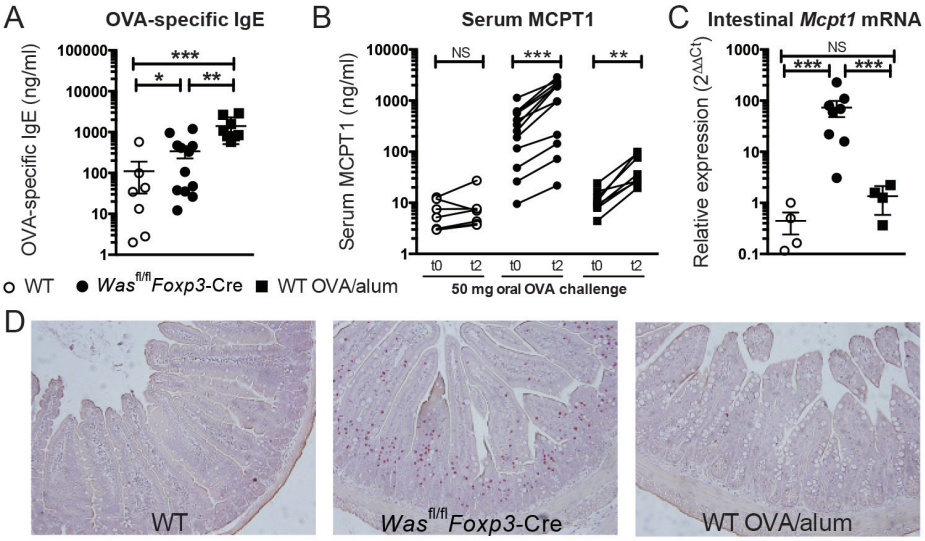

Figure 8.6. Spontaneous oral sensitization to OVA occurs in *Was*$^{fl/fl}$*Foxp3*-Cre mice. (**A**) Adjuvant-free induction of OVA-specific IgE in *Was*$^{fl/fl}$*Foxp3*-Cre (black circles) compared with C57Bl6 WT controls (open circles) and OVA/alum-sensitized C57Bl6 WT mice (dark squares). (**B**) Serum MCPT1 levels in response to oral challenge with 50 mg OVA after oral sensitization protocol. (**C**) Small intestinal mRNA levels of *Mcpt1* and (**D**) chloroacetate staining for jejunal mast cells. Symbols represent individual mice. Error bars show SEM. *p<0.05; ** p<0.01; ***p<0.001; NS: not significant as determined by ANOVA with Tukey's multiple comparison on log-transformed data (Panel A and C) or paired t-test for intraindividual analyses (Panel B).

8.5 Discussion

In the present work, we have characterized mice deficient in WASP as a novel experimental model of food allergy. *Was*$^{-/-}$ animals demonstrate spontaneous, i.e. adjuvant-free, generation of IgE antibodies against oral antigens and these antibodies effectively mediate the antigen-dependent responses in IgE effector cells that underlie the typical symptoms of allergic disease. As such, our model recapitulates both the sensitization and effector phases of food allergy, and could thereby prove to be a valuable addition to the experimental arsenal of allergists and immunologists.

The degree to which *Was*$^{-/-}$ mice can be considered a homologous animal model of food allergy is dependent on several factors. First, we need to interrogate whether food allergy in human WAS patients resembles the more common food allergies that arise in the general population outside the context of this rare immunodeficiency. In this study, we have performed a direct comparison of serum obtained from either WAS or age-matched FA patients. Both diseases were associated with equivalent increases in serum IgE, and screening of IgE and IgG4 reactivity against food allergens showed highly comparable profiles between children from either diagnostic category. Importantly, patients with mutations in *WAS* frequently demonstrated IgE reactivity against the 8 allergens that are responsible for the large majority of clinically relevant food allergies in the US (Sicherer and Sampson, 2010; Wang and Sampson, 2011). Similarly, we have shown that the strongest food-specific IgE responses in *Was*$^{-/-}$ mice are mounted against soy and wheat (**Chapter 7**), which also rank among these 8 major human food allergens (Sicherer and Sampson, 2010; Wang and Sampson, 2011). These observations indicate that the mechanisms that drive the allergenicity of these particular food antigens are not only operative in both WAS and FA patients, but also in *Was*$^{-/-}$ mice.

A second argument in favor of significant overlap between common food allergy and the food allergic responses associated with WASP deficiency is the critical involvement of FOXP3^{+} Tregs in disease pathogenesis. Several observations in the literature imply that dysfunction of Tregs accounts for the failure in oral tolerance as observed in food allergic patients (Akdis and Akdis, 2014a; Noval Rivas et al., 2015). We show here that WASP deficiency confined to FOXP3^{+} Tregs is sufficient for intestinal mast cell expansion and oral sensitization to OVA to occur. We previously documented that WASP deficiency in FOXP3^{+} Treg results in a selective loss of their Th2-suppressive capacity *in vivo* and is associated with their increased expression of the Th2-associated transcription factor GATA3. Such Th2-like GATA3^{+}FOXP3^{+} Tregs have been observed in the blood of children with FA and were found to be pathogenic in a murine model of food allergy that depends on augmented signaling through the IL-4 receptor (Noval Rivas et al., 2015). Increased prevalence of this subset of Tregs is thus another shared feature between WAS-associated and common food allergy.

Another potential advantage of the experimental model presented here is that it allows the study of allergen-dependent responses in the context of a polysensitized host. Screening of human patient sera identified IgE reactivity to multiple unrelated food antigens in children with food allergy as well as WAS patients, which is in line with the epidemiological observation that patients commonly suffer from multiple allergies (de Jong et al., 2011; Liu et al., 2010; Sicherer and Sampson, 2014). By selectively inducing IgE against a single model antigen, this pathophysiological aspect of human food allergy is commonly lost in isomorphic, adjuvant-based experimental models. Indeed, we observed that the fraction of OVA-specific IgE was as high as 80% of total IgE in food allergic mice following systemic sensitization with OVA/alum. In stark contrast, spontaneous oral sensitization in *Was*$^{-/-}$ mice only induced OVA-

specific IgE titers that constituted on average <10%, and in a number of mice even <1% of total IgE. In vitro mast cell degranulation assays and in vivo antigen challenge clearly showed that these disparate fractions of antigen-specific to total IgE do not affect the efficiency with which antigen-specific IgE mediates mast cell activation in response to OVA. Since low titers of allergen-specific IgE can sometimes be found in severely food allergic patients (Sampson et al., 2014; Steckelbroeck et al., 2008), this aspect of human food allergic disease may be more faithfully reflected in *Was*$^{-/-}$ mice than in conventional experimental models.

WASP is expressed in all hematopoietic lineages and is functionally linked to a great variety of immune effector functions (Massaad et al., 2013; Thrasher and Burns, 2010). The immunological consequences associated with a complete absence of WASP could therefore be used as an argument against homology of the model. Our studies on the effects of WASP deficiency in mast cells directly illustrate this principle. Although we observed that IgE-mediated degranulation was relatively unperturbed, we found an almost complete abrogation in the production of pro-inflammatory cytokines from IgE-activated *Was*$^{-/-}$ mast cells, as has been previously described for mast cells deficient in either WASP or the WASP-interacting protein (WIP) (Kettner et al., 2004; Pivniouk et al., 2003). *Was*$^{-/-}$ mice are thus susceptible to intestinal and systemic anaphylaxis, but reduced cytokine production from WASP-deficient mast cells is likely to limit intestinal allergic inflammation. In support of this conclusion, we previously found that food allergy is exacerbated in *Was*$^{fl/fl}$*Foxp3*-Cre animals when compared to *Was*$^{-/-}$ mice (**Chapter 7**). Furthermore, we showed here that *Was*$^{-/-}$*Fcer1a*$^{-/-}$ and *Was*$^{-/-}$*Fcer1a*$^{+/-}$ littermates are equally susceptible to OVA sensitization and mucosal mast cell expansion, which was not the case for WT mice deficient in FcεRI. As such, it is possible that the pro-inflammatory function of FcεRI-signaling in mast cells in other models of food allergy (Burton et al., 2014b; Chen et al., 2015) is dependent on WASP. Since *Was*$^{fl/fl}$*Foxp3*-Cre mice can be efficiently sensitized to OVA and will develop robust intestinal mast cell responses, such potentially confounding influences from panhematopoietic WASP deficiency can be circumvented in this genetic system.

Finally, it is worth pointing out that spontaneous oral sensitization to OVA in *Was*$^{fl/fl}$*Foxp3*-Cre was associated with robust expansion of mucosal mast cell in mice on the normally Th2-resistant C57BL/6 background (Lee et al., 2013; Van Gramberg et al., 2013). Indeed, the OVA/alum model is typically used in conjunction with BALB/c mice. We have here demonstrated that although OVA-IgE can be readily induced in C57BL/6 mice following OVA/alum sensitization, these animals proved to be resistant to the mucosal mast cell expansion in the intestine that is associated with repeated oral allergen challenge. Lack of robust models of food allergy in C57BL/6 mice means that a large part of currently available genetic tools are beyond the easy reach of the experimental allergist, since back-crossing strains to BALB/c background is time-intensive and can thus be prohibitively costly. Examples of tools that are generally more widely available in C57BL/6 animals are congenic markers, transgenic TCRs and a great variety of fluorescence reporter systems (The Jackson Laboratory, Bar

Harbor, ME). Furthermore, the International Mouse Phenotyping Consortium, which aims to generate a null mouse for every gene in the mouse genome, works exclusively with mice on the C57BL/6 background (Skarnes et al., 2011). Availability of an adjuvant-free experimental model of oral sensitization in C57BL/6 mice would thus open up a plethora of research tools to address novel hypotheses on the pathogenesis of food allergic reactions.

In summary, we have identified *Was*$^{-/-}$ mice as a genetic model of food allergy. In contrast to commonly available, isomorphic, adjuvant-based experimental strategies, the oral sensitization phase was faithfully recapitulated in addition to IgE-mediated type I hypersensitivity responses upon allergen exposure. Sensitization to food antigens occurred in both *Was*$^{-/-}$ mice as well as WAS patients, and was directed against allergens most relevant to common food allergy. In addition, polysensitization was a common feature in both mice and patients, and aberrant IgE immune responses were shown to be critically dependent on FOXP3^{+} Tregs. Combined, these features all support the conclusion that *Was*$^{-/-}$ mice may serve as a more homologous animal model of human food allergy, which could likely continue to reveal new insights in to the etiology of human food allergy and inspire new therapeutic approaches.

8.6 Acknowledgements

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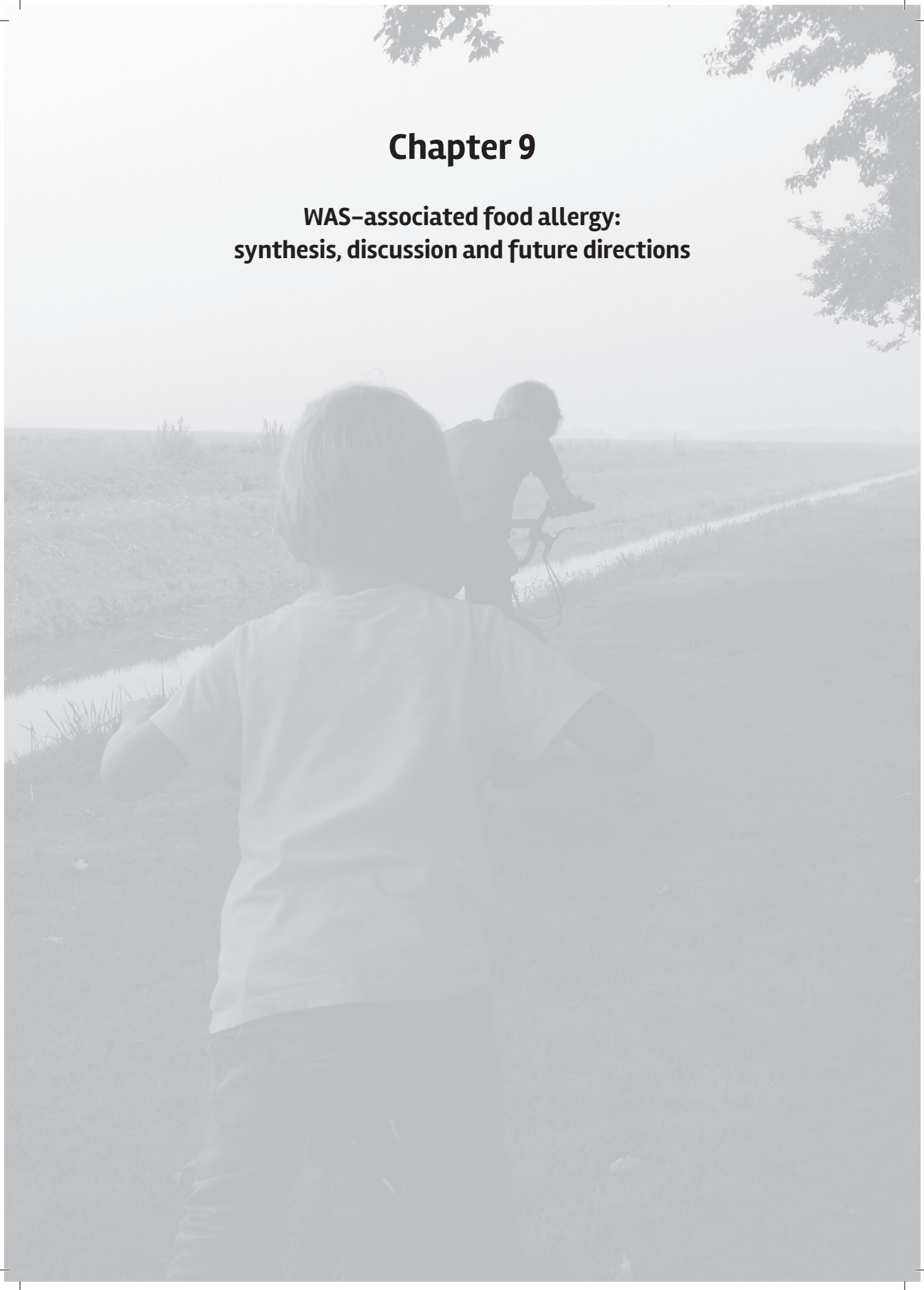
Chapter 9

WAS–associated food allergy: synthesis, discussion and future directions



9.1 Introduction

Despite renewed attention for beneficial roles of IgE-mediated immune responses to allergens (Marichal et al., 2013; Palm et al., 2012; Profet, 1991), it is clear that allergic reactions to innocuous food antigens have highly detrimental effects on the host. In **chapters 6**, **7** and **8**, we have discussed the critical involvement of FOXP3$^+$ CD4$^+$ T cells (Tregs) in regulating the synthesis of IgE. IPEX patients lacking functional Tregs due to mutation of the *FOXP3* gene develop elevated levels of IgE (Bennett and Ochs, 2001), whereas other antibody isotypes (IgM, IgA and IgG) are generally normal (Gambineri et al., 2003). Similarly, we observed increased IgE in a novel humanized mouse model reconstituted with hematopoietic stem cells of an IPEX patient (**Figure 6.6A, page 135**). Treg control over production of IgE is preserved across species, as mice with the *Foxp3^{sf}* mutation also develop greatly increased levels of serum IgE before succumbing to the sequelae of autoimmunity around 21-days of age (**Figure 9.1A** and (Lin et al., 2005)). Besides dysregulated IgE production, the absence of Tregs in mice also leads to significant expansion of intestinal mast cells (**Figure 9.1B-D**), which is commonly observed in patients suffering from food allergy (Caffarelli et al., 1998; Hogan et al., 2012). Since proliferation of mucosal mast cells depends critically on T cell help rather than IgE (Gurish et al., 2004), the intestinal mastocytosis encountered in the context of Treg deficiency signifies aberrant Th2-mediated immune activation (**Figure 9.1E**). As discussed in the introduction of **Chapter 7**, loss of functional Tregs also results in pronounced Th1 and Th17 responses (**Figure 9.1F**), demonstrating that the immune sequelae observed in IPEX patients and *Foxp3^{sf}* mice are the combined effects of aberrant Th1, Th2 and Th17 immune activation.

In **Chapter 7**, we described that patients with the Wiskott-Aldrich syndrome (WAS) suffer from IgE-mediated food allergy (**Figure 7.1, page 159**). Mechanistically, these food allergic responses in the context of WAS could be pinpointed to the selective failure of WASP-deficient FOXP3$^+$ Tregs to restrain Th2-mediated immune activation. Conditional deletion of *Was* in murine FOXP3$^+$ Tregs was sufficient for IgE-mediated responses against common food allergens. In fact, compared to *Was$^{-/-}$* mice, *Was$^{fl/fl}$Foxp3*-Cre animals developed significantly higher levels of serum IgE and exhibited more pronounced expansion of intestinal mast cells. In **Chapters 7** and **8**, reduced effector function of *Was$^{-/-}$* mast cells is offered as a potential explanation for the different susceptibility in allergic disease between these two models (**Figure 7.4, page 164**). However, the question whether WASP deficiency in lineages other than mast cells affects food allergic responses in *Was$^{-/-}$* mice and WAS patients remains to be exhaustively discussed. Since the allergic immune response involves cooperation of many cell types from both the innate as well as adaptive immune arms, analysis of the role of WASP in any of these cellular compartments is required for a thorough understanding of the food allergy that occurs in patients with WAS. Because many of the features of WAS-associated food allergy are shared with common food allergic patients (**Chapter 8**), appreciation of any pro- and anti-allergic factors mediated by WASP deficiency could shed new light on the

general pathogenesis of IgE-mediated responses to food. Lastly, comparative analysis of *Was*$^{-/-}$ and *Was*$^{fl/fl}$*Foxp3*-Cre mice will help better define WASP-deficient animals as a novel experimental model of food allergy (**Chapter 8**). The aim of this chapter is therefore to provide a comprehensive overview of factors that may help explain the more severe food allergy observed in *Was*$^{fl/fl}$*Foxp3*-Cre mice in comparison to *Was*$^{-/-}$ and WT animals.

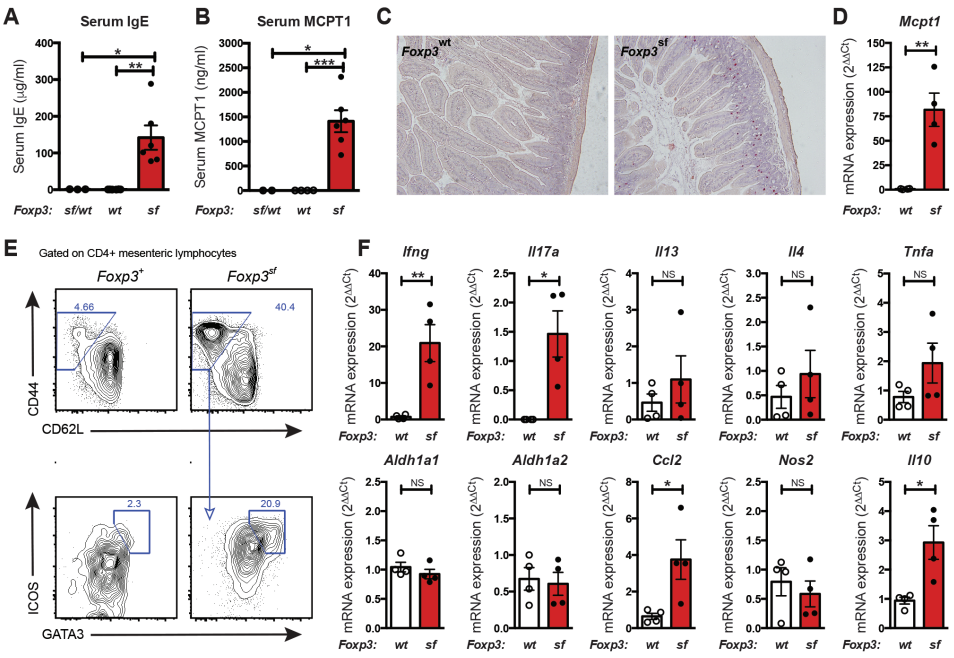


Figure 9.1. ***Foxp3*^{sf} *mice develop hyper IgE responses and expansion of intestinal mast cells.*** Levels of total serum IgE (**A**) and MCPT1 (**B**) in *Foxp3*$^{sf/wt}$ females and their *Foxp3*^{sf} and *Foxp3*^{wt} offspring. (**C**) Chloroacetate esterase staining on jejunal sections in *Foxp3*^{sf} and *Foxp3*^{wt} littermates, and corresponding mRNA expression levels of *Mcpt1* (**D**). Flow cytometry plots of GATA3^{+}ICOS^{+} Th2-type effector cells within the parent gate of CD44^{hi}CD62L^{lo}CD4^{+} effector memory T cells from MLNs of representative samples. (**F**) mRNA expression levels in small intestinal sections as assessed by quantitative RT-PCR. Dots represent individual mice and error bars depict SEM. *p<0.05; ** p<0.01; ***p<0.001; NS: not significant as determined by student t-test.

9.2 Cellular functions of WASP

Structural integrity and modulation of the actin cytoskeleton allows eukaryotic cells not merely to maintain polarization and orientation in the context of a multicellular host, but also to undergo division, migration, locomotion, and cell-cell interactions. Despite this plethora of actin-dependent functions, all external signals that can induce cytoskeletal rearrangements converge on the activation of only three members of the evolutionarily

highly conserved Rho protein family: Cdc42, Rac and Rho. To account for the great variety in effector responses, these activated proteins can interact with a large number of proteins from the WASP family, which serve to activate the Arp2/3 complex to initiate the polymerization of actin filaments (Alberts et al., 2008). WASP itself was the first protein to be identified as a nucleation-promoting factor (NPFs), but several other closely related family members such as neural-WASP (N-WASP) and WAVE1-3 have since been discovered (Massaad et al., 2013). As intermediary factors between Cdc42 and Arp2/3, WASP family proteins thus play a critical role in translating external signals into changes of the actin cytoskeleton. Under normal conditions, WASP is auto-inhibited through its binding to the WASP Interacting Protein (WIP). Upon cellular signaling, however, the WIP/WASP complex can be recruited to the cell surface, where Cdc42 and PIP_2 bind to WASP to liberate its VCA domain from the auto-inhibited state. The VCA domain, in turn, activates the Arp2/3 complex and hence initiates actin polymerization. Auto-inhibition and intracellular delivery by WIP warrant the highly controlled and localized effect of WASP, allowing cytoskeletal rearrangements in a polarized fashion. Once phosphorylated, WASP is rapidly ubiquitinated and degraded, which further limits WASP activity to the site of the external stimulus (Massaad et al., 2013; Matalon et al., 2013; Thrasher and Burns, 2010). Of all other WASP-family members, N-WASP shares the greatest levels of homology with WASP (around 50%), including the VCA domain. While WASP is exclusively expressed in hematopoietic lineages, the expression of N-WASP is ubiquitous, which is reflected in the fact that genetic deletion of N-WASP is embryonically lethal (Westerberg et al., 2012). In the remainder of this chapter, we will review the consequences of WASP-deficiency on the function of immune compartments involved in the allergic response.

9.3 Antigen Presenting Cells

In contrast to B cells, which can be activated by intact antigens in the absence of other immune cells, T cells can only recognize their cognate antigen in the context of MHC proteins on the surface of antigen presenting cells (APCs). Consequently, effective T cell immunity relies on the presence and function of a broad surveillance network of mononuclear phagocytes that continuously sample and process the antigenic environment. Because the mechanisms underlying the uptake of antigens do not necessarily discriminate between antigens of pathogenic origin versus those that are derived from innocuous sources such as food and commensals, APCs provide T cells with additional signals to ensure the appropriate downstream immune response. These signals consist of interactions between various cell-surface receptors and ligands, and also include the cytokine milieu in which the APC-T cell interaction takes place. The efficient crosstalk between APC and T cell is facilitated by the formation of a temporary *immune synapse*, a highly dynamic mechanical structure at the interface between the antigen-specific TCR and peptide-loaded MHC molecule. The integrity

of the immune synapse is strongly dependent on rearrangements in the actin cytoskeleton, which follow from a sequence of events that is initiated upon primary signaling through the TCR and includes the recruitment of nucleation-promoting factors, including WASP, to the immune synapse (Barda-Saad et al., 2005; Matalon et al., 2013). In the absence of WASP on the T cell side, the function of the immune synapse is severely impaired, and *Was*$^{-/-}$ T cells show aberrant antigen-specific interaction with APCs (Matalon et al., 2013). Similarly, APCs critically rely on actin cytoskeletal rearrangements for normal intercellular interactions, as well as for carrying out other functions such as cytokinesis and cell migration (Moulding et al., 2013). The paradigm of APCs functioning as gatekeepers of T cell activation and downstream immune responses raises the question how deficiency of WASP in mononuclear phagocytes contributes to the immunopathology observed in WAS patients and mice deficient in WASP.

As in other hematopoietic lineages, WASP actively regulates the actin cytoskeleton in mononuclear phagocytes (Massaad et al., 2013; Thrasher and Burns, 2010). Its absence results in aberrant podosome formation and chemotaxis of APCs, which has been observed independently in human monocytes (Badolato et al., 1998), macrophages (Linder et al., 1999), and dendritic cells (Burns et al., 2001) from patients with WAS. In contrast to T and B lymphocytes, the survival advantage of WASP-competent cells over WASP-deficient counterparts is less pronounced for the myeloid compartment, with the exception of marginal zone macrophages (Westerberg et al., 2008). However, a non-redundant role for WASP in the function of APCs can be distinguished on at least four different levels:

1 *Phagocytosis and antigen uptake*. Human macrophages that are deficient in WASP show a reduced ability to form the phagocytic cup that is required for the efficient uptake of particles such as bacteria (Tsuboi and Meerloo, 2007). This defect is associated with aberrant presentation of particulate antigens, as *Was*$^{-/-}$ dendritic cells induced lower levels of D011.10 T cell proliferation than WT dendritic cells following co-culture with ovalbumin-expressing bacteria (Westerberg et al., 2003). Conversely, no difference in DO11.10 T cell activation was observed between WASP-deficient and WASP-competent dendritic cells when soluble ovalbumin was used as the antigenic stimulus, suggesting that aberrant antigen uptake and presentation in the absence of WASP is limited to particulate antigens (Westerberg et al., 2003). A recent paper points out another difference in antigen handling between WASP-competent and WASP-deficient dendritic cells: in the absence of WASP, APCs show increased propensity for cross-presentation of exogenous antigen. Cross-presentation refers to the process in which non-self antigens taken up by APCs are presented on MHC class I rather than class II molecules, consequently leading to $CD8^+$ instead of $CD4^+$ T cell responses (Baptista et al., 2016).

2 *Tissue homing and migration*. The podosomes that are deficient in the context of WAS are actin-dependent membrane protrusions that allow cells to attach to and

move through the extracellular matrix. In addition, the absence of WASP leads to aberrant integrin assembly, which affects the ability of mononuclear phagocytes and granulocytes to interact with ICAM-1 on endothelial cells during cell extravasation from the circulation (Moulding et al., 2013). Consequently, *Was*$^{-/-}$ dendritic cells exhibit defective homing to draining lymph nodes (Snapper et al., 2005), which persists after their adoptive transfer into WT hosts and thus represents a cell-intrinsic defect (Bouma et al., 2007). It has been hypothesized that delayed migration of dendritic cells after antigen uptake results in ectopic maturation that hence could contribute to pathogenesis of eczematous skin lesions in WAS patients (Moulding et al., 2013; Thrasher and Burns, 2010), although this remains to be demonstrated *in vivo*.

3 *Immune synapse formation and T cell activation*. As delineated above, the point of contact between T cells and APCs gives rise to an immune synapse, which depends on structural rearrangements in the actin cytoskeleton and provides a platform for sustained signaling through the T cell receptor. The role of WASP and the cytoskeleton in immune synapse function have predominantly been studied on the T cell side of this interface (Barda-Saad et al., 2005; Matalon et al., 2013; Sims et al., 2007). However, normal immune synapse integrity also requires cytoskeletal rearrangements in dendritic cells (Al-Alwan et al., 2001). Priming of WASP-competent $CD4^+$ and $CD8^+$ T cells by WASP-deficient dendritic cells is reduced (Bouma et al., 2007; Pulecio et al., 2008), which was later demonstrated to be due to defects in the formation of the immune synapse (Bouma et al., 2011; Malinova et al., 2015). WT T cells interacting with *Was*$^{-/-}$ dendritic cells showed reduced polarization of the T cell receptor, LFA-1, talin and f-actin to the immune synapse, and this was associated with lower levels of localized tyrosine phosphorylation, indicative of aberrant signaling through the TCR (Bouma et al., 2011). These abnormalities are exacerbated further by defects in integrin function, as actin-mediated localization of ICAM-1 and MHCII to the IS, which promotes the stability of the immune synapse, was recently shown to be WASP dependent (Malinova et al., 2015). In addition to an overall reduced proliferation, T cells interacting with *Was*$^{-/-}$ dendritic cells secrete lower levels of IL2 and undergo Th17 skewing at the expense of Th1 and Th2 lineages, as evidenced by elevated production of IL-17 and lower levels of IFNγ and IL-4 in dendritic cell and T cell co-cultures (Malinova et al., 2015). In a similar assay, reduced induction of Tregs has been observed from *Was*$^{-/-}$ compared to WT dendritic cells (Nguyen et al., 2012).

4 *Interpretation of environmental stimuli.* In addition to the mechanical aspects associated with a loss of WASP-dependent functions in APCs, absence of WASP could interfere with intracellular processes that endow mononuclear phagocytes with their capacities for immune surveillance. As an example of this principle,

> Prete and colleagues have demonstrated that *Was*$^{-/-}$ plasmacytoid dendritic cells are hyperresponsive to TLR9 signaling (Prete et al., 2013). The TLR9 pathway is activated by fragments of bacterial DNA and results in the rapid generation of pro-inflammatory cytokines such as type 1 interferons and IL-12 from endosomes. This heightened state of activation in plasmacytoid dendritic cells was associated with the occurrence of autoimmunity and colitis in *Was*$^{-/-}$ mice. Mechanistically, this study thus reveals a role for WASP-dependent actin cytoskeleton rearrangement in the intracellular trafficking and compartmentalization of pathogen-associated molecular patterns in APCs (Prete et al., 2013).

Oral tolerance to food antigens largely depends on the CD103^{+} subset of dendritic cells in the lamina propria, which capture intestinal antigens and preferentially induce Treg responses through the production of TGFβ and retinoic acid (Berin and Sampson, 2013). This process appears to be confined to the mesenteric lymph nodes, implicating that oral tolerance requires the migration of CD103^{+} dendritic cells from the lamina propria to these secondary lymphoid organs in the intestine. Indeed, surgical removal of MLNs prior to antigen exposure or genetic deletion of the chemokine receptor CCR7 on dendritic cells both abrogate oral tolerance (Worbs et al., 2006). Consequently, it can be hypothesized that migratory defects in WASP-deficient dendritic cells could result in diminished delivery of food antigens to the MLNs in *Was*$^{-/-}$ mice, which in turn could contribute to food allergic sensitization. In support of this theory, lower fractions of CD11c^{+}CD11b^{+}CD103^{+} dendritic cells amongst total dendritic cells have been described in the MLN and lamina propria of *Was*$^{-/-}$*Rag2*$^{-/-}$ compared to *Rag2*$^{-/-}$ mice (Nguyen et al., 2012). In **Chapter 7**, we have described the generation of *Was*$^{fl/fl}$*Itgax*-Cre mice, which express cre-recombinase in association with the dendritic cell-specific CD11c promoter. These mice were found to have serum MCPT1, IgE and IgG1 levels equivalent to *Was*$^{wt/wt}$*Itgax*-Cre littermates (**Figure 7.4A** and results not shown), demonstrating that WASP deficiency confined to CD11c^{+} dendritic cells – and thus including the highly migratory, tolerogenic subset of CD11c^{+}CD103^{+} dendritic cells – is insufficient to initiate the cascade of food allergic sensitization. However, in addition to a potentially causative role in the pathogenesis of WAS-associated food allergy, we should also consider the possibility that WASP-deficiency in the APC compartment contributes to the differential susceptibility to food allergy between *Was*$^{-/-}$ and *Was*$^{fl/fl}$*Foxp3*-Cre mice. As is apparent from the foregoing discussion, it is conceivable that WASP-deficient dendritic cells have a diminished uptake of (particulate) antigens from the intestinal lumen, which could dampen the CD4^{+} T cell activation in an already sensitized host. Moreover, increased cross-presentation of food antigens via MHC I rather than MHC II in the absence of WASP may further reduce CD4^{+} effector T cell responses. In addition, decreased migratory capacity of APCs might reduce the chance of their successful interaction with T cells in the lamina propria. It is furthermore likely that the food antigen-specific effector T cells that interact with *Was*$^{-/-}$ APCs may be hampered in their

activation due to failing structural support of immune synapse stability from the dendritic cell side, leading to diminished signaling through the T cell receptor. Interestingly, Malinova and colleagues have demonstrated *in vitro* that T cells interacting with WASP-deficient dendritic cells preferentially differentiate away from the Th2 lineage and instead assume a Th17 phenotype (Malinova et al., 2015). As such, the absence of WASP from dendritic cells in *Was*$^{-/-}$ but not in *Was*$^{fl/fl}$*Foxp3*-Cre animals may operate as an opposing force to the uncontrolled Th2 immune activation that occurs in the context of WASP-deficient FOXP3^{+} Tregs. In line with this hypothesis is the observation that the Th2 signature of the inflammatory infiltrate that typifies WASP deficiency-associated colitis in mice on the 129SvEv background (Nguyen et al., 2007) is absent in colonic inflammation that follows after transfer of WT naïve T cells into *Was*$^{-/-}$*Rag2*$^{-/-}$ hosts (Nguyen et al., 2012).

In conclusion, it is conceivable that loss of WASP-dependent functions from APCs in *Was*$^{-/-}$ mice contribute to a dampened food allergic phenotype. Formal test of this hypothesis could be attained by generating *Was*$^{fl/fl}$*Foxp3-Cre Itgax*-Cre double knock-out mice, which would allow for the estimation of the effect size in the absence of confounding influence from WASP-deficiency in non-Tregs, non-CD11c^{+} dendritic cells.

9.4 B cells

Similar to the absence of WASP in antigen presenting cells, we must consider WASP deficiency in B cells as a possible explanation for the difference in susceptibility to food allergy between *Was*$^{-/-}$ and *Was*$^{fl/fl}$*Foxp3*-Cre mice. The B cell compartment can be envisioned as a rather distal checkpoint in food allergic sensitization, only initiating and maintaining the synthesis of food-specific IgE and IgG1 when several conditions are met. Since a growing body of evidence suggests that B cell homeostasis is affected in WAS patients and WASP-deficient animals (Massaad et al., 2013; Thrasher and Burns, 2010), lower serum levels of food-specific IgE and IgG1 in *Was*$^{-/-}$ mice could potentially be caused by a loss of WASP-dependent functions in B cells. For the benefit of the current discussion, we will highlight the following B-cell-intrinsic roles for WASP:

1 *Development, differentiation, and survival.* Convincing evidence for a role of WASP in B cell homeostasis came from experiments that used heterozygous *Was*$^{+/-}$ mice, or WT and *Was*$^{-/-}$ mixed-bone-marrow-chimeric, B-cell-deficient μMT animals. In these settings, WASP-competent B cells exhibit a survival advantage over *Was*$^{-/-}$ counterparts (Meyer-Bahlburg et al., 2008; Westerberg et al., 2008). This advantage starts to be evident at the late transitional B cell stage, and becomes particularly striking in the marginal zone B cell populations (Meyer-Bahlburg et al., 2008; Westerberg et al., 2008),

resulting in a significantly reduced number of marginal zone B cells in both *Was*$^{-/-}$ mice and patients with WAS (Westerberg et al., 2005). With the generation of *Was*$^{fl/fl}$*Mb1*-Cre mice, it has become possible to study the effect of the selective loss of WASP from the B cell lineage. These animals demonstrate unperturbed early B cell development in the bone-marrow, but have a reduced number of total B lymphocytes in the spleen and an altered distribution of B cell subsets compared to WT counterparts: both *Was*$^{-/-}$ and *Was*$^{fl/fl}$*Mb1*-Cre mice are characterized by reduced marginal zone and follicular B cells, whereas a relative increase is seen in plasma cells and germinal center B cells (Recher et al., 2012). These data are indicative of a cell-intrinsic role for WASP in the peripheral homeostasis of B-lymphocytes.

2 *Mobility and chemotaxis.* Analogous to T lymphocytes and mononuclear phagocytes, loss of WASP in B cells results in altered cell morphology and reduced cell surface protrusions (Westerberg et al., 2001). Aberrant rearrangement of the actin cytoskeleton likely underlies the observation that *Was*$^{-/-}$ B cells show impaired migration towards B cell-tropic chemokines such as CXCL13 and CXCL12 *in vitro*, because expression levels of the respective chemokine receptors CXCR5 and CXCR4 on B lymphocytes was found to be unaltered (Westerberg et al., 2005). This study has also documented the changes in splenic architecture following immunization with *sheep red blood cell* antigen and found that germinal centers in the spleens of *Was*$^{-/-}$ hosts were more resistant to expansion than in WT counterparts, which could not be overcome by transfer of WT T cells prior to immunization (Westerberg et al., 2005). These results suggest that WASP-deficient B cells are less efficient at mediating a humoral immune response against a foreign antigen. It is currently unclear to which extent alterations in migratory capacity contribute to the observed alterations in distribution of splenic subpopulations of B cells that are observed in *Was*$^{-/-}$ and *Was*$^{fl/fl}$*Mb1*-Cre mice.

3 *Activation and isotype switching.* Whether WASP is required for the activation of B and T lymphocytes was the subject of the first investigations in *Was*$^{-/-}$ mice. Whereas a significant reduction was observed in *Was*$^{-/-}$ T cell proliferation following anti-CD3ε stimulation, stimulation of the B cell receptor (BCR) with anti-IgM antibody resulted in efficient proliferation despite the absence of WASP protein (Snapper et al., 1998). Follow-up studies found that WASP-deficient B cells are actually hyperresponsive to BCR signaling, as evidenced by prolonged Ca^{2+} flux following anti-IgM treatment, which is likely related to a delayed internalization of the BCR (Becker-Herman et al., 2011). In vitro, *Was*$^{-/-}$ B cells furthermore exhibit an increased propensity to undergo class switching to IgG1 after combined stimulation with anti-CD40 antibodies (simulating T cell help) and IL4, or to IgG2b following stimulation

with LPS (Westerberg et al., 2005).[4] The heightened response to BCR ligation in the absence of WASP has been hypothesized to mediate a loss of peripheral B cell tolerance and thereby contribute to WAS-associated autoimmunity. Irradiated μMT animals receiving mixed *Was*$^{-/-}$/μMT bone-marrow spontaneously develop increased numbers of germinal center B cells (defined as B220^{+}PNA^{+}FAS^{+}) in the spleen and IgG autoantibodies in serum, before succumbing to an SLE-like glomerulonephritis. Anti-CD4 treatment of these chimeric mice significantly ameliorated autoimmune pathology, and abrogated spontaneous germinal center formation and production of IgG2b and IgG2c (the C57BL/6 equivalent of IgG2a), although increased levels of IgG3 autoantibodies could still be detected in this setting (Becker-Herman et al., 2011). Aberrant BCR signaling alone is insufficient to explain these results, as loss of TLR-mediated signaling through genetic deletion of the TLR adapter protein MyD88 completely halted autoimmune responses and GC formation, including synthesis of IgG3 autoantibodies (Becker-Herman et al., 2011). These results were later confirmed in *Was*$^{fl/fl}$*Mb1*-Cre mice, which spontaneously develop germinal centers and have elevated serum levels of IgM and IgG with reactivity against a broad variety of self-antigens (Recher et al., 2012). Combined, these data demonstrate that autoimmune humoral immune responses can be initiated and driven by WASP-deficient B cells in the presence of a WASP-competent T cell compartment.

4 *Variation of the BCR repertoire.* A follow-up paper to the study by Becker-Herman et al. has recently shed more light on the role of WASP in B cell homeostasis (Kolhatkar et al., 2015). By employing the Nur77-GFP reporter system, which provides a fluorescent read-out of strength of TCR or BCR signaling (Zikherman et al., 2012), this study provided further confirmation of the increased sensitivity to BCR signaling in *Was*$^{-/-}$ B cells, and went on to demonstrate that combined hyperresponsiveness to BCR and TLR signaling results in alterations in the naïve B cell repertoire. The merit of this work lies in showing that these changes are due to an increase in antigen-mediated, positive selection amongst B cells at the transitional stage, rather than from a failure of negative selection in the bone-marrow. Auto-reactive B cells can be found in healthy mice and humans, and evidence suggests that the selection of these clones is an active process depending on BCR-signaling in response to splenic self-antigens, CD40-mediated T cell help and B cell activating factor (BAFF) (Kolhatkar et al., 2015; Zikherman et al., 2012; Gaudin et al., 2004). The benefit of maintaining potentially self-reactive B cell clones in the naive B cell repertoire has been thought to stem from an increased chance of reactivity against invading pathogens (Male et al., 2006) or to contribute to the pool of natural immunoglobulins to serve

4 LPS functions as a polyclonal stimulator of B cells, and challenge of naïve mature B lymphocytes with LPS in the presence of IL4 is sufficient to induce class-switching to IgG1 and IgE in vitro (Kracker and Radbruch, 2004).

homeostatic functions such as clearance of apoptotic cells (Gaudin et al., 2004; Kolhatkar et al., 2015). In WASP deficiency, this positive selection of splenic B cells is altered, resulting in an enrichment for low-affinity, self-reactive BCRs (Kolhatkar et al., 2015). Importantly, skewing of BCR repertoire and increased auto-reactivity has recently been observed by several groups after sequencing naïve B cells from WAS patients (Castiello et al., 2014; O'Connell et al., 2014; Simon et al., 2014), implicating that altered selection in *Was*$^{-/-}$ mice may also be operative in WAS patients.

5 *Regulatory function.* The study of regulatory subsets amongst T cells has been at the forefront of adaptive immunity research for the last two decades. However, it is becoming clear that a subset of B cells (analogously termed Bregs) also exhibits immunoregulatory functions, including the production of IL10. Bregs may originate from several B cell subpopulations, but appear to be largely controlled by T cell help, as there is no induction of IL10-producing B cells in absence of CD40 or ICAM-1 (Mauri and Bosma, 2012). Importantly, Bregs may support the development of Tregs by acting as an additional source of IL10, as evidenced by reduced numbers of Tregs in μMT mice (Mauri and Bosma, 2012). Parasites can induce Bregs, and consequent recruitment of Tregs may contribute to the modulating effects on allergy by ova of *Schistosoma mansoni* (Khan et al., 2015; Amu et al., 2010). Gerben Bouma and colleagues have recently interrogated the role of regulatory B cells in WASP-deficient mice (Bouma et al., 2014). Both *Was*$^{-/-}$ and *Was*$^{fl/fl}$*Mb1*-Cre animals showed lower frequencies of Bregs in the spleen at baseline. In a model of Th17-mediated experimental arthritis that relies on immunization with BSA in complete Freund's adjuvant followed by intra-articular injection of BSA, *Was*$^{-/-}$ mice were found more susceptible to disease when compared to WT mice, and adoptive transfer of WT Bregs ameliorated inflammation and increased Treg numbers in the inflammatory infiltrate. However, *Was*$^{fl/fl}$*Mb1*-Cre mice showed equivalent susceptibility to arthritis to WT mice, demonstrating that Breg-dysfunction is unlikely to account for the increased autoimmunity in WAS.

Taken together, these results demonstrate that WASP-dependent processes form a critical part in many aspects of B cell biology.

Since *Was*$^{fl/fl}$*Foxp3*-Cre mice showed significantly higher titers of total and food-specific IgE and IgG1, we asked whether WASP deficiency in B cells would account for restrained allergic responses in *Was*$^{-/-}$ animals. As delineated above, murine WASP-deficient B cells have an altered BCR repertoire, exhibit increased proliferation in germinal centers (GCs) and spontaneously secrete higher levels of IgM (Recher et al., 2012). In support of normalization of the B cell compartment in *Was*$^{fl/fl}$*Foxp3*-Cre mice, we observed complete abrogation of the hyper-IgM

phenotype that is seen in *Was*$^{-/-}$ mice (**Figure 9.2A**). These alterations in the peripheral B cell pool were likely to be of consequence for food allergic sensitization, as significantly higher serum titers of anti-soy and anti-wheat IgM in *Was*$^{fl/fl}$*Foxp3*-Cre mice are indicative of a higher fraction of naïve B cells with specificity for food antigens (**Figure 9.2B**). We then screened serum of WAS patients for total and food-specific IgM. In line with previously published data (Thrasher and Burns, 2010), and in contrast with WASP-deficient mice, we observed reduced levels of total IgM in WAS patients compared to those with or without food allergy (**Figure 9.2C**). Anti-soy and anti-wheat IgM titers were similarly decreased in WAS patients compared to those with food allergy (**Figure 9.2D**), strongly resembling the data obtained in *Was*$^{-/-}$ and *Was*$^{fl/fl}$*Foxp3*-Cre mice. These data demonstrate that the naïve B cell repertoire in healthy mice and humans contains lymphocytes with BCRs that exhibit specificity for soy and wheat, and suggest that WASP deficiency affects the number of food-specific B cells. Lower levels of IgM with reactivity against food in *Was*$^{-/-}$ mice did not result from the recruitment of food-specific clonotypes from the naïve to the antigen-experienced, class-switched B cell repertoire, because *Was*$^{fl/fl}$*Foxp3*-Cre mice and food allergic children show normal levels of food-specific IgM. In light of recently published data (Kolhatkar et al., 2015), it seems likely that the skewing of the B cell repertoire that results from enhanced positive selection of transitional WASP-deficient B cells negatively affects the number of food-specific B-cells that are available for food antigen-specific activation. This smaller pool of naïve (IgM^{+}) soy- and wheat-specific B cells might contribute to lower levels of food-specific IgE and IgG1 (derived from class-switched, antigen-experienced B cells) observed in *Was*$^{-/-}$ mice in comparison to *Was*$^{fl/fl}$*Foxp3*-Cre animals.

Differential activation requirements of WASP-deficient and WASP-competent B cells may underlie further differences in humoral immunity between *Was*$^{-/-}$ and *Was*$^{fl/fl}$*Foxp3*-Cre mice. Data from *Was*$^{fl/fl}$*Mb1*-Cre mice showed that the spontaneous germinal center formation observed in *Was*$^{-/-}$ animals occurs as a result of WASP-deficiency in the B cell compartment (Recher et al., 2012). Germinal centers contain antigen-specific B cells that are undergoing active proliferation leading to clonal expansion. Somatic hypermutation results in differential antigen affinity amongst the progeny of the founder B cells, and through competition for limited T cell help, this process results in affinity maturation of the antibody response. Furthermore, T cell-mediated help and cytokine production instructs class switching in proliferating B cells prior to their differentiation into plasma cell or memory B cell precursors, as well as their migration away from the germinal center (Male et al., 2006). Initial antigen exposure of naïve B cells occurs via CD11c^{+} dendritic cells, which enhance their survival by providing soluble B cell activating factor (BAFF) and APRIL, and the cumulative signals received from dendritic cells and T cells at this stage shape the fate of the descendants of a particular B cell many generations later (Goodnow et al., 2010). Interestingly, elevated serum levels of BAFF in WASP deficiency have recently been reported by several independent

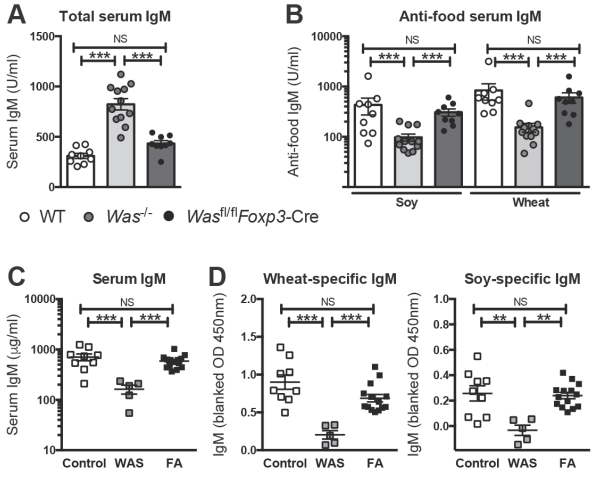


Figure 9.2. ***WASp deficiency in B cells affects food-specific IgM levels in mice and humans.*** (**A**) Total and (**B**) soy and wheat-specific levels of serum IgM in WT (open circles), *Was*$^{-/-}$ (gray circles) and *Was*$^{fl/fl}$*Foxp3*-Cre (black circles) mice. (**C**) Total and (**D**) soy and wheat-specific levels of serum IgM levels in in pediatric WAS patients and age-matched controls without (control) or with food allergy (FA). Dots represent individual mice or patients and error bars depict SEM. *p<0.05; ** p<0.01; ***p<0.001; NS: not significant as determined by one-way ANOVA with Tukey's multiple comparisons test.

groups (Castiello et al., 2014; Crestani et al., 2015; Kolhatkar et al., 2015) and elevated BAFF may therefore drive increased B cell recruitment and survival, although murine data suggest that WASP-deficiency reduces responsiveness to BAFF signaling (Bouma et al., 2014). Commitment to germinal center proliferation instead of differentiation into extrafollicular plasma cells or recirculating memory B cell depends on the migration of B cells towards follicular dendritic cells, a process that is dependent in part on CXCL13-CXCR5 interaction (Goodnow et al., 2010). Notably, migration in response to dendritic-cell-derived CXCL13 was shown to be aberrant in *Was*$^{-/-}$ B cells (Westerberg et al., 2005), which would be predicted to result in less efficient germinal center formation in WASP deficiency. Increased expression of transcription factor *Bcl6* underlies further differentiation into germinal center B cells, whereas expression of Blimp1 and IRF4 promotes a plasma cell fate. This reciprocal engagement of transcription factors is dependent on strength of BCR and CD40 ligation, as well as TLR signals, with increased signal strength all promoting expression of IRF4 instead of BCL6 (Goodnow et al., 2010). These findings can link alterations in responsiveness to BCR and TLR stimuli in WASP-deficient B cells (Becker-Herman et al., 2011; Kolhatkar et al., 2015) to a different germinal center

to plasma cell ratio in *Was*$^{-/-}$ mice, although this paradigm would predict increased signaling to skew the ratio in favor of plasma cells instead of germinal center B cells.

Since germinal centers contribute critically to affinity maturation of B cells, dysfunction in the germinal center response could result in antibodies with reduced antigen affinity. In **Chapter 8**, we used the *in vitro* degranulation of bone-marrow derived mast cells following soluble OVA challenge as a readout for the affinity and functionality of OVA-specific IgE that was spontaneously induced in *Was*$^{-/-}$ hosts in comparison with antibodies obtained from OVA/Alum immunized WT mice (**Figure 8.2D-E, page 191**). Here, we observed no differences in the capacity of antigen-specific IgE to mediate mast cell degranulation, suggesting that affinity maturation is not significantly altered in the absence of WASP and that *Was*$^{-/-}$ mice can make high-quality antibodies.

To further study the role of aberrant germinal center responses in the context of food allergic sensitization, we compared the B cell compartment in intestinal lymphoid organs of *Was*$^{-/-}$ and *Was*$^{fl/fl}$*Foxp3*-Cre animals. Although reduced total B cell counts have been described in the spleens of *Was*$^{-/-}$ and *Was*$^{fl/fl}$*Mb1*-Cre mice (Recher et al., 2012), we found that MLNs from both *Was*$^{-/-}$ and *Was*$^{fl/fl}$*Foxp3*-Cre mice contained increased numbers of B cells. In Peyer's patches, significantly fewer B cells were encountered in *Was*$^{-/-}$ than *Was*$^{fl/fl}$*Foxp3*-Cre mice (**Figure 9.3A**). Within PPs and MLNs, we measured an increase in both absolute and relative numbers of germinal center B cells, which was significantly greater in the PPs of *Was*$^{fl/fl}$*Foxp3*-Cre mice compared to WT and *Was*$^{-/-}$ mice (**Figure 9.3B-C**). These results suggest that WASP-deficiency in B cells may contribute to their altered distribution throughout the GALT and clearly indicate that aberrant germinal center responses also occur in a WASP-competent B cell compartment in the presence of WASP-deficient Tregs.

Failing regulatory mechanisms in the T cell lineage can also result in increased germinal center responses, as evidenced by increased germinal center B cells in PPs (but not spleen or MLNs) of mice deficient in iTregs via deletion of the intronic *Foxp3* enhancer CNS1 (Josefowicz et al., 2012b). We found that *scurfy* mice deficient in all Foxp3 regulatory cells have profound spontaneous GC formation, including in the MLNs, with significantly higher frequencies of germinal center B cells (~15%) compared to *Was*$^{-/-}$ and *Was*$^{fl/fl}$*Foxp3*-Cre mice (up to 5% of B220^{+} B cells) (**Figure 9.3D**). This represents a pathogenic process, as B cells have been shown to critically contribute to autoimmune disease in scurfy mice (Aschermann et al., 2013) and were found to be dysregulated in humanized mice reconstituted with hematopoietic stem cells from an IPEX patient (**Chapter 6**). A number of studies have identified mechanisms that allow Tregs to directly suppress B cell functions. Contact-dependent killing of antigen presenting B cells by cognate CD4^{+}CD25^{+} T cells has been observed (Zhao et al., 2006), and *in vitro* data suggest that this function is defective in WASP-deficient Tregs due to a reduced ability to secrete granzyme B (Adriani et al., 2011), although to which extent this contributes to spontaneous germinal center formation in WASP-deficient mice has not been investigated. The very question how Tregs regulate GC responses resulted in the identification of a

subpopulation of FOXP3^{+} Tregs that co-express transcription factor Bcl6, the lineage-defining master regulator of both follicular helper T cell (Crotty, 2014) and germinal center B cell differentiation (Linterman et al., 2011) (Chung et al., 2011) (Wollenberg et al., 2011) (Sage et al., 2012). These follicular regulatory T cells (T_{FR}) cells express CXCR5, allowing CXCL13-mediated chemotaxis to germinal centers, and exert control over germinal center B cell responses, as *Cxcr5*$^{-/-}$ or *Bcl6*$^{-/-}$ Tregs are unable to restrain aberrant germinal center formation (Chung et al., 2011). Suppression occurs through inhibition of affinity maturation and plasma cell differentiation (Chung et al., 2011) as well as through controlling follicular helper T (T_{FH}) cells activation (Linterman et al., 2011). Dysregulation of T_{FH} or T_{FR} responses form an attractive hypothesis to explain aberrant antibody responses in food allergic *Was*$^{-/-}$ mice, but to date no study has characterized the T_{FH} or T_{FR} compartment in the context of WASP deficiency.

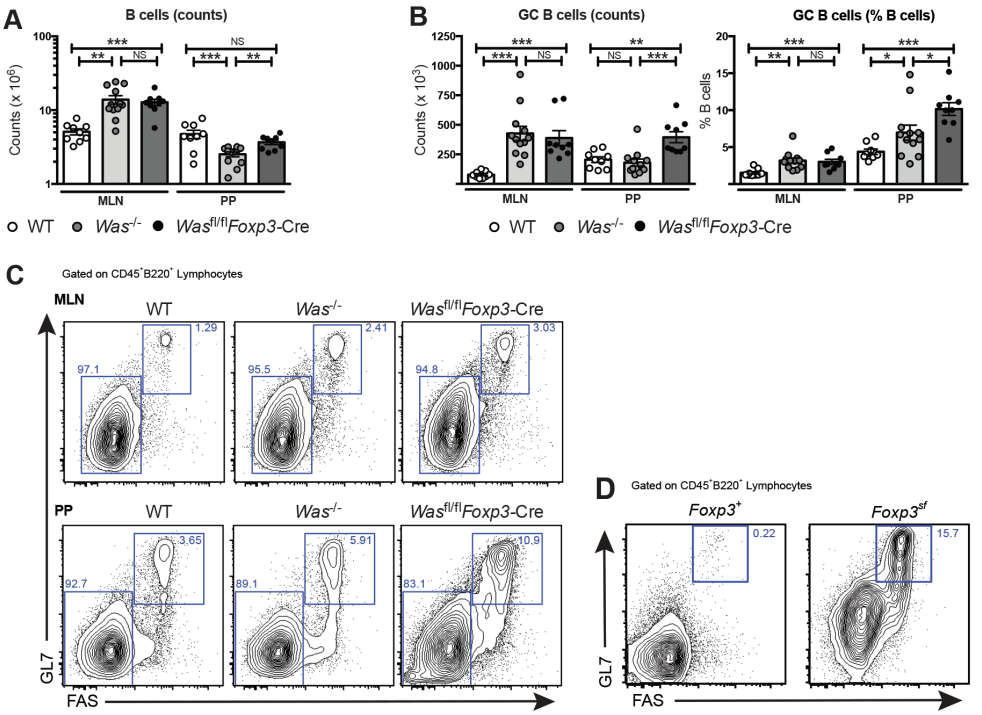


Figure 9.3. ***Deletion of WASP in Foxp3^{+} Tregs results in B-cell lymphoproliferation and aberrant germinal center responses.*** (**A**) Quantification of absolute B220^{+} cell numbers in MLNs and PPs of WT (open circles), *Was*$^{-/-}$ (gray circles) and *Was*$^{fl/fl}$*Foxp3*-Cre (black circles) mice. (**B**) Absolute numbers and relative proportion of germinal center (GC) B cells, with gating strategy shown in representative samples in (**C**). (**D**) GC B cells in MLNs of scurfy mice compared to their littermate controls. Dots represent individual mice and error bars depict SEM. *p<0.05; ** p<0.01; ***p<0.001; NS: not significant as determined by one-way ANOVA with Tukey's multiple comparisons test.

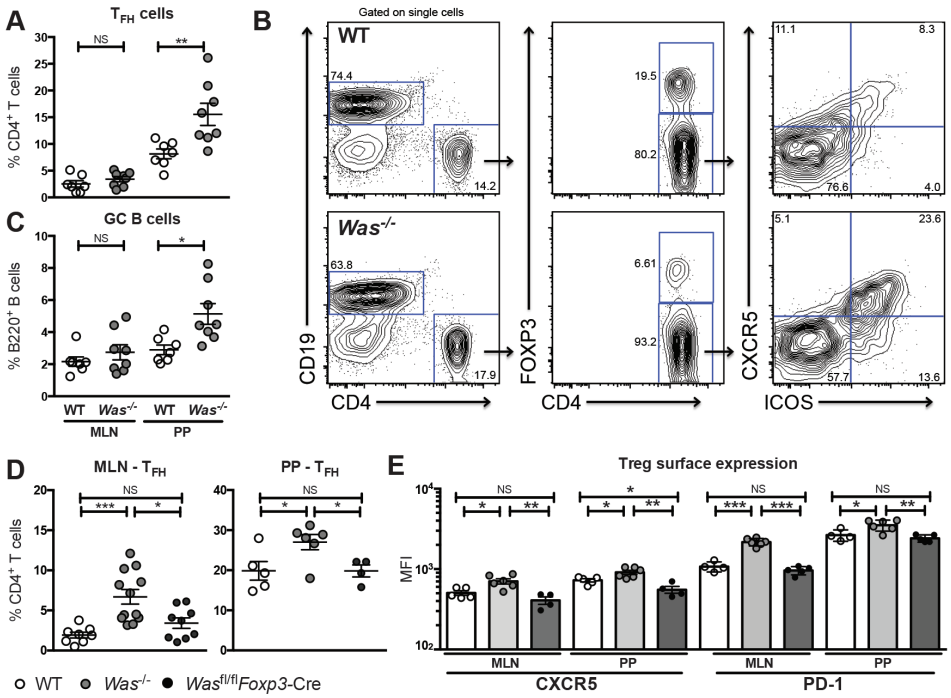


Figure 9.4 ***Follicular helper T cells are increased in Was$^{-/-}$ but not in Was$^{fl/fl}$Foxp3*-Cre mice.** (**A**) Fraction of follicular helper T (T_{FH}) cells amongst CD4$^+$ lymphocytes in WT (open circles) and *Was$^{-/-}$* (gray circles) mice on the BALB/c background in MLN and Peyer's patches (PP), with gating strategy depicted in (**B**). (**C**) Quantification of T_{FH} cells in MLNs and PPs of WT (open circles), *Was$^{-/-}$* (gray circles) and *Was$^{fl/fl}$Foxp3*-Cre (black circles) mice on the C57BL/6 background. (**D**) Surface expression of T_{FH} markers CXCR5 and PD-1 on CD4$^+$Foxp3$^+$ regulatory T cells in WT, *Was$^{-/-}$* and *Was$^{fl/fl}$Foxp3*-Cre animals as determined by flow cytometry. Dots represent individual mice and error bars depict SEM. *p<0.05; ** p<0.01; ***p<0.001; NS: not significant as determined by one-way ANOVA with Tukey's multiple comparisons test.

Using food allergic *Was$^{-/-}$* mice on the BALB/c background (described in **Chapter 7, Figure 7.2, page 161**), we found increased fractions of T_{FH} cells in PPs (defined as ICOS$^+$CXCR5$^+$, (Sage et al., 2012)) when compared to WT controls (**Figure 9.4A-B**). Increased T_{FH} cells in PPs correlated with increased levels of germinal center B cells in this anatomical location, whereas both T_{FH} and germinal center B cells in MLNs were not elevated in the *Was$^{-/-}$* mice in this experiment (**Figure 9.4C**). To assess the association between increased T_{FH} cell differentiation in WASP deficiency and spontaneously occurring food allergic sensitization, we repeated the analysis in WT, *Was$^{-/-}$* and *Was$^{fl/fl}$Foxp3*-Cre on the C57BL/6 background. In line with increased germinal center B cells (**Figure 9.3B**), we observed a consistent increase in the fraction of CXCR5$^+$ T_{FH} in both MLNs and PPs of *Was$^{-/-}$* animals (**Figure 9.4D**). However, increased T_{FH} cells were not observed in *Was$^{fl/fl}$Foxp3*-Cre mice, despite an even greater expanded germinal center B cell compartment in these mice (**Figure 9.3B** and **9.4D**).

These results demonstrate that T_{FH} differentiation is indeed aberrant in *Was*$^{-/-}$ animals, but also show that an increased T_{FH} compartment is dispensable for aberrant germinal center formation and allergic sensitization.

Pronounced T_{FH} differentiation in *Was*$^{-/-}$ but not *Was*$^{fl/fl}$*Foxp3*-Cre mice could indicate that T cell-intrinsic loss of WASP might result in upregulation of T_{FH} markers such as CXCR5. If this were true, then WASP-deficient Tregs would be expected to assume a T_{FR} phenotype. However, although we indeed observed increased expression of T_{FR} surface markers CXCR5 and PD-1 on WASP-deficient Tregs in *Was*$^{-/-}$ mice, this did not extend to Tregs with conditional deletion of WASP (**Figure 9.4E**). These data suggest that enhanced differentiation down the T_{FH} pathway is not driven by T cell-specific loss of WASP, but instead is a consequence of the systemic loss of WASP. One mechanism through which deficiency of WASP can be hypothesized to contribute to T_{FH} development is reduced production of IL-2 by WASP-deficient T cells (**Figure 7.6B, page 167**). IL2-signaling in CD4^{+} T cells prevents Bcl6 expression via activation of STAT5 (Johnston et al., 2012) and local levels of IL2 thereby affect T_{FH} differentiation. Of note, FOXP3+ Treg-mediated suppression of IL2 production was recently shown to result in enhanced germinal center formation and improved anti-influenza antibody responses (León et al., 2014), illustrating that physiological Treg function may also enhance development of germinal centers.

Are dysregulated germinal center responses at all required for the production of food-specific IgE? Research into the origin of IgE-secreting B cells has long been obscured by the difficulty in detecting these rare populations of B cells. However, over the last few years, several IgE-reporter systems have become available, and both M1 prime GFP knock-in mice as well as Verigem IgE reporter animals have clearly revealed IgE^{+} germinal centers following parasitic infection (Talay et al., 2012; Yang et al., 2012). However, studies in MHCII-deficient mice and T cell-deficient mice have also identified 'natural IgE' that does not require secondary lymphoid structures or germinal center formation (McCoy et al., 2006). In addition, non-mutated, germinal-center independent B cells can mediate early IgE responses following immunization prior to germinal-center-derived production of IgE (Wu and Zarrin, 2014). One consideration of particular interest to the discussion of the origin of antigen-specific IgE in the context of WASP deficiency comes from observations in the related primary immunodeficiency that results from mutation in the gene *dedicator of cytokinesis 8 (DOCK8)*. DOCK8 and WASP both interact with Cdc42 to mediate changes in the actin cytoskeleton, and it has been suggested that the two proteins participate in the same signaling pathway (Ham et al., 2013; Notarangelo, 2013). DOCK8 patients frequently develop severe allergies, and the humoral immune response is characterized by low levels of serum IgM, normal IgG and greatly elevated IgE, thus strongly resembling patients with WAS (Zhang et al., 2009) (cf. **Figure 7.1A, page 159**). In strong contrast to mice deficient in WASP, *Dock8*$^{-/-}$ mice do not develop germinal centers, potentially due to failure of B cells to form an immunological synapse (Randall et al., 2009), which would indicate a germinal center-independent origin

of antibodies. However, *Dock8*$^{-/-}$ mice do not develop a hyper IgE phenotype (Notarangelo, 2013), so it would be required to study integrity of germinal center formation in humans in order to answer this question. Complicating matters further is the observation that mice deficient in Cdc42 (Burbage et al., 2015) or WASP-interacting protein (WIP) (Keppler et al., 2015) also fail to generate germinal center responses and are characterized by low serum levels of IgM and IgG. Since WIP interacts with both N-WASP as well as WASP, it is possible that N-WASP can compensate for loss of WASP to explain these seemingly paradoxical observations (Westerberg et al., 2012). Deficiency of WIP results in normal Ig levels in the only reported human patient (Lanzi et al., 2012).

Very recent evidence on the regulation of germinal center responses suggests that loss of FAS activity in B cells (underlying the genetic disease autoimmune lymphoproliferative syndrome) results in a population of unconventional germinal center B cells that underwent somatic hypermutation despite loss of antigen reactivity and continue to become plasma cells that preferentially secrete IgE and autoantibodies (Butt et al., 2015). The synthesis of FASligand (FASl) from WASP-deficient T cells is reduced (Nikolov et al., 2010). However, FAS/FASl interactions do not contribute to the direct killing of B cells by $CD4^+CD25^+$ Tregs (Zhao et al., 2006), so although FAS-mediated dysregulation would provide an attractive hypothesis for aberrant B cell responses in *Was*$^{-/-}$ mice, it would not explain the increase in GC B cells in *Was*$^{fl/fl}$*Foxp3*-Cre mice. Generation of WASP-deficient IgE reporter mice would certainly provide additional insight in the question of the origin of food-specific IgE in *Was*$^{-/-}$ mice.

9.5 Effector T cells

Of all immune cells involved in food allergy, WASP-dependent functions have been most extensively characterized for T lymphocytes and the reader is referred to the following two papers for a comprehensive overview (Cotta-de-Almeida et al., 2015; Matalon et al., 2013). Analogous to the previous discussion on B cells, WASP-dependent functions in T cells can be broadly divided over the following, partly overlapping, domains.

1 *Development and survival.* In *Was*$^{+/-}$ heterozygous mice, around 65% of splenic $CD4^+$ and $CD8^+$ T cells express WASP. For thymic populations of double-negative ($CD4^-$ $CD8^-$) or double positive ($CD4^+CD8^+$) developing T cells, these frequencies were closer to 50%, suggesting that WASP-deficiency is associated with reduced survival in the periphery (Westerberg et al., 2008). This may be due to the fact that WASP-deficient T cells appear to be more susceptible to apoptosis (Cotta-de-Almeida et al., 2015; Rengan et al., 2000), although reduced apoptosis of T cells following TCR-restimulation has also been reported (Nikolov et al., 2010). In humans, secondary mutations in *WAS* that lead to protein re-expression is observed in up to 11% of

patients, and in most cases this somatic mosaicism is restricted to T cells (Cotta-de-Almeida et al., 2015). These revertant T cells show a diversified TCR repertoire and, in line with *Was*$^{+/-}$ mice, also have a selective advantage *in vitro* and *in vivo*, although autoimmunity and immunodeficiency continued to exist (Trifari et al., 2010).

2 *Mobility and chemotaxis*. Like in other hematopoietic lineages, motility defects have been observed in WASP-deficient T cells and it is likely that aberrant migration contributes to immunodeficiency, although this remains to be formally demonstrated (Cotta-de-Almeida et al., 2015) (Haddad et al., 2001). In Transwell plates, *Was*$^{-/-}$ T lymphocytes exhibit reduced CCR7-mediated migration towards CCL19, which may have accounted for the reduced homing of CD4^{+} T cells to PPs in comparison to WT T cells (Snapper et al., 2005). Homing defects were not observed in MLNs and spleen, and since WASP integrates multiple chemotactic signals (Cotta-de-Almeida et al., 2015), the consequences of WASP deficiency likely depend on the broad immunological context. It is unknown whether defects in CXCR5-mediated chemotaxis as observed in *Was*$^{-/-}$ B cells (Westerberg et al., 2005) also extend to T cells, which would be relevant given the increased fractions of T_{FH} and germinal center B cells in *Was*$^{-/-}$ (**Figure 9.4**).

3 *Immune synapse formation and activation.* It has long been known that WASP-deficient T cells show aberrant activation in response to ligation of the TCR with anti-CD3 antibodies (Gallego et al., 1997; Snapper et al., 1998). In Chapter 7, we discussed that WASP is rapidly recruited to the TCR upon ligation (Barda-Saad et al., 2005), and is required for internalization of the TCR complex (Zhang et al., 1999). As we mentioned above in our discussion on APCs, WASP is indispensable for the formation of the immune synapse, where it acts reciprocally with PKCtheta to maintain immune synapse stability (Badour et al., 2003; Sims et al., 2007). The failure to maintain effective immune synapses between T cells and APCs has also been demonstrated in human WAS patients and was associated with reduced Ca^{2+} signaling and aberrant signal integration in WASP-deficient T cells (Calvez et al., 2011).

4 *Th differentiation, effector function and cytokine production.* In addition to aberrant activation of antigen-specific CD4^{+} or CD8^{+} T cells, it has become clear that absence of WASP also affects the differentiation of Th subsets. Skewed Th differentiation is of obvious pertinence to our current discussion, since a dysregulated Th1/Th2 balance has been hypothesized to underlie the atopic features of WAS patients (Cotta-de-Almeida et al., 2015; Massaad et al., 2013). A frequently cited study found that CD4^{+} T cells from WAS patients exhibited a reduced induction of *TBX21* (the gene encoding the Th1 transcription factor T-bet) following stimulation of TCR and CD28,

whereas *GATA3* mRNA levels were similar to those in T cells from healthy donors. This was reflected in significantly reduced production of IFN-γ, IL-2 and TNF-α, but not IL-4 or IL-5, following TCR stimulation *in vitro* (Trifari et al., 2006). Mechanistically, diminished Th1 differentiation was associated with reduced translocation of NFAT to the nucleus (Trifari et al., 2006), which had also been found to underlie diminished *Il2* gene expression in *Was*$^{-/-}$ mice (Cianferoni et al., 2005). Similarly, CD8^{+} cytotoxic T lymphocytes from WAS patients secrete lower levels of IFN-γ, IL-2 and TNF-α (De Meester et al., 2010). However, reduced Th1 immunity in WASP deficiency has been challenged by Deborah Fowell and colleagues (Morales-Tirado et al., 2010), who observed that transfer of *Was*$^{-/-}$ CD4^{+} T cells to *Rag*$^{-/-}$ mice resulted in protection against *Leishmania major*, which depends on IFN-γ production by Th1 cells, but not against the Th2-inducing *Nippostrongylus brasiliensis*. Although WASP-deficient CD4^{+} T cells were found to produce lower levels of IL-4, it is hard to compare the outcomes of these two parasite studies as the *N brasiliensis* model was carried out in TCR Cα-deficient and not *Rag*$^{-/-}$ mice (Morales-Tirado et al., 2010). Further adding to the complex role of WASP in Th differentiation is the recognition of WASP-mediated epigenetic effects (Taylor et al., 2010). In primary Th1-differentiating cells, WASP could be found in the nucleus where it was recruited to the proximal promoter locus of *TBX21* (encoding T-bet) as well as *IFNG* and *STAT1*. In contrast, no binding was observed to the core promoters of either *GATA3* or *RORc* (encoding RORgt) Interestingly, these authors also identified WASP binding to exons 9 and 12 of *FOXP3*, although the significance of this remains to be determined (Taylor et al., 2010).

5 *TCR repertoire variation.* Similar to recent observations in B-lymphocytes, WAS patients show alterations in TCR repertoire compared to age-matched healthy subjects (O'Connell et al., 2014; Wada et al., 2005; Wu et al., 2015). Multiple mechanisms may contribute to this, including the accumulation of expanded T cell clones with specificity for self or persistent infectious agents - or for that matter, allergens – as well as abnormal thymic generation of a diversified naïve T cell pool (Cotta-de-Almeida et al., 2015). Analysis of the TCR-repertoire in *Was*$^{-/-}$ mice could help differentiate these two non-mutually exclusive origins of TCR-skewing, but in contrast to B cells (Kolhatkar et al., 2015), this has not yet been published.

As we argued in **Chapter 7**, it is likely that the loss of WASP in non-regulatory T cells contributes to the differential disease activity observed between *Was*$^{-/-}$ and *Was*$^{fl/fl}$*Foxp3*-Cre mice. Following *ex vivo* stimulation, we found significantly lower IL-4 production by CD4^{+} T cells isolated from MLNs of *Was*$^{-/-}$ mice when compared to *Was*$^{fl/fl}$*Foxp3*-Cre animals (**Figure 7.6F, page 167**). Since we also observed both a relative and absolute decrease in GATA3^{+} T cells (**Figure 7.6D, page 167** and **Figure 7.S4D, page 178**), this reduced IL-4 production

appears to result from diminished Th2 differentiation rather than secretory defects in individual Th2 cells. T cell activation defects due to aberrant formation of immune synapses with food-antigen presenting mononuclear phagocytes provide an additional explanation for a dampened food allergic response in *Was*$^{-/-}$ mice. Lastly, alterations in the TCR repertoire may affect the availability of food antigen-specific T cells in the same way as we hypothesized to occur for B cells, which could reduce the likelihood of antigen-specific proliferation upon encountering food antigens.

9.6 Regulatory T cells

Throughout this chapter, we have considered the WASP-deficient FOXP3^{+} Treg compartment as the one constant determinant between *Was*$^{fl/fl}$*Foxp3*-Cre and *Was*$^{-/-}$ mice. However, we must also consider differences in FOXP3^{+} Tregs themselves as potentially contributing to disparate degrees of food allergy in *Was*$^{fl/fl}$*Foxp3*-Cre and *Was*$^{-/-}$ mice. In **Chapter 7** we found equivalent or even increased absolute and relative fractions of FOXP3^{+} Tregs in *Was*$^{fl/fl}$*Foxp3*-Cre compared to WT animals, whereas Tregs were significantly reduced in *Was*$^{-/-}$ mice (**Figure 7.6A, page 167**). In addition, earlier in this chapter we observed that *Was*$^{-/-}$ but not *Was*$^{fl/fl}$*Foxp3*-Cre Tregs expressed increased levels of the T_{FH} markers CXCR5 and PD-1 (**Figure 9.4E**). Although we identified that Th2 skewing in WASP-deficient Tregs occurred to the same extent in mice of either genotype (**Figure 7.7A, page 169**), it is possible that differences in Treg numbers or phenotype contribute to differential food allergic disease.

Our currently available data are insufficient to explain alterations in the FOXP3^{+} Treg compartment between *Was*$^{-/-}$ and *Was*$^{fl/fl}$*Foxp3*-Cre mice. However, based on the foregoing discussion of WASP deficiency in other hematopoietic lineages, we can envision a number of potential mechanisms:

1 *TCR-signal-mediated changes in Treg development and repertoire*. As discussed in **Chapter 6**, TCR signaling is a critical determinant during T cell development in the thymus. Rather than being deleted through negative selection, T cells that express a TCR with low-affinity for self-antigens will undergo epigenetic changes associated with a regulatory phenotype, including the expression of FOXP3 (Li and Zheng, 2015; Ohkura et al., 2013). Very recently, a role for the conserved region CNS3 of the FOXP3 gene has been identified as an epigenetic switch for Treg commitment in response to suboptimal TCR signals, with CNS3-deficient Tregs showing a skewed TCR repertoire that has higher affinity for self peptides (Feng et al., 2015). Given the abovementioned role of WASP in integrating TCR signals, it can be anticipated that the timing of loss of WASP (i.e. before or after Treg lineage commitment and Foxp3

transcription) will have marked effects on the specificity and regulatory function of Tregs that leave the thymus.

2 *Altered Treg maintenance or induction by Was$^{-/-}$ dendritic cells.* Earlier in this chapter, we discussed the potential contribution of *Was*$^{-/-}$ mononuclear phagocytes on spontaneously occurring food allergy in the context of WASP deficiency. BrdU and Annexin staining following transfer of WT Tregs into *Rag2*$^{-/-}$ or *Was*$^{-/-}$*Rag2*$^{-/-}$ mice revealed equivalent rates of apoptosis, but reduced proliferation of FOXP3^{+} T cells, indicating aberrant Treg maintenance by *Was*$^{-/-}$ innate immune cells (Nguyen et al., 2012). *Was*$^{-/-}$*Rag2*$^{-/-}$ mice showed reduced numbers of tolerogenic, CD11c^{+}CD11b^{+}CD103^{+} dendritic cells (Nguyen et al., 2012), which form the subset of intestinal APCs that migrate to the MLNs to induce Tregs. In this process, the vitamin A metabolite *retinoic acid* has been identified as a critical cofactor that acts together with TGF-β and IL-2 to promote Treg differentiation from naïve T cells at mucosal sites (Coombes et al., 2007; Josefowicz et al., 2012a; Zheng et al., 2010). As depicted in the heatmap data in **Figure 7.5A (page 165)**, we observed striking differences between genotypes in the expression of the vitamin A metabolizing enzymes RALDH1 (*Aldh1a1)* and RALDH2 *(Aldh1a2)* (**Figure 9.5A**). Although expression of *Aldh1a2* is restricted to tolerogenic CD103^{+} dendritic cells (Hall et al., 2011b), we found no differences in transcription of *Itgax* (CD11c) or *Itgae* (CD103) in intestinal tissue, which suggests an equal abundance of CD103 positive dendritic cells in the lamina propria of WT, *Was*$^{-/-}$ and *Was*$^{fl/fl}$*Foxp3*-Cre mice (**Figure 9.5B**). Expression of *Aldh1a1* and *Aldh1a2* was even more reduced in *Was*$^{fl/fl}$*Foxp3*-Cre than *Was*$^{-/-}$ mice, demonstrating that WASP deficiency in dendritic cells is dispensable for downregulation of these enzymes. Given that *Aldh1a1* and *Aldh1a2* associated with mast cell markers in our hierarchical cluster analysis (**Figure 7.5A, page 165**), we asked whether their reduced expression was a consequence of Th2-mediated inflammation. By comparing *Was*$^{-/-}$ mice with WT animals that were sensitized with OVA/alum, we observed that despite equivalent degrees of type 2 inflammation as assessed by expression of *Mcpt1* (**Figure 8.3D, page 193**), lower levels of *Aldh1a1* expression were only seen in *Was*$^{-/-}$ mice (**Figure 9.5C**). Moreover, in WASP-deficient mice, a negative correlation between expression of *Mcpt1* and *Aldh1a1* was observed, whereas in food allergic WT mice this correlation was inverted (**Figure 9.5D**). Combined, these results identify the downregulation of RALDH enzymes in tolerogenic dendritic cells as a feature of *Was*$^{-/-}$ FOXP3^{+} Tregs rather than of WASP deficiency in dendritic cells themselves. Reduced expression of *Aldh1a1* and *Aldh1a2* was not seen in *Foxp3*^{sf} mice (**Figure 9.1F, page 205**), which illustrates that downregulation of RALDH enzymes in *Was*$^{-/-}$ and *Was*$^{fl/fl}$*Foxp3*-Cre mice does not result from a functional deficiency of Tregs. In addition, it should be noted that retinoic acid is required for

normal Ca^{2+} signaling and NFAT translocation following TCR-mediated activation of T cells (Hall et al., 2011b; 2011a). As discussed in the previous section, both these mechanisms are already defective *Was*$^{-/-}$ T cells, thus adding an additional layer of complexity to the interplay between retinoic acid metabolism and failing immune homeostasis. The role of RALDH-positive dendritic cells in WAS-associated allergy deserves further study.

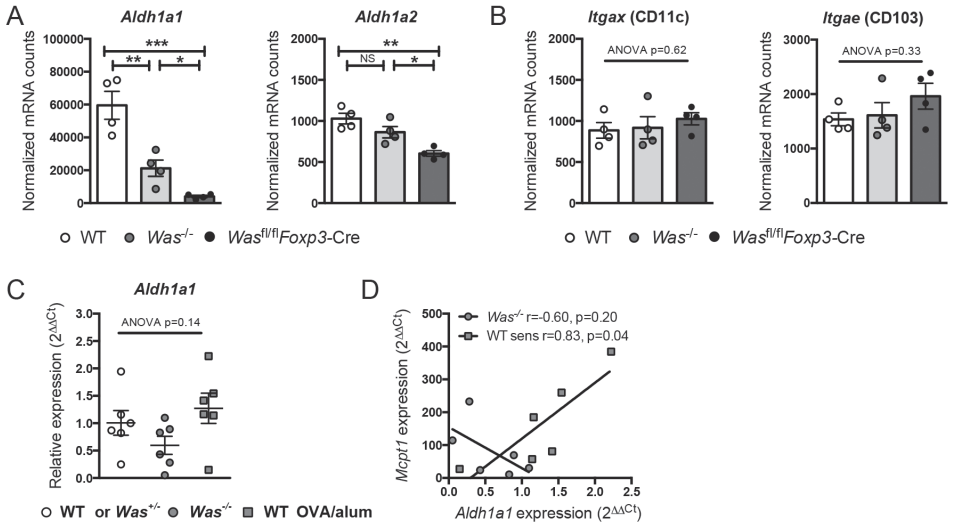


Figure 9.5 ***WASP deficiency in Foxp3*$^{+}$ *Tregs is associated with reduced mRNA expression of RALDH enzymes.*** (**A**) mRNA expression levels of *Aldh1a1* and *Aldh1a2* in jejunum of WT (open circles), *Was*$^{-/-}$ (gray circles) or *Was*$^{fl/fl}$*Foxp3*-Cre (black circles) C57BL/6 mice. (B) mRNA expression levels of *Itgax* and *Itgae* in jejunum of WT, *Was*$^{-/-}$ or *Was*$^{fl/fl}$*Foxp3*-Cre mice. (**C**) *Aldh1a1* mRNA expression in jejunum of OVA/alum sensitized (gray squares) WT mice and OVA-gavaged *Was*$^{-/-}$ (gray circles) or WT/ *Was*$^{+/-}$ littermates (open circles) on the BALB/c background. (**D**) Differential Pearson correlation between intestinal expression of *Aldh1a1* and *Mcpt1* in OVA-gavaged *Was*$^{-/-}$ (gray circles) and OVA/alum sensitized WT BALB/c mice (gray squares). Dots represent individual mice and error bars depict SEM. * p<0.05; ** p<0.01; ***p<0.001; NS: not significant as determined by one-way ANOVA with Tukey's multiple comparisons test.

3 *Altered induction from microbes.* Over the last few years, the composition of the microflora has been increasingly recognized as a critical determinant of Treg repertoire and numbers (Atarashi et al., 2013; Geuking et al., 2011; Lathrop et al., 2011). Consequently, any differences in microbial colonization between *Was*$^{-/-}$ and *Was*$^{fl/fl}$*Foxp3*-Cre mice can potentially result in differences peripheral Treg induction. Similarly, RALDH levels are lower in germ-free or MyD88-deficient mice (Hall et al., 2011b), suggesting that differential expression of *Aldh1a1* and *Aldh1a2* could be the result of alterations in the microflora. The composition of the microflora will be discussed in a later section in this chapter.

4 *Reduced induction from regulatory B cells*. As previously discussed in this chapter, regulatory B cells are a recently identified subset of IL-10 producing cells capable of recruiting Tregs. Considering that Bregs are decreased in *Was*$^{-/-}$ mice and that this reduction is due to a B cell-intrinsic defect (Bouma et al., 2014), it is likely that Breg cells occur in normal frequencies in *Was*$^{fl/fl}$*Foxp3*-Cre mice and may thus contribute to increased Treg numbers in these mice.

5 *Alterations in baseline IL-2 levels.* As discussed in the previous section, the failure to maintain immune synapse stability (Sims et al., 2007) and aberrant NFAT signaling following TCR-mediated activation (Cianferoni et al., 2005) are associated with reduced IL-2 production from WASP-deficient T cells (**Figure 7.6B, page 167**). IL-2 is critical for the formation of Tregs, both during thymic development as well as for their peripheral induction (Josefowicz et al., 2012a; Li and Zheng, 2015). As described in **Chapter 7**, the relative IL-2 deficiency in *Was*$^{-/-}$ mice has been hypothesized to contribute to Treg dysfunction, because exogenous IL-2 increased suppressive function of WASP-deficient Tregs in vitro (Humblet-Baron et al., 2007; Maillard et al., 2007). Although we have clearly demonstrated that a correction of defective IL-2 production in *Was*$^{fl/fl}$*Foxp3*-Cre mice (**Figure 7.6B, page 167)** is insufficient to resolve immune dysregulation *in vivo* (**Chapter 7**)*,* it is conceivable that lower levels of IL-2 in *Was*$^{-/-}$ animals result in reduced thymic and peripheral Treg differentiation. Indeed, STAT5 overexpression is associated with increased numbers of FOXP3^{+} Tregs (Burchill et al., 2008). Along the same line of argumentation, and as also discussed in the B cell paragraph of this chapter, it is becoming clear that STAT5-mediated IL-2 signaling through IL-2Rα (CD25) negatively affects Tfh differentiation through inhibition of *Bcl6* transcription (Ballesteros-Tato et al., 2012; Johnston et al., 2012; Nurieva et al., 2012). Reduced IL-2 levels in *Was*$^{-/-}$ but not *Was*$^{fl/fl}$*Foxp3*-Cre mice could thus be envisioned to skew the differentiation of T cells away from Treg and Th1 and towards T_{FH} in *Was*$^{-/-}$, which would explain both the increased fractions of Tfh as well as reduced Tregs in these animals compared to *Was*$^{fl/fl}$*Foxp3*-Cre mice. Similarly, differential IL-2 levels could be expected to cause an upregulation of Tfh markers CXCR5 and PD-1 in WASP-deficient Tregs in *Was*$^{-/-}$ but not *Was*$^{fl/fl}$*Foxp3*-Cre mice (**Figure 9.4E, page 218**). Combined, our results thus support a model in which WASP-deficient Tregs are affected in number and phenotype by differential levels of extrinsic IL-2 *in vivo*. Adding further significance to differential baseline levels of IL-2, Hondowicz et al. recently showed that IL-2 is required for the formation of allergen-specific Th2 cells in lung tissue (Hondowicz et al., 2015). Since these cells were recruited from the CXCR5^{-} population of T cells, the increased fraction of CXCR5^{+} T cells as well as lower concentrations of IL-2 could both contribute to dampened allergic disease in *Was*$^{-/-}$ compared to *Was*$^{fl/fl}$*Foxp3*-Cre mice.

Although it is likely that a combination of these proposed mechanisms underlie differences in the Treg compartment between *Was*$^{-/-}$ and *Was*$^{fl/fl}$*Foxp3*-Cre mice, it is clear that none of these are causative of food allergic sensitization. In fact, one important conclusion from the work presented in **Chapter 7** is that immune dysregulation occurs in *Was*$^{fl/fl}$*Foxp3*-Cre mice despite normal Treg numbers (**Figure 7.6A, page 167**), activation status (CD44^{hi}CD62L^{lo}, **Figure 7.6D, page 167**) and site of induction (Neuropilin-1, **Figure 7.S4B, page 178**). Furthermore, WT levels of immunoregulatory proteins CTLA4, GITR, and CD103 expression have been previously observed on the surface of *Was*$^{-/-}$ Tregs (Maillard et al., 2007; Marangoni et al., 2007). The only difference that we observed between WT and *Was*$^{-/-}$ Tregs – obtained from either *Was*$^{-/-}$ or *Was*$^{fl/fl}$*Foxp3*-Cre mice – were the increased levels of Th2 transcription factor GATA3 in Tregs with an effector-like, CD44^{hi}CD62L^{lo}, phenotype. GATA3^{+} Tregs were recently found to promote food allergy in both humans and mice (Noval Rivas et al., 2015). However, the mechanism of how WASP deficiency results in enhanced expression of GATA3 remains undefined. In the discussion of **Chapter 7**, we have started speculating on this question, and in the following section, that argument will be expanded.

Immune dysfunction in WAS patients constitutes features of both immunodeficiency (i.e. increased susceptibility to opportunistic infections and malignancies) and immune dysregulation (i.e. increased autoimmunity and allergy). Intuitively, this amounts to a paradoxical condition, because it implies simultaneous hypo- and hyper-activation of the immune system. Yet the combination of immunodeficiency and immune dysregulation can also be found in a wide variety of primary T cell immunodeficiencies other than WAS (Notarangelo, 2013), suggesting that both features can be consequences of a single pathogenic mechanism. This notion is supported by genetic mouse models of T cell-mediated immunodeficiencies, where a range of different mutations that affect T cell effector function concurrently result in autoimmunity and dysregulated IgE and IgG1 responses (see for example Table 2 in (Liston et al., 2008)). A recurring feature amongst many of the genetic defects in these different mouse models is that they directly affect TCR signaling. For example, as mentioned in **Chapter 7**, mutations in the TCR-associated scaffold protein LAT result in reduced Ca^{2+} signaling and IL-2 production upon TCR ligation of isolated T cells *in vitro*, but also lead to profound Th2-skewed lymphoproliferation and hyper IgE responses in these animals *in vivo* (Aguado et al., 2002; Sommers et al., 2002). Similarly, mice with compound heterozygous mutations in the TCR-dependent tyrosine kinase ZAP70 demonstrate reduced Ca^{2+} signaling and aberrant immune responses to *Bordetella pertussis*, while also developing elevated serum IgE/IgG1 and autoantibodies (Siggs et al., 2007). These mouse models show that mutations that interfere with TCR signaling can result in the same combination of immunodeficiency and Th2-type immune dysregulation that is observed in WASP-deficient mice.

How to link aberrant TCR signals to the development of Th2 effector responses? It is becoming clear that the strength of the TCR:pMHC interaction is a determinant for subsequent

Th differentiation (Datta and Milner, 2011). This concept was founded on the observations from the laboratories of Kim Bottomly and Anne O'Garra that Th2 differentiation results from low-affinity TCR stimulation, whereas higher affinity interactions are more likely to give rise to Th1 cells (Datta and Milner, 2011; Hosken et al., 1995; Leitenberg et al., 1998). Recently, it was corroborated further using intra-vital microscopy that allowed for quantification of T cell–dendritic cell interaction times and synapse size, which were shown to exert greater influence on the outcome of T cells than presence of Th1 or Th2 skewing cytokines or adjuvants (van Panhuys et al., 2014). Furthermore, weak TCR signaling is also hypothesized to underlie early cell-fate decisions that differentiate between a Th1 and Tfh response (Tubo and Jenkins, 2014). In this scheme, shorter p:MHCII dwell times (i.e. lower signal quality) specifically promote Tfh over Th1 differentiation via preferential expression of Bcl-6 instead of Blimp-1 (Tubo and Jenkins, 2014; Tubo et al., 2013). An attractive feature of this theory is that it helps explain the Th2 skew that is observed in immunodeficiencies characterized as 'leaky SCID', of which the Omenn syndrome is a well-known example. Like SCID, Omenn syndrome can be caused by mutations in the RAG enzymes that mediate VDJ recombination in developing T cells. In strong contrast to the former, T cell development is not completely abrogated (hence the leakiness). However, the T cells that do form in Omenn syndrome patients display a severely restricted TCR repertoire. The lack of high-affinity interactions with pMHCII molecules on APCs causes a selective Th2 skew (Milner et al., 2010). In mice, a restricted TCR repertoire results in eosinophilia and Th2-type inflammation (Milner et al., 2007), and Omenn patients have elevated IgE and increased levels of serum IL-4 (Ozcan et al., 2008).

Given the involvement of WASP in TCR-mediated signaling and T cell activation (Matalon et al., 2013) and our results that show increased Th2 and Tfh differentiation in *Was*$^{-/-}$ mice (**Chapter 7** and **Figure 9.4**), it is tempting to speculate that absence of WASP from T cells affects TCR:pMHCII interactions in a way that promotes Th2 and Tfh development over Th1 differentiation. Indeed, aberrant TCR signaling in itself has been hypothesized to underlie the immune dysregulation, Th2 skewing and atopic features observed in WAS patients (Datta and Milner, 2011). Furthermore, the recently demonstrated skewing of the TCR repertoire in patients with WAS (O'Connell et al., 2014; Wada et al., 2005; Wu et al., 2015) can be envisioned to contribute further to a reduced quality of TCR:pMHCII interactions via an Omenn-like mechanism. However, by identifying increased allergic disease in *Was*$^{fl/fl}$*Foxp3*-Cre mice compared to *Was*$^{-/-}$ animals, we have shown that WAS deficiency in naïve effector T cells is dispensable for aberrant Th2 skewing. Atopy in the context of WAS is not due to aberrant priming of T cells, but results instead from a selective loss of Th2 suppressive capability in WASP-deficient Tregs. Nevertheless, it is very well possible that the abovementioned mechanisms that link aberrant TCR:pMHCII interactions to a Th2-like differentiation are also operative in WASP-deficient FOXP3^{+} T cells to give rise to a Th2-skewed population of Tregs which preferentially co-express GATA3.

In addition to the here suggested effect on transcription factor expression, emerging evidence demonstrates that TCR-signaling is also required for normal Treg function. In model systems that allow for the abrogation of TCR-expression after T cells have started expressing FOXP3 (and thus have committed to the Treg lineage), continuous TCR activation was found indispensable for the prevention of autoimmunity (Levine et al., 2014; Vahl et al., 2014). Very recently, the role of the TCR on Tregs was illuminated further by intravital microscopy studies, which identified that *in vivo*, pSTAT5^{+} Tregs cluster together with IL-2 producing self-reactive CD4^{+} T cells. Deletion of the TCR on Tregs abrogated their migration to these self-reactive T cells, which was associated with rapid-onset autoimmunity (Liu et al., 2015). These results strongly suggest that antigen-specific activation of Tregs via their TCR serves as an important chemotaxis signal, and misinterpretation of this signal in the absence of WASP may be what underlies lymphoproliferation and allergic responses in *Was*$^{-/-}$ and *Was*$^{fl/fl}$*Foxp3*-Cre mice. It is furthermore conceivable that a failure of WASP-deficient Tregs to migrate in response to TCR-mediated signals is exacerbated further by the negative effect of loss of WASP on cell motility. Intravital studies in *Was*$^{fl/fl}$*Foxp3*-Cre mice would be required to further test this hypothesis, and to delineate the mechanism that renders WASP-deficient Tregs pathogenic.

9.7 IgE Effector cells

Loss of WASP-dependent functions from IgE effector cells would be the most straightforward explanation for the alleviated food allergic phenotype observed in *Was*$^{-/-}$ mice when compared to *Was*$^{fl/fl}$*Foxp3*-Cre animals. If WASP-deficiency cripples the acute hypersensitivity response, this would translate directly in a reduction of histamine-mediated allergic symptoms. In **Chapter 7**, we described that anaphylaxis to food antigens had never occurred in a cohort of 25 individuals with mutations in *WAS*, and showed that these patients have reduced IgE-dependent and IgE-independent cutaneous mast cell responses (**Figure 7.1D-E, page 159**). This was in line with murine data, because mice deficient in WASP (Pivniouk et al., 2003) or WASP interacting protein (Kettner et al., 2003) were found to be protected against IgE-mediated anaphylaxis following passive immunization with hapten-specific IgE. However, in **Chapter 8** we demonstrated that *Was*$^{-/-}$ mice on the BALB/c background were susceptible to allergic diarrhea (**Figure 8.3B, page 193**) and systemic anaphylaxis (**Figure 8.3E-F**) following OVA challenge. These immune responses were mediated at least in part by IgE, as oral challenge with OVA resulted in the rapid increase in serum MCPT1, which is a selective biomarker of IgE-dependent degranulation of mucosal mast cells ((Khodoun et al., 2011) and **Chapter 8**). How to reconcile these discrepant findings? First of all, the study by Pivniouk and colleagues used *Was*$^{-/-}$ mice on a C57BL/6-129SvEv mixed genetic background, but 129SvEv as WT controls. As delineated in detail in Chapter 7, C57BL/6 are notoriously resistant to Th2-mediated immunity, including allergic responses, and incomplete back-

crossing may therefore have contributed to the observed differential susceptibility to anaphylaxis. A second, not mutually exclusive explanation could be that the elevated levels of endogenous IgE in *Was*$^{-/-}$ mice block FcεRI binding sites for exogenous NP-specific IgE, resulting in reduced mast cell sensitization before antigen challenge the next day. Following our protocol of repeated oral sensitizations (**Figure 8.2B, page 191**), OVA-specific IgE becomes part of the endogenous IgE pool and has sufficient time to saturate the surface IgE receptors of mast cells. Using i*n vitro* culture of bone-marrow derived mast cells from *Was*$^{-/-}$ and WT mice of both the BALB/c and C57BL/6 genetic background, we further confirmed that IgE-mediated degranulation was almost completely intact in the absence of WASP (**Figure 8.4B, page 195**). Despite normal acute degranulation, *Was*$^{-/-}$ mast cells significantly differed from WT counterparts in their ability to release inflammatory cytokines in response to IgE-mediated signals (**Figure 8.4D)**, demonstrating that WASP-deficiency does alter mast cell function. In addition to signaling events downstream of IgE:FcεRI, WASP is also implicated in the response to ligation of KIT with stem cell factor (Mani et al., 2009), which explains why WASP-competent mast cells show a selective advantage in culture over *Was*$^{-/-}$ counterparts and may affect homeostasis of intestinal mast cells after initial expansion.

It is tempting to ascribe the reduced allergic inflammation in *Was*$^{-/-}$ mice to altered IgE-mediated cytokine production from WASP-deficient mast cells, but it is currently not clear to which extent mast-cell-derived cytokines contribute to intestinal allergy. We showed in **Chapter 8** that the expansion of mucosal mast cells in WASP-deficient mice occurs independently of IgE:FcεRI signaling, because *Was*$^{-/-}$*Fcer1a*$^{-/-}$ and *Was*$^{-/-}$*Fcer1a*$^{+/-}$ littermates were equally susceptible to OVA sensitization and mucosal mast cell expansion (**Figure 8.5, page 196**). This is in line with results from experiments in IgE-deficient mice, which show reduced, but nevertheless still pronounced expansion of mucosal mast cells and elevated serum MCPT1 following infection with *T. Spiralis*. (Gurish et al., 2004). In contrast, the absence of T cells in *nu/nu* mice completely abrogates mucosal mast cell expansion in *T. Spiralis*-infected mice (Ruitenberg and Elgersma, 1976), suggesting that T-cell-derived mediators are likely the most critical determinant of mucosal mast cell expansion (Burton et al., 2012; Chu et al., 2014; Reber et al., 2012). Recently it was shown that mast-cell derived IL-9 acts to significantly promote mucosal (MCPT1^{+}) mast cells expansion in response to IL-33 (Chen et al., 2015; Forbes et al., 2008). *Il9* transcripts were below the level of detection in *Was*$^{-/-}$ as well as WT-derived cultured mast cells, irrespective of IgE-mediated stimulation (results not shown). Although this likely reflects the failure of these cultured cells to adopt a mucosal phenotype *in vitro*, the fact that we did not observe considerable increases in the abundance of *Il9* or *Il33* mRNA in jejunal sections of *Was*$^{-/-}$ and *Was*$^{fl/fl}$*Foxp3*-Cre, and that these genes furthermore did not associate with mast cell markers in our hierarchical cluster analysis (**Figure 7.5A, page 165**) argues against significant involvement of mast-cell-derived IL-9 in mucosal mast cell expansion in the context of WASP-deficiency. Conversely, we found that mast cell expansion could be completely abrogated by the absence of lymphocytes in

Was$^{-/-}$*Rag2*$^{-/-}$, or through deletion of IL-4 in *Was*$^{-/-}$*Il4*$^{-/-}$ mice (**Figure 7.S3A, page 177** and **Figure 7.6G, page 167** respectively). Reduced IL-4 production from WASP-deficient T cells (**Figure 7.6F**) may therefore be quantitatively more important on limiting mast cell expansion than mast-cell intrinsic defects in cytokine production.

Basophils comprise the other arm of IgE-mediated effector responses. Given their low abundance in blood in combination with a mast-cell-like phenotype, basophils have long been considered a more or less redundant immune population (Karasuyama et al., 2011). Not surprisingly, data regarding the role of WASP in basophil function are limited to one study on one human WAS patient whose basophils showed normal histamine release following IgE-dependent (anti-IgE) and IgE-independent (calcium ionophore A23187) stimulation (Marone et al., 1986). Scientific interest in basophils has greatly increased in recent years and with the availability of new genetic tools, the role of basophils in relation to other immune populations and IgE-mediated diseases is now becoming more crystallized (Karasuyama et al., 2011; 2009; Siracusa et al., 2013; Voehringer, 2013). Although basophils express FcεRI, several reports indicate that basophils are completely dispensable for acute, IgE-mediated hypersensitivity responses, which are mediated by mast cells (Sawaguchi et al., 2012; Tsujimura et al., 2008). In contrast, basophils were shown to contribute critically to IgG-mediated anaphylaxis and basophil depletion before challenge rescued mice from death (Tsujimura et al., 2008). Furthermore, in an IgE-dependent fashion, basophils contribute to chronic, late phase allergic inflammation independent from involvement of mast cells and T cells (Mukai et al., 2005). Data from *Mcpt8*-Cre mice allowing constitutive deletion of basophils confirmed a reduced severity of chronic allergic responses and recall responses following secondary challenge with *Nippostrongylus brasiliensis*, but found that basophils are dispensable during a primary parasite infection and for the induction of IgE and IgG1 responses against allergens (Ohnmacht et al., 2010). Work on the role of basophils in intestinal immunity and food allergy has come from a number of papers from the group of David Artis, who identified that epithelial cell-derived TSLP promotes a subpopulation of IL-3 independent basophils particularly capable of supporting Th2 cells during allergic responses (Siracusa et al., 2011). Translation of this finding to human allergic disease was provided by patients with EoE, in whom polymorphisms in the *TSLP* are associated with increased risk (Noti et al., 2013; Rothenberg et al., 2010) (and see **Chapters 1, 2, 3 and 5**). Surprisingly, TSLP was dispensable for mediating the increased basophil numbers that were observed in mice treated with antibiotics. Rather, basophil expansion appeared to be regulated directly by IgE, because no increase in basophils was seen following antibiotic treatment of *Rag*$^{-/-}$, *Il4*$^{-/-}$ or *Igh7*$^{-/-}$ (IgE-deficient) mice and exogenous IgE expanded basophils in *Rag*$^{-/-}$ hosts (Hill et al., 2012). However, a role for TSLP was identified in a model of food allergic sensitization initiated via allergen exposure to atopic dermatitis-like skin lesions. In this model, TSLP-expanded basophils contributed to mast cell expansion in the intestine, as evidenced by reduced *Mcpt1*

and *Mcpt2* expression in *Tslpr*$^{-/-}$ mice or after DTR-mediated basophil depletion, although mRNA expression of basophil-specific *Mcpt8* was not assessed (Noti et al., 2014). In **Chapter 5** we have seen that this mechanism is also operative in murine EoE following epicutaneous sensitization. Combined these results suggest that basophils contribute to intestinal allergic responses, and the consequences of WASP-deficiency in these cells therefore deserve further study.

Blood basophils (gated as CD45^{mid}CD49b^{+}, see **Figure 8.5A, page 196**) made up a significantly greater fraction of peripheral blood mononuclear cells (PBMCs) in *Was*$^{-/-}$ than in WT or *Was*$^{fl/fl}$*Foxp3*-Cre animals (**Figure 9.6A**). In line with our earlier results (**Figure 7.S1B, page 175**), significantly different levels of serum IgE between these groups (**Figure 7.4C, page 164**) were reflected in the degree of IgE loading on the basophil cell surface, which was strongest in *Was*$^{fl/fl}$*Foxp3*-Cre mice (**Figure 9.6B**). To assess whether the increased proportion of blood basophils in *Was*$^{-/-}$ animals resulted from a basophil-intrinsic effect of WASP deficiency, we determined absolute basophil numbers in blood of *Was*$^{-/-}$*Rag2*$^{-/-}$ and *Rag2*$^{-/-}$ mice. Since RAG2 deficiency extends to both T and B cells, no IgE loading was detected on the surface of basophils (**Figure 9.6C**). In the absence of adaptive immunity, WASP-deficient basophils were reduced compared to WT basophils (**Figure 9.6D**), suggesting that loss of WASP is associated with reduced rather than increased basophil survival. Comparison of *Was*$^{-/-}$*Rag2*$^{-/-}$ and *Rag2*$^{-/-}$ mice also allowed for analysis of *in vivo* expression levels of FcεRI in the absence of confounding influence of different levels of IgE. Here we observed a slight reduction in FcεRI expression on the surface of WASP-deficient basophils **Figure 9.6E**), although the biological relevance of this difference is unlikely to be large.

Based on abovementioned data on the regulation of basophils by IgE (Hill et al., 2012), we could have expected that uncontrolled Th2 responses in *Was*$^{fl/fl}$*Foxp3*-Cre mice would be associated with a larger effect on blood basophil numbers. However, study of *Was*$^{-/-}$*Il4*$^{-/-}$ animals further confirmed that increased serum IgE and Th2-mediated intestinal inflammation in WASP deficiency did not affect total basophil numbers (**Figure 9.6F**). However, these results do not rule out the possibility that loss of WASP-dependent functions from basophils contributes to differential intestinal inflammation between *Was*$^{-/-}$ and *Was*$^{fl/fl}$*Foxp3*-Cre mice. In the gene expression data presented in **Figure 7.5A (page 165)**, we observed increased expression of basophil-specific *Mcpt8* in jejunum from *Was*$^{fl/fl}$*Foxp3*-Cre, but not *Was*$^{-/-}$ mice (**Figure 9.6G**), which implicates that WASP-competent, but not WASP-deficient basophils contribute to the immune infiltrate in allergic intestinal inflammation. We therefore assessed basophil responses following antigen challenge in WT and *Was*$^{-/-}$ hosts. In OVA/alum-sensitized BALB/c animals (following the sensitization scheme depicted in **Figure 8.2A, page 191**), OVA challenge by gavage is associated with the rapid disappearance of basophils from the systemic circulation, followed by almost complete repletion in the following 24 hours (**Figure 9.6H**). The kinetics of this response are apparently similar to the increase in serum MCPT1 that signifies the IgE-mediated activation of mucosal mast cells (**Figure 9.6H**).

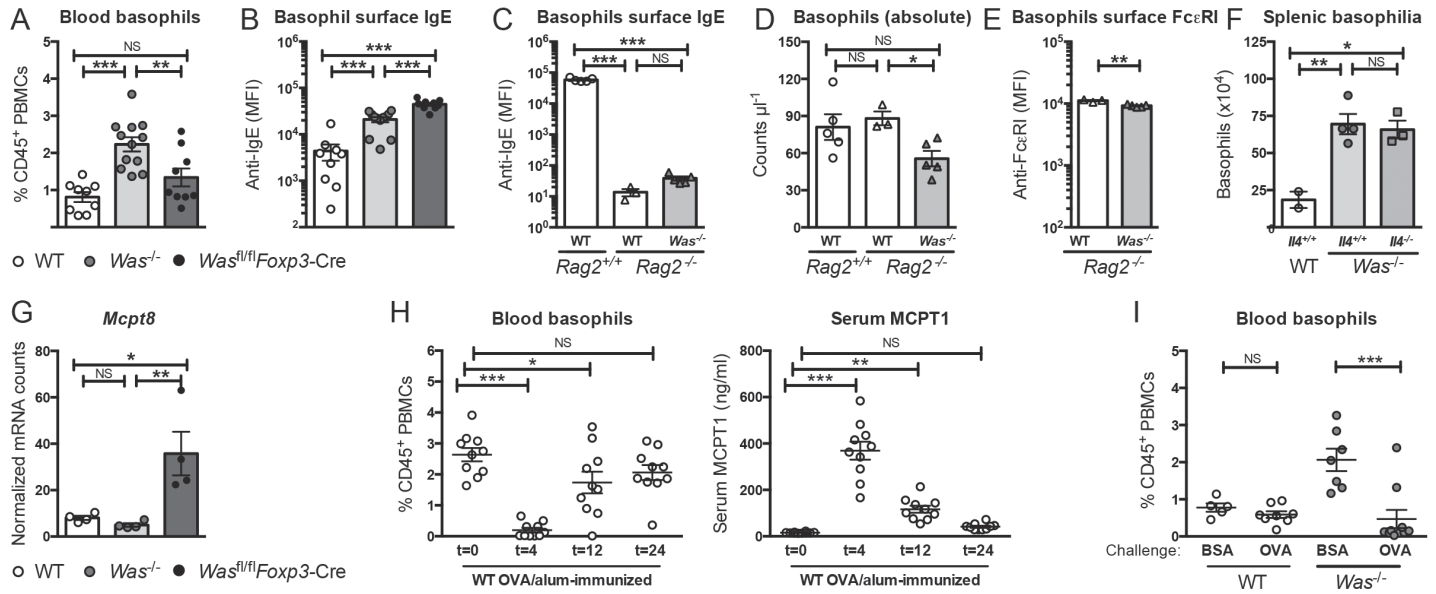


Figure 9.6 ***Was*$^{-/-}$ *and Was*$^{fl/fl}$*Foxp3-Cre mice demonstrate differential involvement of basophils.*** (**A**) Quantification of basophils (defined as CD45^{mid}CD49b^{+}) in blood of WT (open circles), *Was*$^{-/-}$ (gray circles) or *Was*$^{fl/fl}$*Foxp3*-Cre (black circles) C57BL/6 mice. (**B**) Levels of surface IgE-loading expressed as mean fluorescence index (MFI) on flow cytometry. (**C-E**) Analysis of the basophils in *Rag2*$^{-/-}$ and *Was*$^{-/-}$*Rag2*$^{-/-}$ animals on the 129SvEv background. (**F**) Total number of spleen basophils in *Was*$^{-/-}$*Il4*$^{-/-}$ and *Was*$^{-/-}$ or WT mice. (**G**) mRNA expression level of basophil marker *Mcpt8* in jejunum of WT, *Was*$^{-/-}$ or *Was*$^{fl/fl}$*Foxp3*-Cre mice. (**H**) Kinetics of basophil and serum MCPT1 responses following oral OVA challenge in OVA/alum-sensitized mice. (**I**) Blood basophil levels in WT and *Was*$^{-/-}$ mice following a protocol of adjuvant-free oral sensitization and challenged with OVA or irrelevant antigen BSA. Dots represent individual mice and error bars depict SEM. * p<0.05; ** p<0.01; ***p<0.001; NS: not significant as determined by one-way ANOVA with Tukey's multiple comparisons test or t-test (E and I).

Using *Was*$^{-/-}$ mice that were sensitized to OVA using the adjuvant-free protocol depicted in **Figure 8.2B (page 191)**, we observed that antigen-specific basophil responses were intact in the absence of WASP (**Figure 9.6I**). In combination with our data in **Chapter 8** that in the absence of WASP, IgG-mediated anaphylaxis, which has been described to be basophil-dependent (Tsujimura et al., 2008) is intact (**Figure 8.5F, page 196**), these results indicate that allergen-specific activation of basophils occurs relatively unperturbed in *Was*$^{-/-}$ hosts. However, aberrant migration of granulocytes has been reported in WASP deficiency (Snapper et al., 2005), and reduced migration of WASP-deficient basophils to sites of allergic inflammation could therefore provide a potential explanation for absence of *Mcpt8* mRNA expression that is not ruled out by our current set of data.

Mast cells and basophils are increasingly recognized as important regulators of inflammatory responses rather than mere IgE effector cells in the context of allergic responses and anti-parasite immunity (Galli and Tsai, 2012; Karasuyama et al., 2011; Voehringer, 2013). Further research will likely lead to identification of novel regulatory functions. If some of these functions are found to be dependent on WASP, then additional roles for IgE effector cells in modifying disease severity in WASP-deficient mice may be uncovered. Combined deletion of WASP from Tregs and mast cells or basophils could provide a first step towards identification of such roles.

9.8 Microflora

Research has begun to illuminate the many ways in which commensal microbes shape intestinal immune responses (Belkaid and Hand, 2014; Honda and Littman, 2012; Hooper et al., 2012). Each of these interactions bear the potential to become confounding variables in experiments that do not account for baseline differences in microbial populations (Eberl, 2015; Macpherson and McCoy, 2015; Moon et al., 2015; Ivanov et al., 2009). Colonization status therefore deserves to be considered as a factor that may contribute to differential disease between *Was*$^{-/-}$ and *Was*$^{fl/fl}$*Foxp3*-Cre mice.

The study of the microbiome in the context of primary immunodeficiencies is still in its infancy. For WAS patients, the only currently available data pertain to bacteria and fungi on the skin, which were studied in relation to patients with atopic dermatitis as well as two other PIDs associated with eczematous skin disease: autosomal dominant hyper IgE syndrome and DOCK8 deficiency (Oh et al., 2013). In all three immunodeficiencies, a reduction in diversity of the cutaneous microbiome was observed, but this could have been a function of eczematous inflammation, as diversity was also reduced in AD patients compared to healthy controls. However, PID patients also demonstrated increased ecological permissiveness, manifesting as colonization with species that were not found on the skin of AD or healthy patients. These data thus link immune dysfunction to dysbiosis in human skin (Oh et al., 2013). For WASP-

deficient mice, the importance of the microflora was demonstrated by showing that *Was*$^{-/-}$ animals on the 129SvEv background do not develop colitis in the absence of *Helicobacter* species (Nguyen et al., 2013), but so far no microbial sequencing data has been published. Importantly, the great majority of experiments that make up the currently available WAS literature have not been performed in littermate-controlled or cohoused setting; in many instances, control mice were obtained directly from repository laboratories rather than bred and maintained in tandem with *Was*$^{-/-}$ animals at the research institutes.

In **Chapter 7**, we demonstrated that food-allergic sensitization in *Was*$^{-/-}$ mice occurs in the absence of a commensal flora (**Figure 7.3, page 163**). In sharp contrast to colonic inflammation (Nguyen et al., 2013), we can therefore conclude that WAS-associated food allergy is not caused by a pathogenic host-microbe interaction. Our finding, however, does not rule out a modulatory effect from intestinal bacteria on disease pathogenesis. Indeed, we observed that the humoral immune response to food antigens is shaped by commensals, since SPF-housed mice developed higher food-specific IgA, and lower IgG1, than GF-housed counterparts (**Figure 7.3E**). These observations fit within the emerging paradigm that microbial colonization status is an extremely important determinant in shaping host IgE immune responses (Berni Canani et al., 2015). GF WT mice have previously been shown to have elevated levels of polyclonal, natural IgE (McCoy et al., 2006) and were found to be more susceptible to oral adjuvant-induced sensitization to peanuts (Cahenzli et al., 2013). Using recolonization experiments with increasingly complex microfloras, these authors demonstrated the existence of a critical complexity threshold above which the spontaneous hyper IgE phenotype of WT GF mice could be reversed (Cahenzli et al., 2013). Furthermore, species of *Clostridia* have been described to promote tolerance in cholera-toxin-mediated IgE sensitization to food (Stefka et al., 2014).

To assess whether differential disease activity between WT, *Was*$^{-/-}$ and *Was*$^{fl/fl}$*Foxp3*-Cre mice was associated with alterations in the microflora, we performed 16S rRNA gene sequencing on stool samples obtained from six-week-old animals that had been co-housed for three weeks after weaning. Following isolation of genomic DNA (Mobio PowerMag DNA Isolation Kit), bacterial 16S V4 rDNA regions were enriched and barcoded through amplification with fusion primers, then concentrated with solid-phase reversible immobilization, and finally quantified by electrophoresis (Agilent 2100 Bioanalyzer). 16S rDNA was analyzed with MiSeq® reagent cartridges.

First, we considered the amount of microbial variation within samples obtained from different genotypes (alpha diversity). At a sequencing depth of 150 000 sequences, alpha diversity for individual mice ranged from 519 to 966 operational taxonomic units (OTUs), and in many samples, richness continued to increase even in the last increment in sequencing depth. However, the variation between mice belonging to the same genotype was greater than that observed between genotypes, and ANOVA analysis at different sequencing depths revealed no significant differences in alpha diversity between WT, *Was*$^{-/-}$ and *Was*$^{fl/fl}$*Foxp3*-Cre mice (**Figure 9.7A**). As a control, we also measured alpha diversity in *Was*$^{-/-}$ mice that had

been treated with oral antibiotics. Broad-spectrum antibiotics supplemented to the drinking water resulted in a 4-fold reduction in alpha diversity in fecal pellets, demonstrating that our methods reliably detected biological variation.

Alpha diversity addresses the fraction of unique OTUs, but does not consider the origin of these OTUs in relation to taxonomic classification. We next assessed how the OTUs in fecal samples were distributed over different bacterial *phyla*. In mice from all three genotypes, *Bacteroidetes* species made up over 50% of OTUs, followed by *Firmicutes* and *Proteobacteria*, which is typical for the murine as well as human intestine (Cho and Blaser, 2012; Honda and Littman, 2012). Among the 9 most abundant phyla, we observed no significant differences in distribution between groups, with the exception *Cyanobacteria*, which were lower in *Was*$^{-/-}$ than in *Was*$^{fl/fl}$*Foxp3*-Cre mice (**Figure 9.7B**). At the two subsequent taxonomic levels, we found that the top 9 most abundant *classes* and *orders* respectively did not differ significantly between mice of either genotype (ANOVA p>0.05). However, at the level of bacterial *families*, we started observing differences in the distribution between the most abundant taxons, with relatively increased abundances of both *Prevotellaceae* and *Bacteroidaceae* in *Was*$^{fl/fl}$*Foxp3*-Cre mice in comparison with co-housed WT and *Was*$^{-/-}$ counterparts (**Figure 9.7C**). It is noteworthy that expansion of *Prevotellaceae* has been described as a pathogenic factor that results in reduced IL-18 signaling, inflammasome dysfunction, and increased susceptibility to DSS colitis (Elinav et al., 2011). As is apparent from **Figure 7.5A (page 165)**, reduced *Il18* expression clustered strongly with increased mast cell markers and Th2-type cytokines in *Was*$^{fl/fl}$*Foxp3*-Cre mice, which suggests that dysbiosis could be an upstream determinant of food allergic disease in these animals. Irrespective of these interesting speculations, our combined results indicate that the composition of the microbiome is significantly different between mice with different genotypes.

To be able to consider the whole composition of the microbiome rather than merely the most abundant members at each taxonomic level, we calculated Weighted UniFrac dissimilarity scores that allow for graphical analysis of complex relationships between samples using a two-dimensional Prinicipal Coordinate Analysis (PCoA). Furthermore, to allow identification of significant alterations at the level of the whole microbiome we utilized the Adonis permutation test, which estimates the likelihood of the encountered distribution by comparing it to randomly generated permutations. Using these approaches, we first asked whether the microbiome differed between mice with (*Was*$^{-/-}$ and *Was*$^{fl/fl}$*Foxp3*-Cre mice) and without (WT) spontaneously occurring food allergy. Principal component analysis based on 238 taxa that demonstrated significant differences in abundance across at least one of the groups revealed that food allergic status separated samples into distinct clusters, which suggests that disease status is associated with alterations in the microflora (**Figure 9.7D**). To visualize these findings in more detail, we generated a circular heatmap image that plots the relative abundance of an OTU, expressed as coloric representation of the Z-score obtained from the distribution of that particular OTU amongst all three genotypes,

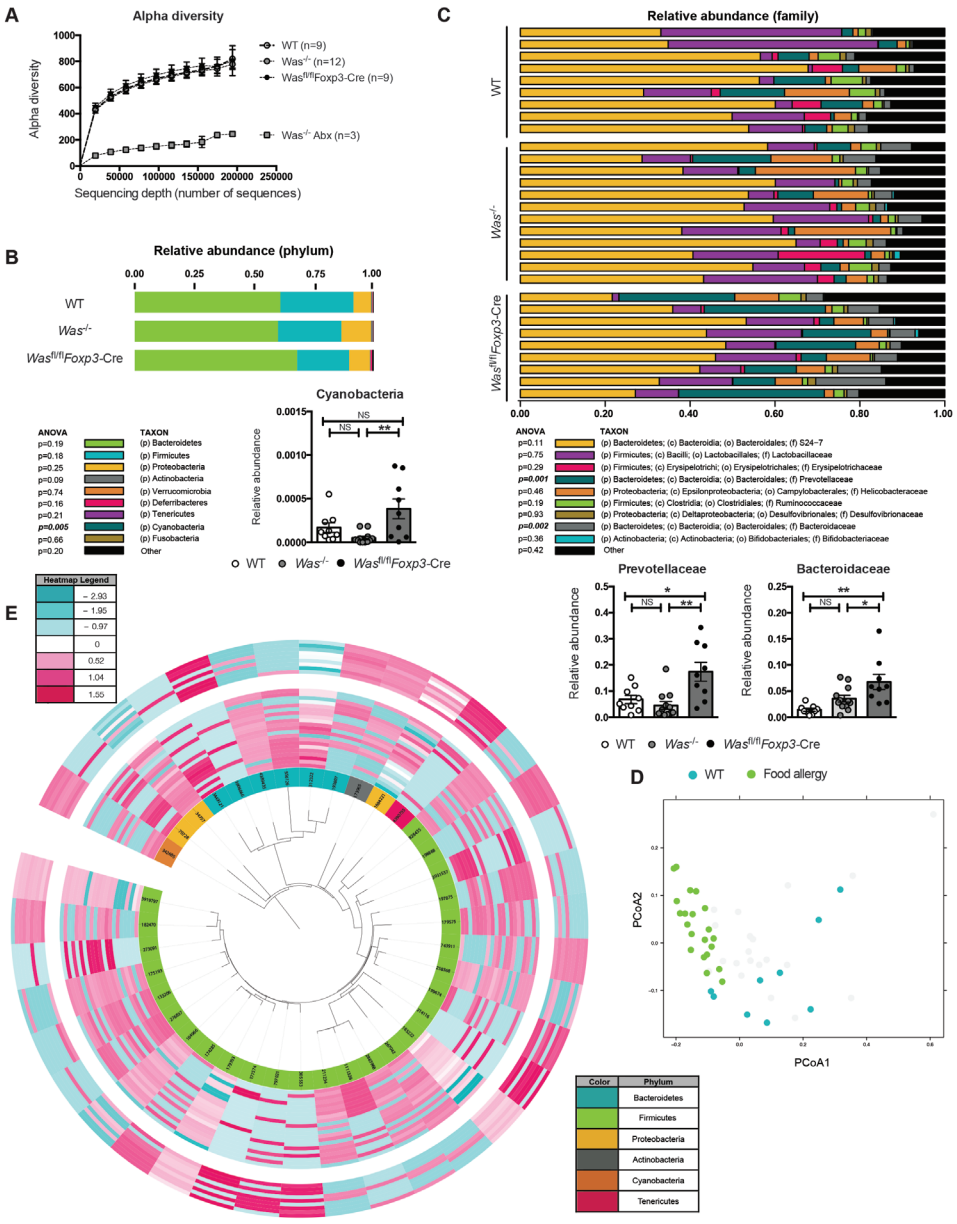


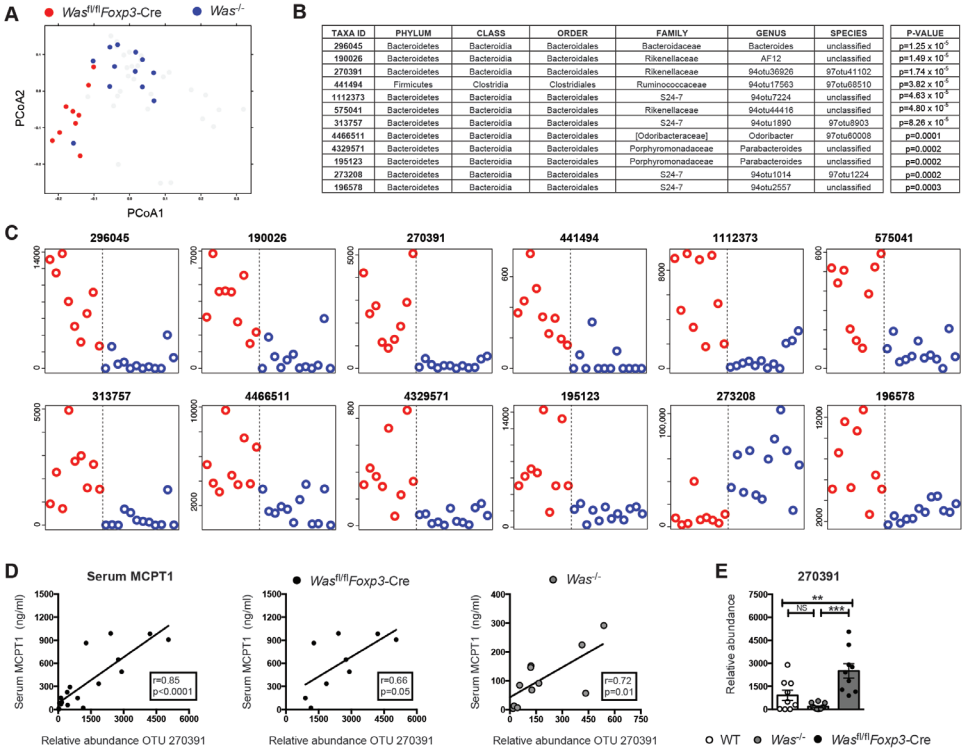
Figure 9.7 ***Spontaneously occurring food allergy in WASP deficiency is associated with a distinct microbiota signature.*** Results of 16S rDNA sequencing with MiSeq®. (**A**) Alpha diversity defined as number of unique operational taxonomic units (OUTs) for different number of sequences read in fecal pellets obtained from WT (open circles), *Was*$^{-/-}$ (gray circles) or *Was*$^{fl/fl}$*Foxp3*-Cre (black circles) mice. Antibiotic-treated *Was*$^{-/-}$ mice (gray squares) serve as an internal control. (**B**) Relative fractions of the 9 bacterial phyla present in highest abundance, with results of ANOVA test indicated for every phylum analyzed. For *Cyanobacteria*, subanalysis between genotypes is shown. (**C**) Relative abundance of the 9 most abundant bacterial families. Each bar represents an individual sample. ANOVA results

and taxonomic classification are denoted for every family in the color legend. (**D**) Principal coordinate analysis (PCoA) depicting the average weighted UniFrac distance between samples with (*Was*$^{-/-}$ and *Was*$^{fl/fl}$*Foxp3*-Cre) and without (WT) food allergy. (**E**) Circular hierarchical cluster dendrogram depicting the relative abundance (Z-score) of 38 taxa belonging to 33 families between samples with (inner ring) and without (outer ring) food allergy, with the color legend showing the phylum to which the particular family belongs. Dots represent individual mice and error bars depict SEM. *p<0.05; ** p<0.01; ***p<0.001; NS: not significant as determined by one-way ANOVA with Tukey's multiple comparis

as a hierarchical cluster based on the average linkage (**Figure 9.7E**, cf. hierarchical cluster diagram in **Figure 7.5A, page 165**). For data reduction, only the most significantly different OTU for each bacterial *family* (n=33) is shown, except for 5 families in which different OTUs demonstrated opposite directions of change and an example of each was selected. Combined, these results show that food allergy associated with WASP-deficiency in mice is associated with a microbiota signature that differs from co-housed WT control mice.

We next asked whether differences in the microbiome were associated with the differential disease activity observed in *Was*$^{-/-}$ and *Was*$^{fl/fl}$*Foxp3*-Cre mice. Significant differences in abundance were observed for 295 taxa, and the PCoA based on the Weighted UniFrac distance between samples clearly separated both genotypes except for one *Was*$^{-/-}$ outlier (Adonis p=0.003, **Figure 9.8A**), demonstrating that *Was*$^{-/-}$ and *Was*$^{fl/fl}$*Foxp3*-Cre mice had a distinct microbial signature. The 12 taxa for which the difference in abundance between the two groups reached the highest statistical significance are listed in the table in **Figure 9.8B**. Except for the *Ruminococcaceae* (OTU number 441494), all these OTUs belonged to members of the order *Bacteroidales*. Strikingly, for 11 out of 12 species (p=0.003) that differed most significantly between species, the greater abundance was observed in *Was*$^{fl/fl}$*Foxp3*-Cre and not in *Was*$^{-/-}$ mice (**Figure 9.8C**). Finally, we assessed whether any of these differences correlated with differential disease activity in *Was*$^{-/-}$ and *Was*$^{fl/fl}$*Foxp3*-Cre mice. Having identified serum MCPT1 as a non-invasive biomarker of intestinal mast cell expansion and activation (**Chapter 7 and 8**), we performed correlation analysis between serum MCPT1 levels obtained at the time of stool collection and the 12 OTUs in **Figure 9.8B**. Not surprisingly given the strong difference in serum MCPT1 between *Was*$^{-/-}$ and *Was*$^{fl/fl}$*Foxp3*-Cre mice (**Figure 7.4B, page 164**), we observed statistically significant correlations between MCPT1 and all 12 relative OTU abundances. However, if an altered abundance of a particular OTU was truly related to disease activity, we reasoned that a significant correlation should also uphold in a within-genotype analysis. Such a relationship was found for OTU 270391, belonging to a species of the *Rikanellaceae* family not further named than *97otu41102* (see **Figure 9.8B**): its relative abundance correlated positively and significantly with serum levels of MCPT1 in two independent analyses of *Was*$^{fl/fl}$*Foxp3*-Cre as well as *Was*$^{-/-}$ animals, thus strengthening a true association between the abundance of this species with intestinal disease severity and Th2-immune skewing (**Chapter 7**). Importantly, the species *97otu41102* does not depend on increased intestinal mast cells, because it was also found in the microflora of a subset of WT

animals that do not have elevated serum MCPT1 (**Figure 9.8E**). These results suggest that *97otu41102* may play a pro-allergic role in a genetically susceptible host and illustrate how alterations in the microflora could contribute to differential disease activity between *Was*$^{-/-}$ and *Was*$^{fl/fl}$*Foxp3*-Cre animals.



TAXA ID	PHYLUM	CLASS	ORDER	FAMILY	GENUS	SPECIES	P-VALUE
296045	Bacteroidetes	Bacteroidia	Bacteroidales	Bacteroidaceae	Bacteroides	unclassified	$p=1.25 \times 10^{-5}$
190026	Bacteroidetes	Bacteroidia	Bacteroidales	Rikenellaceae	AF12	unclassified	$p=1.49 \times 10^{-4}$
270391	Bacteroidetes	Bacteroidia	Bacteroidales	Rikenellaceae	94otu36926	97otu41102	$p=1.74 \times 10^{-5}$
441494	Firmicutes	Clostridia	Clostridiales	Ruminococcaceae	94otu17563	97otu68510	$p=3.82 \times 10^{-5}$
1112373	Bacteroidetes	Bacteroidia	Bacteroidales	S24-7	94otu7224	unclassified	$p=4.63 \times 10^{-5}$
575041	Bacteroidetes	Bacteroidia	Bacteroidales	Rikenellaceae	94otu44416	unclassified	$p=4.80 \times 10^{-5}$
313757	Bacteroidetes	Bacteroidia	Bacteroidales	S24-7	94otu1890	97otu8903	$p=8.26 \times 10^{-5}$
4466511	Bacteroidetes	Bacteroidia	Bacteroidales	[Odoribacteraceae]	Odoribacter	97otu60008	p=0.0001
4329571	Bacteroidetes	Bacteroidia	Bacteroidales	Porphyromonadaceae	Parabacteroides	unclassified	p=0.0002
195123	Bacteroidetes	Bacteroidia	Bacteroidales	Porphyromonadaceae	Parabacteroides	unclassified	p=0.0002
273208	Bacteroidetes	Bacteroidia	Bacteroidales	S24-7	94otu1014	97otu1224	p=0.0002
196578	Bacteroidetes	Bacteroidia	Bacteroidales	S24-7	94otu2557	unclassified	p=0.0003

Figure 9.8 ***Differential disease activity between Was$^{-/-}$ and Was$^{fl/fl}$Foxp3-Cre mice is associated with differences in the intestinal microbiota.*** (**A**) PCoA depicting the average weighted UniFrac distance between samples obtained from *Was*$^{-/-}$ and *Was*$^{fl/fl}$*Foxp3*-Cre mice. (**B**) Table listing the 12 taxa that were differed most significantly in relative abundance between *Was*$^{-/-}$ and *Was*$^{fl/fl}$*Foxp3*-Cre mice, with distribution of and relative abundance depicted in (**C**). (**D**) Pearson correlation analyses between the relative abundance of OTU 270391 and concurrently obtained serum MCPT1 levels in all food allergic (left), *Was*$^{fl/fl}$*Foxp3*-Cre mice (middle) and *Was*$^{-/-}$ (right) mice. (**E**) Relative abundance of OTU 270391 in WT mice in relation to *Was*$^{-/-}$ and *Was*$^{fl/fl}$*Foxp3*-Cre animals. Dots represent individual mice and error bars depict SEM. ** p<0.01; ***p<0.001; NS: not significant as determined by one-way ANOVA with Tukey's multiple comparisons test.

9.9 Other cell types

In this chapter, we have reviewed how WASP deficiency in a range of cellular immune compartments could affect the propensity for spontaneous food allergy in Was$^{-/-}$ mice.

Although spanning the breadth of the allergic response from allergic sensitization (microbiome, APCs) to the function of IgE effector cells, we have limited our discussion to cell types on which we have generated experimental data during the studies presented in this thesis. However, our current understanding of the pathogenesis of food allergic responses remains incomplete, and other immune cells that have hitherto not been discussed in this chapter are likely to play a modulatory role in WASP-deficiency associated food allergy. Three examples are:

1 *Invariant chain natural killer T (iNKT) cells*. iNKT cells have been implicated in the pathogenesis of allergic asthma and may act as an important link between microbial signals and immune homeostasis (Olszak et al., 2012). Indeed, in **Chapter 4** of this thesis, we have uncovered a role for iNKT cells in the pathogenesis of eosinophilic esophagitis in pediatric patients. In *Was*$^{-/-}$ mice and WAS patients, iNKT cells in liver and spleen are significantly decreased compared to healthy controls (Astrakhan et al., 2009) and have a functional impairment in their production of IL-4 and IFN-γ (Locci et al., 2009). Although not directly tested, it seems likely that *Was*$^{fl/fl}$*Foxp3*-Cre animals have normal development of iNKT cells, and differential involvement of iNKT cells should be considered in explaining the difference in allergic responses between *Was*$^{-/-}$ and *Was*$^{fl/fl}$*Foxp3*-Cre animals.

2 *Innate lymphoid cells.* Innate lymphoid cells have recently been recognized as important contributors to mucosal immune responses. Although devoid of antigen-specific TCRs, innate lymphocytes type 2 (ILC2s) express GATA3 and are capable of producing Th2-type cytokines in response to epithelial cell-derived alarmins such as IL-25 and IL-33 (Spits et al., 2013). Through expression of MHCII, ILC2s may further promote type-2 mucosal immune responses by priming of Th2 lymphocytes (Gasteiger and Rudensky, 2014; Oliphant et al., 2014). To date, no studies have been published on the effects of WASP deficiency on the function of ILCs, although this is a line of investigation that is actively pursued in our laboratory (personal communication Amlan Biswas, PhD and Scott Snapper, MD PhD).

3 *Eosinophils.* Eosinophilic infiltration is an important component of type-2 mucosal immunity, as extensively discussed in **Part 1** of this thesis. Our mRNA expression data (**Figure 7.5A, page 165**) demonstrated increased intestinal expression of *Il5* mRNA. Since IL-5 serves as a major chemotactic factor for eosinophils, an altered contribution of eosinophils might play a role in food allergic responses in *Was*$^{-/-}$ mice. However, no published data currently exist on the consequences of WASP deficiency on the function of eosinophils.

Table 9.1

Cell type	Result from intracellular loss of WASP	Presumed pro (↑) or anti (↓) allergic effect	Reference
APCs	Diminished uptake of (particulate) food antigens	↓	Westerberg et al. 2003
	Increased cross-presentation of exogenous antigens	↓	Baptista et al. 2016
	Aberrant tissue migration and homing (reduced interaction with T cells)	↓	Snapper et al. 2005; Bouma et al. 2007
	Ectopic maturation of DCs (hypothesized)	↑	Thrasher and Burns 2010; Moulding et al. 2013
	Instability of immune synapse: reduced IL-2 production and Th17 skew away from Th2/Th1	↓	Malinova et al. 2015; Bouma et al. 2011
	Reduced induction of Tregs from *Was*$^{-/-}$ DCs	↑	Nguyen et al. 2012
	Hyperresponsiveness to TLR9 signaling	(?)	Prete et al. 2013
	Increased production of B Cell Activating Factor (BAFF)	↑	Castiello et al. 2014; Crestani et al. 2015; Kolhatkar et al. 2015
B cells	Reduced number of B cells in spleen	(?)	Recher et al. 2012
	Relative increase in GC B cells and plasma cells	↑	Recher et al. 2012
	Increased class-switching to IgG1 following anti-CD40 and IL-4	↑	Westerberg et al. 2005
	BCR repertoire enriched for low-affinity, self-reactive BCRs	(?)	Kolhatkar et al. 2015
	Reduced regulatory B cells in spleen	↑	Bouma et al. 2014
	Lower levels of food-specific IgM	↓	**Figure 9.2, page 215**
	Reduced B cell responsiveness to BAFF signaling	↓	Bouma et al. 2014
	Reduced CXCR5-mediated chemotaxis	↓	Westerberg et al. 2005
T cells	Reduced FASl production and reduced killing of B cells	↑	Nikolov et al. 2010
	Increased T_{FH} differentiation	↑	**Figure 9.4, page 218**
	Increased propensity for apoptosis	(?)	Rengan et al. 2000; Nikolov et al. 2010
	Reduced homing to PPs	(?)	Snapper et al. 2005

	Low IL-2: decreased formation of tissue resident Th2 cells	↓	Hondowicz et al. 2015
T cells (ctd.)	Low IL-2: decreased induction of Foxp3$^+$ Tregs	↑	Josefowicz et al. 2012; Li and Zheng 2015
	Alterations in TCR repertoire	(?)	O'Connell et al. 2014; Wu et al. 2015; Wada et al. 2005
	Reduced IL-4 production from Th2 cells	↓	Morales-Tirado et al. 2010; **Figure 7.6F page 167**
	Aberrant activation following anti-TCR/CD3 stimulation	(?)	Gallego et al. 1997; Snapper et al. 1998; Calvez et al. 2011
	Defective immune synapse formation (T cell side)	(?)	Sims et al. 2007; Badour et al. 2003
Tregs	Loss of Th2 suppression and assumption of a GATA3$^+$ Th2 phenotype	↑	**Chapter 7**
	Reduced expression of RALDH enzymes in intestinal DCs	↑	**Figure 9.5, page 225**
	Increased GC B cell responses (direct or indirect effect?)	↑	**Figure 9.3, page 217**
	Defective granzyme B-dependent killing of B cells	↑	Adriani et al. 2011
Mast cells	Reduced pro-inflammatory cytokine production following IgE-mediated stimulation	↓	Pivniouk et al. 2003; **Figure 8.4D, page 195**
	Reduced IgE-independent activation following morphine challenge in human WAS	↓	**Figure 7.1E, page 159**
Basophils	Reduced survival of circulating basophils	↓	**Figure 9.6D, page 233**
	Reduced expression of surface FceRI on basophils	(?)	**Figure 9.6E, page 233**
Microbiome	Reduced growth of *97otu41102* in a *Was*$^{-/-}$ host	↓	**Figure 9.8D, page 239**
	Reduced *Prevotellacea* species in a *Was*$^{-/-}$ host	↓	**Figure 9.7C, page 237**
iNKT cells	Reduced in number and impairment of function	↓	Astrakham et al. 2009; Locci et al. 2009

9.10 Food allergy in *Was*$^{-/-}$ and *Was*$^{fl/fl}$*Foxp3*–Cre mice follows different natural courses

Up to this point, our discussion of the differential disease susceptibility between *Was*$^{-/-}$ and *Was*$^{fl/fl}$*Foxp3*-Cre mice has focused on the question how cell type-specific features of WASP deficiency may confer a pro- or anti-food allergic effect (summarized in **Table 9.1**). At the level of the organism, the severity of food allergy reflects the balance between all of these effects that are simultaneously operative. Given the large number of factors that are potentially involved, stochastic variation within this equation can easily be envisioned to result in considerable heterogeneity, even amongst genetically identical littermates. Furthermore, it is likely that this balance is dynamic, with individual variables gaining or losing biological significance in the course of disease. Such a notion is supported by human data showing that food allergies to certain allergens (e.g. milk, soy and wheat) are frequently outgrown during the first years of life (Longo et al., 2013; Sicherer and Sampson, 2014). Therefore, the study of severity of allergy over time in *Was*$^{-/-}$ and *Was*$^{fl/fl}$*Foxp3*-Cre mice may provide an additional dimension to our current discussion.

Figure 9.9A demonstrates the serum MCPT1 levels in a cohort of WT, *Was*$^{-/-}$ and *Was*$^{fl/fl}$*Foxp3*-Cre mice that was longitudinally followed to an age of 5 months. Whereas MCPT1 levels stayed consistently elevated in *Was*$^{fl/fl}$*Foxp3*-Cre animals between 2 and 5 months of age, there was a significant decline over time in *Was*$^{-/-}$ mice. Food allergy in these latter animals may thus be susceptible to spontaneous resolution as is commonly seen in pediatric patients. These differences in the natural course of disease are further proof for a dampened allergic phenotype in *Was*$^{-/-}$ compared to *Was*$^{fl/fl}$*Foxp3*-Cre animals.

9.11 Results from heterozygous *Was*$^{fl/wt}$*Foxp3*–Cre mice shed further light on WAS–associated food allergic disease

Obligate female carriers of a defective *WAS* gene do not suffer from any hematological abnormalities or immunodeficiency. In humans, this has been attributed to non-random inactivation of the X chromosome in hematopoietic (CD34^{+}) stem cells, which therefore only give rise to WASP-competent hematopoietic lineages (Wengler et al., 1995). Indeed, if the genetic mechanism responsible for such non-random inactivation fails, features of WAS can be observed in a female patient (Parolini et al., 1998). In mice, the selective advantage of WASP becomes apparent only after the CD34^{+} stem cell phase and has been demonstrated to affect different hematopoietic cells to a different degree (Westerberg et al., 2008). FOXP3^{+} Tregs are amongst the cellular subsets for which this selective advantage is strongest, with >90% of Tregs in *Was*$^{+/-}$ heterozygotes being WASP competent (Westerberg et al., 2008). Given the critical role of WASP-deficient Tregs in the pathogenesis of allergic disease, it is therefore not

surprising that heterozygous *Was*$^{+/-}$ females are protected from the spontaneous expansion of intestinal mast cells (**Figures 7.2, page 161** and **8.3**, **page 193**, and **Figure 9.9B**). However, whether this is true for heterozygous *Was*$^{wt/fl}$*Foxp3*-Cre mice has not been examined. In this genetic system, expression of WASP from T cells is only lost after transcription of *Foxp3* is initiated. Because WASP confers its selective advantage during the transition from the CD4$^-$ CD8$^-$ double negative to CD4$^+$CD8$^+$ double positive stage in the thymus (Cotta-de-Almeida et al., 2007; Westerberg et al., 2008), it seems likely that a higher fraction of Tregs in *Was*$^{wt/fl}$*Foxp3*-Cre animals are WASP-deficient.

Female mice possessing only one *Was*^{fl} allele in combination with the *Foxp3*-Cre transgene develop increased serum MCPT1 levels (**Figure 9.9D**). Although average values are significantly lower than those observed in homozygous females or hemizygous males, serum MCPT1 is consistently elevated over WT controls (**Figure 9.9D**) and intestinal mast cell numbers are increased (**Figure 9.9E**). Interestingly, this is only observed in heterozygotes born from *Was*$^{fl/fl}$*Foxp3*-Cre mothers (termed *Was*$^{wt/fl}$*Foxp3*-Cre mice, see **Figure 9.9C**). Genetically indistinguishable *Was*$^{fl/wt}$*Foxp3*-Cre mice that inherited the *Was*^{fl} allele from the father do not develop elevated MCPT1 levels (**Figure 9.9D**).

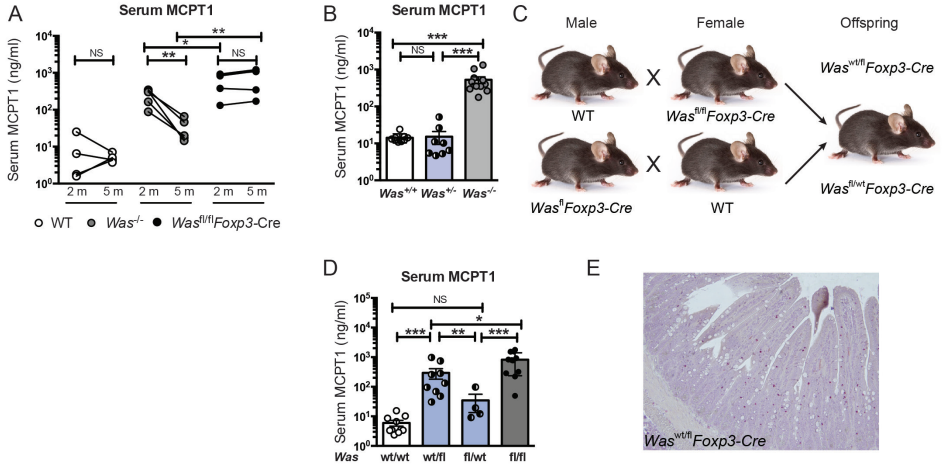


Figure 9.9 ***Differential disease susceptibility Was$^{-/-}$ and Was$^{fl/fl}$Foxp3-Cre becomes more apparent over time and affects immune homeostasis in heterozygous mice.*** (**A**) Serum levels of MCPT1 at 2 months and 5 months in WT (open circles), *Was*$^{-/-}$ (gray circles) or *Was*$^{fl/fl}$*Foxp3*-Cre (black circles) C57BL/6 mice. (**B**) Serum MCPT1 in *Was*$^{+/+}$ (WT), *Was*$^{+/-}$ and *Was*$^{-/-}$ mice on the BALC/c background. (**C**) Breeding strategy to generate *Was*$^{wt/fl}$*Foxp3*-Cre and *Was*$^{fl/wt}$*Foxp3*-Cre heterozygous animals. (**D**) Serum MCPT1 levels in *Was*$^{wt/fl}$*Foxp3*-Cre and *Was*$^{fl/wt}$*Foxp3*-Cre heterozygous animals in comparison with WT and *Was*$^{fl/fl}$*Foxp3*-Cre mice. (**E**) Representative chloroacetate esterase staining on jejunal sections from a *Was*$^{wt/fl}$*Foxp3*-Cre heterozygous female. Dots represent individual mice and error bars depict SEM. * p<0.05; ** p<0.01; ***p<0.001; NS: not significant as determined by (paired) t-test (A) or one-way ANOVA with Tukey's multiple comparisons test (B and D).

Additional animal studies are required to solidify these findings and illuminate the factors responsible for this difference between *Was*$^{wt/fl}$*Foxp3*-Cre and *Was*$^{fl/wt}$*Foxp3*-Cre mice, starting with quantification of the ratios of WASP-competent vs. WASP-deficient Tregs. However, it is tempting to draw the following preliminary conclusions from the already available data:

1 *WASP-deficient FOXP3*$^{+}$ *Tregs can cause allergic disease in the presence of WT counterparts.* These data favor a pathogenic, pro-inflammatory role for WASP-deficient Tregs in the pathogenesis of WAS-associated allergy, which is critically different from the elevated IgE and mast cell expansion that occurs in the absence of *any* Tregs in IPEX patients or *scurfy* mice. Such a 'gain-of-pathogenic-function' hypothesis could also help explain the isolated loss of Th2 suppression while suppression of Th1 and Th17 responses in *Was*$^{-/-}$ mice remain unperturbed.

2 *The genetic susceptibility to food allergy caused by loss of WASP in FOXP3*$^{+}$ *Tregs is influenced by maternal factors.* Effects of genetic imprinting notwithstanding, the fact that *Was*$^{wt/fl}$*Foxp3*-Cre and *Was*$^{fl/wt}$*Foxp3*-Cre mice are genetically similar yet develop different allergic phenotypes suggests that pups born to a food allergic mother have a higher chance of becoming food allergic themselves. Attractive targets for further exploration are maternal factors such as composition of breast milk (e.g. IgA, IgG or cytokine content) or the intestinal microbiome, which could be pinpointed further by additional cross-foster experiments. Although this conclusion increases the complexity of the mechanism of immune dysregulation that results from loss of WASP in Tregs, it also emphasizes the translational value of a model of WASP-deficiency in Tregs in study of allergic disease.

9.12 Concluding remarks

This chapter provides a synthesis of the pathogenesis of food allergy in the context of WASP deficiency. Building on the large body of existing WAS literature, it reviews the experimental evidence described in the research papers that make up the previous three chapters of this thesis, and provides an additional nine figures of data not incorporated in another chapter. The overarching goal of the discussion presented here has been to explain why spontaneously occurring food allergies are more pronounced in *Was*$^{fl/fl}$*Foxp3*-Cre than *Was*$^{-/-}$ mice. As delineated in the introduction of this chapter, this question is relevant for the following three reasons:

1 *Analysis of the role of WASP in all of the cellular compartments involved in the pathogenesis of allergic disease is required for a thorough understanding of the food allergy that occurs in patients with WAS.* We have found that WAS-associated food allergy hinges on dysfunction of the FOXP3^{+} Treg compartment and that the most severe allergy is seen in mice that only lack WASP in this particular cell type (**Chapter 7**). Human WAS patients, however, are WASP deficient in all hematopoietic lineages, and thereby more closely resemble *Was*$^{-/-}$ mice than the more allergic *Was*$^{fl/fl}$*Foxp3*-Cre animals. Understanding the factors that confer partial protection against fulminant allergic responses could aid in identifying patients at risk for allergy and thereby result in improved clinical management of patients with WAS. Appreciation of the interplay between pro- and anti-allergic effects of individual cell types will further help gauge the immune status of individuals that have undergone treatment with gene-corrected autologous hematopoietic stem cells that results in mixed chimerism (Hacein-Bey-Abina et al., 2015; Mukherjee and Thrasher, 2013), or in patients with (lineage-specific) chimerism following hematopoietic stem cell transplantation (Moratto et al., 2011). Additionally, secondary mutations leading to chimeric re-expression of WASP (Trifari et al., 2010) may also affect the allergic balance.

2 *Many of the features of WAS-associated food allergy are shared with common food allergic patients and improved understanding of its mechanisms could therefore shed new light on the general pathogenesis of IgE-mediated responses to food.* In stark contrast to WAS, which has an incidence of <1 in 100 000 births (Puck and Candotti, 2006), food allergy currently affects 4-8 out of a 100 children in Westernized societies (Longo et al., 2013; Sicherer and Sampson, 2014). A fundamental question underlying the research presented in this thesis is therefore to which extent the mechanistic insight into the pathogenesis of WAS-associated food allergy can be extrapolated to common food allergies. In **Chapter 7** we demonstrated that WAS patients are susceptible to similar food allergies as those observed in the general population. Furthermore, increased levels of GATA3^{+} FOXP3^{+} Tregs were recently described in pediatric cow's milk allergic patients (Noval Rivas et al., 2015). Although their mechanistic origin may differ, our identification of GATA3^{+} FOXP3^{+} Tregs in WASP-deficient mice and patients could indicate a final common pathway of disease pathogenesis that is shared between patients with food allergies and WAS-associated food allergic disease.

3 *In depth analysis of the differences between Was*$^{-/-}$ *and Was*$^{fl/fl}$*Foxp3-Cre mice helps to better define WASP-deficient animals as an experimental model of food allergy*. In **Chapters 7 and 8**, we have defined *Was*$^{-/-}$ animals as the first spontaneous

mouse model of food allergy with a direct human equivalent. *Was*$^{-/-}$ and *Was*$^{fl/fl}$*Foxp3*-Cre mice develop IgE antibodies against allergens that are responsible for IgE-mediated allergies in children (e.g. wheat and soy), whereas epidemiologically less allergenic foods such as corn were also better tolerated in mice. These findings implicate considerable translational potential of this novel model in determining what renders particular foods allergenic. In addition, many recent high-impact trials published on pediatric food allergy deal with questions regarding the timing of introduction of allergens as well as the effect of allergen avoidance on established allergies (Perkin et al., 2016; Toit et al., 2015; 2016). Although highly instructive from a preventative health perspective, unraveling the underlying mechanism of failed oral tolerance to allergens requires experimentally controlled settings and laboratory animals. In answering these questions, it is pertinent that the disease models used do not rely on an adjuvant-induced break of oral tolerance, but instead faithfully mimic the kinetics and route of sensitization as occurs in humans. The data presented in **Figure 9.9A** suggest that *Was*$^{-/-}$ mice can outgrow their food allergies, which provides further proof of a more humanized system. As suggested throughout this chapter as well as in the discussion of **Chapter 8**, crossing *Was*$^{fl/fl}$*Foxp3*-Cre or *Was*$^{-/-}$ mice with strains deficient in other immunological targets (e.g. basophil-deficient *Mcpt8*$^{-/-}$, B cell-deficient μMT mice or mice genetically deficient in any of the cytokines or chemokines) or a range of reporter animals holds great promise for further illuminating the pathophysiology of food allergic disease. Lastly, advances in humanized mouse models such as presented in **Chapter 6** could very likely increase the human relevance of these experiments even further.

9.13 Acknowledgements

I am indebted to the members of the Fiebiger and Snapper laboratories for the helpful discussions over the years that helped shape the ideas in this chapter. The microbiome studies were performed in collaboration with Luisa Chan from Second Genome and Maciej Chichlowski from Mead Johnson Nutrition Company. Financial support for these studies came from a postdoctoral fellowship from the Mead Johnson Nutrition Company.

References

Aalberse, R.C., Platts-Mills, T.A., and Rispens, T. (2016). The Developmental History of IgE and IgG4 Antibodies in Relation to Atopy, Eosinophilic Esophagitis, and the Modified TH2 Response. Curr Allergy Asthma Rep *16*, 45–49.

Abonia, J.P., Blanchard, C., Butz, B.B., Rainey, H.F., Collins, M.H., Stringer, K., Putnam, P.E., and Rothenberg, M.E. (2010). Involvement of mast cells in eosinophilic esophagitis. J. Allergy Clin. Immunol. *126*, 140–149.

Aceves, S.S. (2015). Eosinophilic esophagitis. Immunology and Allergy Clinics of North America *35*, 145–159.

Aceves, S.S., and Ackerman, S.J. (2009). Relationships between eosinophilic inflammation, tissue remodeling, and fibrosis in eosinophilic esophagitis. Immunology and Allergy Clinics of North America *29*, 197–211–xiii–xiv.

Adriani, M., Jones, K.A., Uchiyama, T., Kirby, M.R., Silvin, C., Anderson, S.M., and Candotti, F. (2011). Defective inhibition of B-cell proliferation by Wiskott-Aldrich syndrome protein-deficient regulatory T cells. Blood *117*, 6608–6611.

Agea, E., Russano, A., Bistoni, O., Mannucci, R., Nicoletti, I., Corazzi, L., Postle, A.D., De Libero, G., Porcelli, S.A., and Spinozzi, F. (2005). Human CD1-restricted T cell recognition of lipids from pollens. J. Exp. Med. *202*, 295–308.

Aguado, E., Richelme, S., Nuñez-Cruz, S., Miazek, A., Mura, A.-M., Richelme, M., Guo, X.-J., Sainty, D., He, H.-T., Malissen, B., et al. (2002). Induction of T helper type 2 immunity by a point mutation in the LAT adaptor. Science *296*, 2036–2040.

Akbari, O., Stock, P., Meyer, E., Kronenberg, M., Sidobre, S., Nakayama, T., Taniguchi, M., Grusby, M.J., DeKruyff, R.H., and Umetsu, D.T. (2003). Essential role of NKT cells producing IL-4 and IL-13 in the development of allergen-induced airway hyperreactivity. Nat. Med. *9*, 582–588.

Akdis, C.A., and Akdis, M. (2009). Mechanisms and treatment of allergic disease in the big picture of regulatory T cells. J. Allergy Clin. Immunol. *123*, 735–46–quiz747–8.

Akdis, C.A., and Akdis, M. (2014a). Mechanisms of immune tolerance to allergens: role of IL-10 and Tregs. J. Clin. Invest. *124*, 4678–4680.

Akdis, M., and Akdis, C.A. (2014b). Mechanisms of allergen-specific immunotherapy: multiple suppressor factors at work in immune tolerance to allergens. J. Allergy Clin. Immunol. *133*, 621–631.

Akei, H.S., Mishra, A., Blanchard, C., and Rothenberg, M.E. (2005). Epicutaneous antigen exposure primes for experimental eosinophilic esophagitis in mice. Gastroenterology *129*, 985–994.

Al-Alwan, M.M., Rowden, G., Lee, T.D., and West, K.A. (2001). The dendritic cell cytoskeleton is critical for the formation of the immunological synapse. J. Immunol. *166*, 1452–1456.

Albert, M.H., Notarangelo, L.D., and Ochs, H.D. (2011). Clinical spectrum, pathophysiology and treatment of the Wiskott-Aldrich syndrome. Curr. Opin. Hematol. *18*, 42–48.

Alberts, B., Johnson, A., Lewis, J., Raff, M., Roberts, K., and Walter, P. (2008). Molecular Biology of the Cell (Garland Science).

Alt, F.W., Oltz, E.M., Young, F., Gorman, J., Taccioli, G., and Chen, J. (1992). VDJ recombination. Immunol. Today *13*, 306–314.

Amu, S., Saunders, S.P., Kronenberg, M., Mangan, N.E., Atzberger, A., and Fallon, P.G. (2010). Regulatory B cells prevent and reverse allergic airway inflammation via FoxP3-positive T regulatory cells in a murine model. J. Allergy Clin. Immunol. *125*, 1114–1124.e1118.

Arias, Á., and Lucendo, A.J. (2014). Dietary therapies for eosinophilic esophagitis. Expert Rev Clin Immunol *10*, 133–142.

Arias, Á., González-Cervera, J., Tenias, J.M., and Lucendo, A.J. (2014). Efficacy of dietary interventions for inducing histologic remission in patients with eosinophilic esophagitis: a systematic review and meta-analysis. Gastroenterology *146*, 1639–1648.

Aschermann, S., Lehmann, C.H.K., Mihai, S., Schett, G., Dudziak, D., and Nimmerjahn, F. (2013). B cells are critical for autoimmune pathology in Scurfy mice. Proc. Natl. Acad. Sci. U.S.a. *110*, 19042–19047.

Assa'ad, A.H., Putnam, P.E., Collins, M.H., Akers, R.M., Jameson, S.C., Kirby, C.L., Buckmeier, B.K., Bullock, J.Z., Collier, A.R., Konikoff, M.R., et al. (2007). Pediatric patients with eosinophilic esophagitis: an 8-year follow-up. J. Allergy Clin. Immunol. *119*, 731–738.

Astrakhan, A., Ochs, H.D., and Rawlings, D.J. (2009). Wiskott-Aldrich syndrome protein is required for homeostasis and function of invariant NKT cells. The Journal of Immunology *182*, 7370–7380.

Atarashi, K., Tanoue, T., Oshima, K., Suda, W., Nagano, Y., Nishikawa, H., Fukuda, S., Saito, T., Narushima, S., Hase, K., et al. (2013). Treg induction by a rationally selected mixture of Clostridia strains from the human microbiota. Nature *500*, 232–236.

Attwood, S.E.A., Lewis, C.J., Bronder, C.S., Morris, C.D., Armstrong, G.R., and Whittam, J. (2003). Eosinophilic oesophagitis: a novel treatment using Montelukast. Gut *52*, 181–185.

Attwood, S.E., Smyrk, T.C., Demeester, T.R., and Jones, J.B. (1993). Esophageal eosinophilia with dysphagia. A distinct clinicopathologic syndrome. Dig Dis Sci *38*, 109–116.

Bacchetta, R., Passerini, L., Gambineri, E., Dai, M., Allan, S.E., Perroni, L., Dagna-Bricarelli, F., Sartirana, C., Matthes-Martin, S., Lawitschka, A., et al. (2006). Defective regulatory and effector T cell functions in patients with FOXP3 mutations. J. Clin. Invest. *116*, 1713–1722.

Badolato, R., Sozzani, S., Malacarne, F., Bresciani, S., Fiorini, M., Borsatti, A., Albertini, A., Mantovani, A., Ugazio, A.G., and Notarangelo, L.D. (1998). Monocytes from Wiskott-Aldrich patients display reduced chemotaxis and lack of cell polarization in response to monocyte chemoattractant protein-1 and formyl-methionyl-leucyl-phenylalanine. J. Immunol. *161*, 1026–1033.

Badour, K., Zhang, J., Shi, F., McGavin, M.K.H., Rampersad, V., Hardy, L.A., Field, D., and Siminovitch, K.A. (2003). The Wiskott-Aldrich syndrome protein acts downstream of CD2 and the CD2AP and PSTPIP1 adaptors to promote formation of the immunological synapse. Immunity *18*, 141–154.

Baenziger, S., Tussiwand, R., Schlaepfer, E., Mazzucchelli, L., Heikenwalder, M., Kurrer, M.O., Behnke, S., Frey, J., Oxenius, A., Joller, H., et al. (2006). Disseminated and sustained HIV infection in CD34+ cord blood cell-transplanted Rag2-/-gamma c-/- mice. Proc. Natl. Acad. Sci. U.S.a. *103*, 15951–15956.

Ballesteros-Tato, A., León, B., Graf, B.A., Moquin, A., Adams, P.S., Lund, F.E., and Randall, T.D. (2012). Interleukin-2 inhibits germinal center formation by limiting T follicular helper cell differentiation. Immunity *36*, 847–856.

Bannert, C., Bidmon-Fliegenschnee, B., Stary, G., Hotzy, F., Stift, J., Nurko, S., Szépfalusi, Z., Fiebiger, E., and Dehlink, E. (2012). Fc-Epsilon-RI, the High Affinity IgE-Receptor, Is Robustly Expressed in the Upper Gastrointestinal Tract and Modulated by Mucosal Inflammation. PLoS ONE *7*, e42066.

Baptista, M.A.P., Keszei, M., Oliveira, M., Sunahara, K.K.S., Andersson, J., Dahlberg, C.I.M., Worth, A.J., Liedén, A., Kuo, I.-C., Wallin, R.P.A., et al. (2016). Deletion of Wiskott-Aldrich syndrome protein triggers Rac2 activity and increased cross-presentation by dendritic cells. Nat Commun *7*, 12175.

Barda-Saad, M., Braiman, A., Titerence, R., Bunnell, S.C., Barr, V.A., and Samelson, L.E. (2005). Dynamic molecular interactions linking the T cell antigen receptor to the actin cytoskeleton. Nat Immunol *6*, 80–89.

Becker-Herman, S., Meyer-Bahlburg, A., Schwartz, M.A., Jackson, S.W., Hudkins, K.L., Liu, C., Sather, B.D., Khim, S., Liggitt, D., Song, W., et al. (2011). WASp-deficient B cells play a critical, cell-intrinsic role in triggering autoimmunity. J. Exp. Med. *208*, 2033–2042.

Belkaid, Y., and Hand, T.W. (2014). Role of the Microbiota in Immunity and Inflammation. Cell *157*, 121–141.

Bellinghausen, I., Reuter, S., Martin, H., Maxeiner, J., Luxemburger, U., Türeci, Ö., Grabbe, S., Taube, C., and Saloga, J. (2012). Enhanced production of CCL18 by tolerogenic dendritic cells is associated with inhibition of allergic airway reactivity. J. Allergy Clin. Immunol. *130*, 1384–1393.

Bennett, C.L., and Ochs, H.D. (2001). IPEX is a unique X-linked syndrome characterized by immune dysfunction, polyendocrinopathy, enteropathy, and a variety of autoimmune phenomena. Curr. Opin. Pediatr. *13*, 533–538.

Bennett, C.L., Christie, J., Ramsdell, F., Brunkow, M.E., Ferguson, P.J., Whitesell, L., Kelly, T.E., Saulsbury, F.T., Chance, P.F., and Ochs, H.D. (2001). The immune dysregulation, polyendocrinopathy, enteropathy, X-linked syndrome (IPEX) is caused by mutations of FOXP3. Nat. Genet. *27*, 20–21.

Berglund, G., Finnström, O., Johansson, S.G., and Möller, K.L. (1968). Wiskott-Aldrich syndrome. A study of 6 cases with determination of the immunoglobulins A, D, G, M and ND. Acta Paediatr Scand *57*, 89–97.

Berin, M.C., and Mayer, L. (2009). Immunophysiology of experimental food allergy. Mucosal Immunol *2*, 24–32.

Berin, M.C., and Sampson, H.A. (2013). Mucosal immunology of food allergy. Curr. Biol. *23*, R389–R400.

Berni Canani, R., Gilbert, J.A., and Nagler, C.R. (2015). The role of the commensal microbiota in the regulation of tolerance to dietary allergens. Current Opinion in Allergy and Clinical Immunology *15*, 243–249.

Biswas, S., Chang, H., Sarkis, P.T.N., Fikrig, E., Zhu, Q., and Marasco, W.A. (2011). Humoral immune responses in humanized BLT mice immunized with West Nile virus and HIV-1 envelope proteins are largely mediated via human CD5+ B cells. Immunology *134*, 419–433.

Blair, P.J., Bultman, S.J., Haas, J.C., Rouse, B.T., Wilkinson, J.E., and Godfrey, V.L. (1994). CD4+CD8- T cells are the effector cells in disease pathogenesis in the scurfy (sf) mouse. J. Immunol. *153*, 3764–3774.

Blanchard, C., Mingler, M.K., McBride, M., Putnam, P.E., Collins, M.H., Chang, G., Stringer, K., Abonia, J.P., Molkentin, J.D., and Rothenberg, M.E. (2008). Periostin facilitates eosinophil tissue infiltration in allergic lung and esophageal responses. Mucosal Immunol *1*, 289–296.

Blanchard, C., Mingler, M.K., Vicario, M., Abonia, J.P., Wu, Y.Y., Lu, T.X., Collins, M.H., Putnam, P.E., Wells, S.I., and Rothenberg, M.E. (2007). IL-13 involvement in eosinophilic esophagitis: transcriptome analysis and reversibility with glucocorticoids. J. Allergy Clin. Immunol. *120*, 1292–1300.

Blanchard, C., Stucke, E.M., Burwinkel, K., Caldwell, J.M., Collins, M.H., Ahrens, A., Buckmeier, B.K., Jameson, S.C., Greenberg, A., Kaul, A., et al. (2010). Coordinate interaction between IL-13 and epithelial differentiation cluster genes in eosinophilic esophagitis. The Journal of Immunology *184*, 4033–4041.

Blanchard, C., Stucke, E.M., Rodriguez-Jimenez, B., Burwinkel, K., Collins, M.H., Ahrens, A., Alexander, E.S., Butz, B.K.B., Jameson, S.C., Kaul, A., et al. (2011). A striking local esophageal cytokine expression profile in eosinophilic esophagitis. J. Allergy Clin. Immunol. *127*, 208–217, 217.e1–.e7.

Blanchard, C., Wang, N., and Rothenberg, M.E. (2006a). Eosinophilic esophagitis: pathogenesis, genetics, and therapy. J. Allergy Clin. Immunol. *118*, 1054–1059.

Blanchard, C., Wang, N., Stringer, K.F., Mishra, A., Fulkerson, P.C., Abonia, J.P., Jameson, S.C., Kirby, C., Konikoff, M.R., Collins, M.H., et al. (2006b). Eotaxin-3 and a uniquely conserved gene-expression profile in eosinophilic esophagitis. J. Clin. Invest. *116*, 536–547.

Blumberg, R., and Powrie, F. (2012). Microbiota, disease, and back to health: a metastable journey. Sci Transl Med *4*, 137rv7.

Bock, S.A., Muñoz-Furlong, A., and Sampson, H.A. (2007). Further fatalities caused by anaphylactic reactions to food, 2001-2006. J. Allergy Clin. Immunol. *119*, 1016–1018.

Bossuyt, P.M., Reitsma, J.B., Bruns, D.E., Gatsonis, C.A., Glasziou, P.P., Irwig, L.M., Lijmer, J.G., Moher, D., Rennie, D., de Vet, H.C.W., et al. (2003). Towards complete and accurate reporting of studies of diagnostic accuracy: The STARD Initiative. pp. 40–44.

Bouma, G., Burns, S., and Thrasher, A.J. (2007). Impaired T-cell priming in vivo resulting from dysfunction of WASp-deficient dendritic cells. Blood *110*, 4278–4284.

Bouma, G., Carter, N.A., Recher, M., Malinova, D., Adriani, M., Notarangelo, L.D., Burns, S.O., Mauri, C., and Thrasher, A.J. (2014). Exacerbated experimental arthritis in Wiskott-Aldrich syndrome protein deficiency: modulatory role of regulatory B cells. Eur. J. Immunol. *44*, 2692–2702.

Bouma, G., Mendoza-Naranjo, A., Blundell, M.P., de Falco, E., Parsley, K.L., Burns, S.O., and Thrasher, A.J. (2011). Cytoskeletal remodeling mediated by WASp in dendritic cells is necessary for normal immune synapse formation and T-cell priming. Blood *118*, 2492–2501.

Brainard, D.M., Seung, E., Frahm, N., Cariappa, A., Bailey, C.C., Hart, W.K., Shin, H.-S., Brooks, S.F., Knight, H.L., Eichbaum, Q., et al. (2009). Induction of robust cellular and humoral virus-specific adaptive immune responses in human immunodeficiency virus-infected humanized BLT mice. J. Virol. *83*, 7305–7321.

Brandt, E.B., Strait, R.T., Hershko, D., Wang, Q., Muntel, E.E., Scribner, T.A., Zimmermann, N., Finkelman, F.D., and Rothenberg, M.E. (2003). Mast cells are required for experimental oral allergen-induced diarrhea. J. Clin. Invest. *112*, 1666–1677.

Branum, A.M., and Lukacs, S.L. (2009). Food allergy among children in the United States. Pediatrics *124*, 1549–1555.

Brennan, P.J., Brigl, M., and Brenner, M.B. (2013). Invariant natural killer T cells: an innate activation scheme linked to diverse effector functions. Nat Rev Immunol *13*, 101–117.

Brightbill, H.D., Jeet, S., Lin, Z., Yan, D., Zhou, M., Tan, M., Nguyen, A., Yeh, S., Delarosa, D., Leong, S.R., et al. (2010). Antibodies specific for a segment of human membrane IgE deplete IgE-producing B cells in humanized mice. J. Clin. Invest. *120*, 2218–2229.

Brown-Whitehorn, T., and Liacouras, C.A. (2007). Eosinophilic esophagitis. Curr. Opin. Pediatr. *19*, 575–580.

Bryce, P.J., Falahati, R., Kenney, L.L., Leung, J., Bebbington, C., Tomasevic, N., Krier, R.A., Hsu, C.-L., Shultz, L.D., Greiner, D.L., et al. (2016). Humanized mouse model of mast cell-mediated passive cutaneous anaphylaxis and passive systemic anaphylaxis. J. Allergy Clin. Immunol.

Burbage, M., Keppler, S.J., Gasparrini, F., Martínez-Martín, N., Gaya, M., Feest, C., Domart, M.-C., Brakebusch, C., Collinson, L., Bruckbauer, A., et al. (2015). Cdc42 is a key regulator of B cell differentiation and is required for antiviral humoral immunity. J. Exp. Med. *212*, 53–72.

Burchill, M.A., Yang, J., Vang, K.B., Moon, J.J., Chu, H.H., Lio, C.-W.J., Vegoe, A.L., Hsieh, C.-S., Jenkins, M.K., and Farrar, M.A. (2008). Linked T cell receptor and cytokine signaling govern the development of the regulatory T cell repertoire. Immunity *28*, 112–121.

Burns, S., Thrasher, A.J., Blundell, M.P., Machesky, L., and Jones, G.E. (2001). Configuration of human dendritic cell cytoskeleton by Rho GTPases, the WAS protein, and differentiation. Blood *98*, 1142–1149.

Burton, O.T., Darling, A.R., Zhou, J.S., Noval-Rivas, M., Jones, T.G., Gurish, M.F., Chatila, T.A., and Oettgen, H.C. (2012). Direct effects of IL-4 on mast cells drive their intestinal expansion and increase susceptibility to anaphylaxis in a murine model of food allergy. Mucosal Immunol.

Burton, O.T., and Oettgen, H.C. (2011). Beyond immediate hypersensitivity: evolving roles for IgE antibodies in immune homeostasis and allergic diseases. Immunol. Rev. *242*, 128–143.

Burton, O.T., Logsdon, S.L., Zhou, J.S., Medina-Tamayo, J., Abdel-Gadir, A., Noval Rivas, M., Koleoglou, K.J., Chatila, T.A., Schneider, L.C., Rachid, R., et al. (2014a). Oral immunotherapy induces IgG antibodies that act through FcγRIIb to suppress IgE-mediated hypersensitivity. J. Allergy Clin. Immunol. *134*, 1310–1317.e1316.

Burton, O.T., Noval Rivas, M., Zhou, J.S., Logsdon, S.L., Darling, A.R., Koleoglou, K.J., Roers, A., Houshyar, H., Crackower, M.A., Chatila, T.A., et al. (2014b). Immunoglobulin E Signal Inhibition during Allergen Ingestion Leads to Reversal of Established Food Allergy and Induction of Regulatory T Cells. Immunity *41*, 141–151.

Butt, D., Chan, T.D., Bourne, K., Hermes, J.R., Nguyen, A., Statham, A., O'Reilly, L.A., Strasser, A., Price, S., Schofield, P., et al. (2015). FAS Inactivation Releases Unconventional Germinal Center B Cells that Escape Antigen Control and Drive IgE and Autoantibody Production. Immunity *42*, 890–902.

Bønnelykke, K., Matheson, M.C., Pers, T.H., Granell, R., Strachan, D.P., Alves, A.C., Linneberg, A., Curtin, J.A., Warrington, N.M., Standl, M., et al. (2013). Meta-analysis of genome-wide association studies identifies ten loci influencing allergic sensitization. Nat. Genet. *45*, 902–906.

Caffarelli, C., Romanini, E., Caruana, P., Street, M.E., and de' Angelis, G. (1998). Clinical food hypersensitivity: the relevance of duodenal immunoglobulin E-positive cells. Pediatr. Res. *44*, 485–490.

Cahenzli, J., Köller, Y., Wyss, M., Geuking, M.B., and McCoy, K.D. (2013). Intestinal Microbial Diversity during Early-Life Colonization Shapes Long-Term IgE Levels. Cell Host and Microbe *14*, 559–570.

Calvez, R., Lafouresse, F., De Meester, J., Galy, A., Valitutti, S., and Dupré, L. (2011). The Wiskott-Aldrich syndrome protein permits assembly of a focused immunological synapse enabling sustained T-cell receptor signaling. Haematologica *96*, 1415–1423.

Castiello, M.C., Bosticardo, M., Pala, F., Catucci, M., Chamberlain, N., van Zelm, M.C., Driessen, G.J., Pac, M., Bernatowska, E., Scaramuzza, S., et al. (2014). Wiskott-Aldrich Syndrome protein deficiency perturbs the homeostasis of B-cell compartment in humans. J. Autoimmun. *50*, 42–50.

Chang, H., Biswas, S., Tallarico, A.S., Sarkis, P.T.N., Geng, S., Panditrao, M.M., Zhu, Q., and Marasco, W.A. (2012). Human B-cell ontogeny in humanized NOD/SCID γc(null) mice generates a diverse yet auto/poly- and HIV-1-reactive antibody repertoire. Genes Immun. *13*, 399–410.

Chang, S.-Y., Cha, H.-R., Chang, J.-H., Ko, H.-J., Yang, H., Malissen, B., Iwata, M., and Kweon, M.-N. (2010). Lack of retinoic acid leads to increased langerin-expressing dendritic cells in gut-associated lymphoid tissues. Gastroenterology *138*, 1468–78–1478.e1–6.

Chen, C.-Y., Lee, J.-B., Liu, B., Ohta, S., Wang, P.-Y., Kartashov, A.V., Mugge, L., Abonia, J.P., Barski, A., Izuhara, K., et al. (2015). Induction of Interleukin-9-Producing Mucosal Mast Cells Promotes Susceptibility to IgE-Mediated Experimental Food Allergy. Immunity *43*, 788–802.

Chen, Z., Benoist, C., and Mathis, D. (2005). How defects in central tolerance impinge on a deficiency in regulatory T cells. Proc. Natl. Acad. Sci. U.S.a. *102*, 14735–14740.

Cheng, L.E., Wang, Z.-E., and Locksley, R.M. (2010). Murine B cells regulate serum IgE levels in a CD23-dependent manner. The Journal of Immunology *185*, 5040–5047.

Cherian, S., Smith, N.M., and Forbes, D.A. (2006). Rapidly increasing prevalence of eosinophilic oesophagitis in Western Australia. Archives of Disease in Childhood *91*, 1000–1004.

Cho, I., and Blaser, M.J. (2012). The human microbiome: at the interface of health and disease. Nat. Rev. Genet. *13*, 260–270.

Chu, D.K., Mohammed-Ali, Z., Jiménez-Saiz, R., Walker, T.D., Goncharova, S., Llop-Guevara, A., Kong, J., Gordon, M.E., Barra, N.G., Gillgrass, A.E., et al. (2014). T helper cell IL-4 drives intestinal Th2 priming to oral peanut antigen, under the control of OX40L and independent of innate-like lymphocytes. Mucosal Immunol.

Chung, Y., Tanaka, S., Chu, F., Nurieva, R.I., Martinez, G.J., Rawal, S., Wang, Y.-H., Lim, H., Reynolds, J.M., Zhou, X.-H., et al. (2011). Follicular regulatory T cells expressing Foxp3 and Bcl-6 suppress germinal center reactions. Nat. Med. *17*, 983–988.

Cianferoni, A., and Spergel, J. (2016). Eosinophilic Esophagitis: A Comprehensive Review. Clin Rev Allergy Immunol *50*, 159–174.

Cianferoni, A., and Spergel, J.M. (2014). Immunotherapeutic approaches for the treatment of eosinophilic esophagitis. Immunotherapy *6*, 321–331.

Cianferoni, A., Massaad, M., Feske, S., la Fuente, de, M.A., Gallego, L., Ramesh, N., and Geha, R.S. (2005). Defective nuclear translocation of nuclear factor of activated T cells and extracellular signal-regulated kinase underlies deficient IL-2 gene expression in Wiskott-Aldrich syndrome. J. Allergy Clin. Immunol. *116*, 1364–1371.

Clayton, F., Fang, J.C., Gleich, G.J., Lucendo, A.J., Olalla, J.M., Vinson, L.A., Lowichik, A., Chen, X., Emerson, L., Cox, K., et al. (2014). Eosinophilic esophagitis in adults is associated with IgG4 and not mediated by IgE. Gastroenterology *147*, 602–609.

Coombes, J.L., Siddiqui, K.R.R., Arancibia-Cárcamo, C.V., Hall, J., Sun, C.-M., Belkaid, Y., and Powrie, F. (2007). A functionally specialized population of mucosal CD103+ DCs induces Foxp3+ regulatory T cells via a TGF-beta and retinoic acid-dependent mechanism. J. Exp. Med. *204*, 1757–1764.

Cotta-de-Almeida, V., Dupré, L., Guipouy, D., and Vasconcelos, Z. (2015). Signal Integration during T Lymphocyte Activation and Function: Lessons from the Wiskott-Aldrich Syndrome. Front Immunol *6*, 47.

Cotta-de-Almeida, V., Westerberg, L., Maillard, M.H., Onaldi, D., Wachtel, H., Meelu, P., Chung, U.-I., Xavier, R., Alt, F.W., and Snapper, S.B. (2007). Wiskott Aldrich syndrome protein (WASP) and N-WASP are critical for T cell development. Proc. Natl. Acad. Sci. U.S.a. *104*, 15424–15429.

Covassin, L., Laning, J., Abdi, R., Langevin, D.L., Phillips, N.E., Shultz, L.D., and Brehm, M.A. (2011). Human peripheral blood CD4 T cell-engrafted non-obese diabetic-scid IL2r**γ** (null) H2-Ab1 (tm1Gru) Tg (human leucocyte antigen D-related 4) mice: a mouse model of human allogeneic graft-versus-host disease. Clin. Exp. Immunol. *166*, 269–280.

Crestani, E., Volpi, S., Candotti, F., Giliani, S., Notarangelo, L.D., Chu, J., Aldave Becerra, J.C., Buchbinder, D., Chou, J., Geha, R.S., et al. (2015). Broad spectrum of autoantibodies in patients with Wiskott-Aldrich syndrome and X-linked thrombocytopenia. J. Allergy Clin. Immunol.

Crotty, S. (2014). T Follicular Helper Cell Differentiation, Function, and Roles in Disease. Immunity *41*, 529–542.

Cuss, A.K., Avery, D.T., Cannons, J.L., Yu, L.J., Nichols, K.E., Shaw, P.J., and Tangye, S.G. (2006). Expansion of functionally immature transitional B cells is associated with human-immunodeficient states characterized by impaired humoral immunity. J. Immunol. *176*, 1506–1516.

Daikh, B.E., Ryan, C.K., and Schwartz, R.H. (2003). Montelukast reduces peripheral blood eosinophilia but not tissue eosinophilia or symptoms in a patient with eosinophilic gastroenteritis and esophageal stricture. Ann. Allergy Asthma Immunol. *90*, 23–27.

Danner, R., Chaudhari, S.N., Rosenberger, J., Surls, J., Richie, T.L., Brumeanu, T.-D., and Casares, S. (2011). Expression of HLA class II molecules in humanized NOD.Rag1KO.IL2RgcKO mice is critical for development and function of human T and B cells. PLoS ONE *6*, e19826.

Datta, S., and Milner, J.D. (2011). Altered T-cell receptor signaling in the pathogenesis of allergic disease. J. Allergy Clin. Immunol. *127*, 351–354.

Davis, B.P., and Rothenberg, M.E. (2016). Mechanisms of Disease of Eosinophilic Esophagitis. Annu Rev Pathol *11*, 365–393.

de Jong, A.B., Dikkeschei, L.D., and Brand, P.L.P. (2011). Sensitization patterns to food and inhalant allergens in childhood: a comparison of non-sensitized, monosensitized, and polysensitized children. Pediatr Allergy Immunol *22*, 166–171.

De Meester, J., Calvez, R., Valitutti, S., and Dupré, L. (2010). The Wiskott-Aldrich syndrome protein regulates CTL cytotoxicity and is required for efficient killing of B cell lymphoma targets. J. Leukoc. Biol. *88*, 1031–1040.

DeBrosse, C.W., Franciosi, J.P., King, E.C., Butz, B.K.B., Greenberg, A.B., Collins, M.H., Abonia, J.P., Assa'ad, A., Putnam, P.E., and Rothenberg, M.E. (2011). Long-term outcomes in pediatric-onset esophageal eosinophilia. J. Allergy Clin. Immunol. *128*, 132–138.

Dehlink, E., Baker, A.H., Yen, E., Nurko, S., and Fiebiger, E. (2010). Relationships between levels of serum IgE, cell-bound IgE, and IgE-receptors on peripheral blood cells in a pediatric population. PLoS ONE *5*, e12204.

Dehlink, E., Platzer, B., Baker, A.H., LaRosa, J., Pardo, M., Dwyer, P., Yen, E.H., Szépfalusi, Z., Nurko, S., and Fiebiger, E. (2011). A soluble form of the high affinity IgE receptor, Fc-epsilon-RI, circulates in human serum. PLoS ONE *6*, e19098.

Dellon, E.S. (2014). Diagnostics of eosinophilic esophagitis: clinical, endoscopic, and histologic pitfalls. Dig Dis *32*, 48–53.

Dellon, E.S., and Liacouras, C.A. (2014). Advances in clinical management of eosinophilic esophagitis. Gastroenterology *147*, 1238–1254.

Dellon, E.S., Chen, X., Miller, C.R., Woosley, J.T., and Shaheen, N.J. (2012). Diagnostic utility of major basic protein, eotaxin-3, and leukotriene enzyme staining in eosinophilic esophagitis. Am J Gastroenterology *107*, 1503–1511.

Dellon, E.S., Speck, O., Woodward, K., Gebhart, J.H., Madanick, R.D., Levinson, S., Fritchie, K.J., Woosley, J.T., and Shaheen, N.J. (2013). Clinical and endoscopic characteristics do not reliably differentiate PPI-responsive esophageal eosinophilia and eosinophilic esophagitis in patients undergoing upper endoscopy: a prospective cohort study. Am J Gastroenterology *108*, 1854–1860.

Dhainaut, M., Coquerelle, C., Uzureau, S., Denoeud, J., Acolty, V., Oldenhove, G., Galuppo, A., Sparwasser, T., Thielemans, K., Pays, E., et al. (2015). Thymus-derived regulatory T cells restrain pro-inflammatory Th1 responses by downregulating CD70 on dendritic cells. Embo J. *34*, 1336–1348.

Dickel, H., Gambichler, T., Kamphowe, J., Altmeyer, P., and Skrygan, M. (2010). Standardized tape stripping prior to patch testing induces upregulation of Hsp90, Hsp70, IL-33, TNF-γ and IL-8/CXCL8 mRNA: new insights into the involvement of 'alarmins'. Contact Derm. *63*, 215–222.

Diegelmann, J., Seiderer, J., Niess, J.-H., Haller, D., Göke, B., Reinecker, H.-C., and Brand, S. (2010). Expression and regulation of the chemokine CXCL16 in Crohn's disease and models of intestinal inflammation. Inflamm Bowel Dis *16*, 1871–1881.

Divekar, R., and Kita, H. (2015). Recent advances in epithelium-derived cytokines (IL-33, IL-25, and thymic stromal lymphopoietin) and allergic inflammation. Current Opinion in Allergy and Clinical Immunology *15*, 98–103.

Doherty, T.A., Baum, R., Newbury, R.O., Yang, T., Dohil, R., Aquino, M., Doshi, A., Walford, H.H., Kurten, R.C., Broide, D.H., et al. (2015). Group 2 innate lymphocytes (ILC2) are enriched in active eosinophilic esophagitis. J. Allergy Clin. Immunol. *136*, 792–794.e793.

Dombrowicz, D., Flamand, V., Miyajima, I., Ravetch, J.V., Galli, S.J., and Kinet, J.P. (1997). Absence of Fc epsilonRI alpha chain results in upregulation of Fc gammaRIII-dependent mast cell degranulation and anaphylaxis. Evidence of competition between Fc epsilonRI and Fc gammaRIII for limiting amounts of FcR beta and gamma chains. J. Clin. Invest. *99*, 915–925.

Drazen, J.M., Israel, E., and O'Byrne, P.M. (1999). Treatment of asthma with drugs modifying the leukotriene pathway. N. Engl. J. Med. *340*, 197–206.

Eberl, G. (2015). Addressing the experimental variability associated with the microbiota. Mucosal Immunol *8*, 487–490.

Eder, W., Ege, M.J., and Mutius, von, E. (2006). The asthma epidemic. N. Engl. J. Med. *355*, 2226–2235.

Egawa, M., Mukai, K., Yoshikawa, S., Iki, M., Mukaida, N., Kawano, Y., Minegishi, Y., and Karasuyama, H. (2013). Inflammatory monocytes recruited to allergic skin acquire an anti-inflammatory M2 phenotype via basophil-derived interleukin-4. Immunity *38*, 570–580.

Ege, M.J., Mayer, M., Normand, A.-C., Genuneit, J., Cookson, W.O.C.M., Braun-Fahrländer, C., Heederik, D., Piarroux, R., Mutius, von, E., GABRIELA Transregio 22 Study Group (2011). Exposure to environmental microorganisms and childhood asthma. N. Engl. J. Med. *364*, 701–709.

Eigenmann, P.A., Sicherer, S.H., Borkowski, T.A., Cohen, B.A., and Sampson, H.A. (1998). Prevalence of IgE-mediated food allergy among children with atopic dermatitis. Pediatrics *101*, E8.

Elinav, E., Strowig, T., Kau, A.L., Henao-Mejia, J., Thaiss, C.A., Booth, C.J., Peaper, D.R., Bertin, J., Eisenbarth, S.C., Gordon, J.I., et al. (2011). NLRP6 inflammasome regulates colonic microbial ecology and risk for colitis. Cell *145*, 745–757.

Erwin, E.A., James, H.R., Gutekunst, H.M., Russo, J.M., Kelleher, K.J., and Platts-Mills, T.A.E. (2010). Serum IgE measurement and detection of food allergy in pediatric patients with eosinophilic esophagitis. Ann. Allergy Asthma Immunol. *104*, 496–502.

Fallon, P.G., Sasaki, T., Sandilands, A., Campbell, L.E., Saunders, S.P., Mangan, N.E., Callanan, J.J., Kawasaki, H., Shiohama, A., Kubo, A., et al. (2009). A homozygous frameshift mutation in the mouse Flg gene facilitates enhanced percutaneous allergen priming. Nat. Genet. *41*, 602–608.

Feehley, T., and Nagler, C.R. (2014). Cellular and molecular pathways through which commensal bacteria modulate sensitization to dietary antigens. Curr. Opin. Immunol. *31*, 79–86.

Feng, Y., van der Veeken, J., Shugay, M., Putintseva, E.V., Osmanbeyoglu, H.U., Dikiy, S., Hoyos, B.E., Moltedo, B., Hemmers, S., Treuting, P., et al. (2015). A mechanism for expansion of regulatory T-cell repertoire and its role in self-tolerance. Nature *528*, 132–136.

Figueroa, D.J., Borish, L., Baramki, D., Philip, G., Austin, C.P., and Evans, J.F. (2003). Expression of cysteinyl leukotriene synthetic and signalling proteins in inflammatory cells in active seasonal allergic rhinitis. Clin. Exp. Allergy *33*, 1380–1388.

Fogg, M.I., Ruchelli, E., and Spergel, J.M. (2003). Pollen and eosinophilic esophagitis. J. Allergy Clin. Immunol. *112*, 796–797.

Fontenot, J.D., Gavin, M.A., and Rudensky, A.Y. (2003). Foxp3 programs the development and function of CD4+CD25+ regulatory T cells. Nat Immunol *4*, 330–336.

Forbes, E.E., Groschwitz, K., Abonia, J.P., Brandt, E.B., Cohen, E., Blanchard, C., Ahrens, R., Seidu, L., McKenzie, A., Strait, R., et al. (2008). IL-9- and mast cell-mediated intestinal permeability predisposes to oral antigen hypersensitivity. J. Exp. Med. *205*, 897–913.

Föger, N., Jenckel, A., Orinska, Z., Lee, K.-H., Chan, A.C., and Bulfone-Paus, S. (2011). Differential regulation of mast cell degranulation versus cytokine secretion by the actin regulatory proteins Coronin1a and Coronin1b. J. Exp. Med. *208*, 1777–1787.

Freeman, J.D., Warren, R.L., Webb, J.R., Nelson, B.H., and Holt, R.A. (2009). Profiling the T-cell receptor beta-chain repertoire by massively parallel sequencing. Genome Res. *19*, 1817–1824.

Furuta, G.T., and Katzka, D.A. (2015). Eosinophilic Esophagitis. N. Engl. J. Med. *373*, 1640–1648.

Furuta, G.T., Kagalwalla, A.F., Lee, J.J., Alumkal, P., Maybruck, B.T., Fillon, S., Masterson, J.C., Ochkur, S., Protheroe, C., Moore, W., et al. (2013). The oesophageal string test: a novel, minimally invasive method measures mucosal inflammation in eosinophilic oesophagitis. Gut *62*, 1395–1405.

Furuta, G.T., Liacouras, C.A., Collins, M.H., Gupta, S.K., Justinich, C., Putnam, P.E., Bonis, P., Hassall, E., Straumann, A., Rothenberg, M.E., et al. (2007). Eosinophilic esophagitis in children and adults: a systematic review and consensus recommendations for diagnosis and treatment. Gastroenterology *133*, 1342–1363.

Fuss, I.J., Heller, F., Boirivant, M., Leon, F., Yoshida, M., Fichtner-Feigl, S., Yang, Z., Exley, M., Kitani, A., Blumberg, R.S., et al. (2004). Nonclassical CD1d-restricted NK T cells that produce IL-13 characterize an atypical Th2 response in ulcerative colitis. J. Clin. Invest. *113*, 1490–1497.

Gaj, T., Gersbach, C.A., and Barbas, C.F. (2013). ZFN, TALEN, and CRISPR/Cas-based methods for genome engineering. Trends Biotechnol. *31*, 397–405.

Gallego, M.D., Santamaría, M., Peña, J., and Molina, I.J. (1997). Defective actin reorganization and polymerization of Wiskott-Aldrich T cells in response to CD3-mediated stimulation. Blood *90*, 3089–3097.

Galli, S.J., and Tsai, M. (2012). IgE and mast cells in allergic disease. Nat. Med. *18*, 693–704.

Gambineri, E., Perroni, L., Passerini, L., Bianchi, L., Doglioni, C., Meschi, F., Bonfanti, R., Sznajer, Y., Tommasini, A., Lawitschka, A., et al. (2008). Clinical and molecular profile of a new series of patients with immune dysregulation, polyendocrinopathy, enteropathy, X-linked syndrome: inconsistent

correlation between forkhead box protein 3 expression and disease severity. J. Allergy Clin. Immunol. *122*, 1105–1112.e1.

Gambineri, E., Torgerson, T.R., and Ochs, H.D. (2003). Immune dysregulation, polyendocrinopathy, enteropathy, and X-linked inheritance (IPEX), a syndrome of systemic autoimmunity caused by mutations of FOXP3, a critical regulator of T-cell homeostasis. Curr Opin Rheumatol *15*, 430–435.

Garn, H., Neves, J.F., Blumberg, R.S., and Renz, H. (2013). Effect of barrier microbes on organ-based inflammation. J. Allergy Clin. Immunol. *131*, 1465–1478.

Gasteiger, G., and Rudensky, A.Y. (2014). PERSPECTIVES. Nat Rev Immunol *14*, 631–639.

Gaudin, E., Hao, Y., Rosado, M.M., Chaby, R., Girard, R., and Freitas, A.A. (2004). Positive selection of B cells expressing low densities of self-reactive BCRs. J. Exp. Med. *199*, 843–853.

Geiss, G.K., Bumgarner, R.E., Birditt, B., Dahl, T., Dowidar, N., Dunaway, D.L., Fell, H.P., Ferree, S., George, R.D., Grogan, T., et al. (2008). Direct multiplexed measurement of gene expression with color-coded probe pairs. Nat. Biotechnol. *26*, 317–325.

Germanov, E., Veinotte, L., Cullen, R., Chamberlain, E., Butcher, E.C., and Johnston, B. (2008). Critical role for the chemokine receptor CXCR6 in homeostasis and activation of CD1d-restricted NKT cells. J. Immunol. *181*, 81–91.

Geuking, M.B., Cahenzli, J., Lawson, M.A.E., Ng, D.C.K., Slack, E., Hapfelmeier, S., McCoy, K.D., and Macpherson, A.J. (2011). Intestinal bacterial colonization induces mutualistic regulatory T cell responses. Immunity *34*, 794–806.

Giacomin, P.R., Siracusa, M.C., Walsh, K.P., Grencis, R.K., Kubo, M., Comeau, M.R., and Artis, D. (2012). Thymic stromal lymphopoietin-dependent basophils promote Th2 cytokine responses following intestinal helminth infection. The Journal of Immunology *189*, 4371–4378.

Godfrey, V.L., Wilkinson, J.E., and Russell, L.B. (1991). X-linked lymphoreticular disease in the scurfy (sf) mutant mouse. Am. J. Pathol. *138*, 1379–1387.

Gonsalves, N., Furuta, G.T., and Atkins, D. (2016). Eosinophilic Gastrointestinal Disorders Affect More Than Just the Esophagus. Journal of Pediatric Gastroenterology and Nutrition *62*, 1–2.

Gonsalves, N., Yang, G.-Y., Doerfler, B., Ritz, S., Ditto, A.M., and Hirano, I. (2012). Elimination diet effectively treats eosinophilic esophagitis in adults; food reintroduction identifies causative factors. Gastroenterology *142*, 1451–9.e1–quize14–5.

Goodnow, C.C., Vinuesa, C.G., Randall, K.L., Mackay, F., and Brink, R. (2010). Control systems and decision making for antibody production. Nat Immunol *11*, 681–688.

Gorantla, S., Sneller, H., Walters, L., Sharp, J.G., Pirruccello, S.J., West, J.T., Wood, C., Dewhurst, S., Gendelman, H.E., and Poluektova, L. (2007). Human immunodeficiency virus type 1 pathobiology studied in humanized BALB/c-Rag2-/-gammac-/- mice. J. Virol. *81*, 2700–2712.

Gould, H.J., and Sutton, B.J. (2008). IgE in allergy and asthma today. Nat Rev Immunol *8*, 205–217.

Greenhawt, M., Aceves, S.S., Spergel, J.M., and Rothenberg, M.E. (2013). The management of eosinophilic esophagitis. J Allergy Clin Immunol Pract *1*, 332–40–quiz341–2.

Gupta, S.K., Peters-Golden, M., Fitzgerald, J.F., Croffie, J.M., Pfefferkorn, M.D., Molleston, J.P., Corkins, M.R., and Lim, J.R. (2006). Cysteinyl leukotriene levels in esophageal mucosal biopsies of children with eosinophilic inflammation: are they all the same? Am J Gastroenterology *101*, 1125–1128.

Gurish, M.F., Bryce, P.J., Tao, H., Kisselgof, A.B., Thornton, E.M., Miller, H.R., Friend, D.S., and Oettgen, H.C. (2004). IgE enhances parasite clearance and regulates mast cell responses in mice infected with Trichinella spiralis. J. Immunol. *172*, 1139–1145.

Hacein-Bey-Abina, S., Gaspar, H.B., Blondeau, J., Caccavelli, L., Charrier, S., Buckland, K., Picard, C., Six, E., Himoudi, N., Gilmour, K., et al. (2015). Outcomes following gene therapy in patients with severe Wiskott-Aldrich syndrome. Jama *313*, 1550–1563.

Haddad, E., Zugaza, J.L., Louache, F., Debili, N., Crouin, C., Schwarz, K., Fischer, A., Vainchenker, W., and Bertoglio, J. (2001). The interaction between Cdc42 and WASP is required for SDF-1-induced T-lymphocyte chemotaxis. Blood *97*, 33–38.

Hall, J.A., Cannons, J.L., Grainger, J.R., Santos, Dos, L.M., Hand, T.W., Naik, S., Wohlfert, E.A., Chou, D.B., Oldenhove, G., Robinson, M., et al. (2011a). Essential role for retinoic acid in the promotion of CD4(+) T cell effector responses via retinoic acid receptor alpha. Immunity *34*, 435–447.

Hall, J.A., Grainger, J.R., Spencer, S.P., and Belkaid, Y. (2011b). The role of retinoic acid in tolerance and immunity. Immunity *35*, 13–22.

Ham, H., Guerrier, S., Kim, J., Schoon, R.A., Anderson, E.L., Hamann, M.J., Lou, Z., and Billadeau, D.D. (2013). Dedicator of cytokinesis 8 interacts with talin and Wiskott-Aldrich syndrome protein to regulate NK cell cytotoxicity. The Journal of Immunology *190*, 3661–3669.

Han, H., Thelen, T.D., Comeau, M.R., and Ziegler, S.F. (2014). Thymic stromal lymphopoietin-mediated epicutaneous inflammation promotes acute diarrhea and anaphylaxis. J. Clin. Invest. *124*, 5442–5452.

Harris, J.K., Fang, R., Wagner, B.D., Choe, H.N., Kelly, C.J., Schroeder, S., Moore, W., Stevens, M.J., Yeckes, A., Amsden, K., et al. (2015). Esophageal microbiome in eosinophilic esophagitis. PLoS ONE *10*, e0128346.

He, R., Oyoshi, M.K., Garibyan, L., Kumar, L., Ziegler, S.F., and Geha, R.S. (2008). TSLP acts on infiltrating effector T cells to drive allergic skin inflammation. Proc. Natl. Acad. Sci. U.S.a. *105*, 11875–11880.

Henson, M., and Burks, A.W. (2012). The future of food allergy therapeutics. Semin Immunopathol *34*, 703–714.

Hill, D.A., Siracusa, M.C., Abt, M.C., Kim, B.S., Kobuley, D., Kubo, M., Kambayashi, T., Larosa, D.F., Renner, E.D., Orange, J.S., et al. (2012). Commensal bacteria-derived signals regulate basophil hematopoiesis and allergic inflammation. Nat. Med. *18*, 538–546.

Hinds, D.A., McMahon, G., Kiefer, A.K., Do, C.B., Eriksson, N., Evans, D.M., St Pourcain, B., Ring, S.M., Mountain, J.L., Francke, U., et al. (2013). A genome-wide association meta-analysis of self-reported allergy identifies shared and allergy-specific susceptibility loci. Nat. Genet. *45*, 907–911.

Hogan, S.P., Wang, Y.-H., Strait, R., and Finkelman, F.D. (2012). Food-induced anaphylaxis: mast cells as modulators of anaphylactic severity. Semin Immunopathol *34*, 643–653.

Honda, K., and Littman, D.R. (2012). The microbiome in infectious disease and inflammation. Annu. Rev. Immunol. *30*, 759–795.

Hondowicz, B.D., An, D., Schenkel, J.M., Kim, K.S., Steach, H.R., Krishnamurty, A.T., Keitany, G.J., Garza, E.N., Fraser, K.A., Moon, J.J., et al. (2015). Interleukin-2-Dependent Allergen-Specific Tissue-Resident Memory Cells Drive Asthma. Immunity 1–13.

Hooper, L.V., Littman, D.R., and Macpherson, A.J. (2012). Interactions between the microbiota and the immune system. Science *336*, 1268–1273.

Hosken, N.A., Shibuya, K., Heath, A.W., Murphy, K.M., and O'Garra, A. (1995). The effect of antigen dose on CD4+ T helper cell phenotype development in a T cell receptor-alpha beta-transgenic model. J. Exp. Med. *182*, 1579–1584.

Howe, S.J., Mansour, M.R., Schwarzwaelder, K., Bartholomae, C., Hubank, M., Kempski, H., Brugman, M.H., Pike-Overzet, K., Chatters, S.J., de Ridder, D., et al. (2008). Insertional mutagenesis combined with acquired somatic mutations causes leukemogenesis following gene therapy of SCID-X1 patients. J. Clin. Invest. *118*, 3143–3150.

Hruz, P., Straumann, A., Bussmann, C., Heer, P., Simon, H.-U., Zwahlen, M., Beglinger, C., Schoepfer, A.M., Swiss EoE study group (2011). Escalating incidence of eosinophilic esophagitis: a 20-year prospective, population-based study in Olten County, Switzerland. J. Allergy Clin. Immunol. *128*, 1349–1350.e5.

Humblet-Baron, S., Sather, B., Anover, S., Becker-Herman, S., Kasprowicz, D.J., Khim, S., Nguyen, T., Hudkins-Loya, K., Alpers, C.E., Ziegler, S.F., et al. (2007). Wiskott-Aldrich syndrome protein is required for regulatory T cell homeostasis. J. Clin. Invest. *117*, 407–418.

Huseby, E.S., White, J., Crawford, F., Vass, T., Becker, D., Pinilla, C., Marrack, P., and Kappler, J.W. (2005). How the T cell repertoire becomes peptide and MHC specific. Cell *122*, 247–260.

Huter, E.N., Punkosdy, G.A., Glass, D.D., Cheng, L.I., Ward, J.M., and Shevach, E.M. (2008). TGF-beta-induced Foxp3+ regulatory T cells rescue scurfy mice. Eur. J. Immunol. *38*, 1814–1821.

Irvine, A.D., McLean, W.H.I., and Leung, D.Y.M. (2011). Filaggrin mutations associated with skin and allergic diseases. N. Engl. J. Med. *365*, 1315–1327.

Ishikawa, F., Yasukawa, M., Lyons, B., Yoshida, S., Miyamoto, T., Yoshimoto, G., Watanabe, T., Akashi, K., Shultz, L.D., and Harada, M. (2005). Development of functional human blood and immune systems in NOD/SCID/IL2 receptor {gamma} chain(null) mice. Blood *106*, 1565–1573.

Ito, R., Takahashi, T., Katano, I., Kawai, K., Kamisako, T., Ogura, T., Ida-Tanaka, M., Suemizu, H., Nunomura, S., Ra, C., et al. (2013). Establishment of a human allergy model using human IL-3/GM-CSF-transgenic NOG mice. The Journal of Immunology *191*, 2890–2899.

Ivanov, I.I., Atarashi, K., Manel, N., Brodie, E.L., Shima, T., Karaoz, U., Wei, D., Goldfarb, K.C., Santee, C.A., Lynch, S.V., et al. (2009). Induction of intestinal Th17 cells by segmented filamentous bacteria. Cell *139*, 485–498.

Jain, N., Nguyen, H., Chambers, C., and Kang, J. (2010). Dual function of CTLA-4 in regulatory T cells and conventional T cells to prevent multiorgan autoimmunity. Proc. Natl. Acad. Sci. U.S.a. *107*, 1524–1528.

Jensen, E.T., Kappelman, M.D., Kim, H., Ringel-Kulka, T., and Dellon, E.S. (2013). Early life exposures as risk factors for pediatric eosinophilic esophagitis. Journal of Pediatric Gastroenterology and Nutrition *57*, 67–71.

Jin, H., Oyoshi, M.K., Le, Y., Bianchi, T., Koduru, S., Mathias, C.B., Kumar, L., Le Bras, S., Young, D., Collins, M., et al. (2009). IL-21R is essential for epicutaneous sensitization and allergic skin inflammation in humans and mice. J. Clin. Invest. *119*, 47–60.

Johnston, R.J., Choi, Y.S., Diamond, J.A., Yang, J.A., and Crotty, S. (2012). STAT5 is a potent negative regulator of TFH cell differentiation. J. Exp. Med. *209*, 243–250.

Josefowicz, S.Z., Lu, L.-F., and Rudensky, A.Y. (2012a). Regulatory T cells: mechanisms of differentiation and function. Annu. Rev. Immunol. *30*, 531–564.

Josefowicz, S.Z., Niec, R.E., Kim, H.Y., Treuting, P., Chinen, T., Zheng, Y., Umetsu, D.T., and Rudensky, A.Y. (2012b). Extrathymically generated regulatory T cells control mucosal TH2 inflammation. Nature *482*, 395–399.

Judd, L.M., Heine, R.G., Menheniott, T.R., Buzzelli, J., O'Brien-Simpson, N., Pavlic, D., O'Connor, L., Gazali, Al, K., Hamilton, O., Scurr, M., et al. (2016). Elevated IL-33 expression is associated with pediatric eosinophilic esophagitis, and exogenous IL-33 promotes eosinophilic esophagitis development in mice. AJP: Gastrointestinal and Liver Physiology *310*, G13–G25.

Jung, D., and Alt, F.W. (2004). Unraveling V(D)J recombination; insights into gene regulation. Cell *116*, 299–311.

Jyonouchi, S., Smith, C.L., Saretta, F., Abraham, V., Ruymann, K.R., Modayur-Chandramouleeswaran, P., Wang, M.-L., Spergel, J.M., and Cianferoni, A. (2014). Invariant natural killer T cells in children with eosinophilic esophagitis. Clin. Exp. Allergy *44*, 58–68.

Jyonouchi, S., Abraham, V., Orange, J.S., Spergel, J.M., Gober, L., Dudek, E., Saltzman, R., Nichols, K.E., and Cianferoni, A. (2011). Invariant natural killer T cells from children with versus without food allergy exhibit differential responsiveness to milk-derived sphingomyelin. J. Allergy Clin. Immunol. *128*, 102–109.e113.

Kagalwalla, A.F., Sentongo, T.A., Ritz, S., Hess, T., Nelson, S.P., Emerick, K.M., Melin-Aldana, H., and Li, B.U.K. (2006). Effect of six-food elimination diet on clinical and histologic outcomes in eosinophilic esophagitis. Clin. Gastroenterol. Hepatol. *4*, 1097–1102.

Karasuyama, H., Mukai, K., Obata, K., Tsujimura, Y., and Wada, T. (2011). Nonredundant roles of basophils in immunity. Annu. Rev. Immunol. *29*, 45–69.

Karasuyama, H., Mukai, K., Tsujimura, Y., and Obata, K. (2009). Newly discovered roles for basophils: a neglected minority gains new respect. Nat Rev Immunol *9*, 9–13.

Katzka, D.A. (2014). Unmet diagnostic needs in eosinophilic esophagitis. Dig Dis *32*, 139–142.

Keppler, S.J., Gasparrini, F., Burbage, M., Aggarwal, S., Frederico, B., Geha, R.S., Way, M., Bruckbauer, A., and Batista, F.D. (2015). Wiskott-Aldrich Syndrome Interacting Protein Deficiency Uncovers the Role of the Co-receptor CD19 as a Generic Hub for PI3 Kinase Signaling in B Cells. Immunity *43*, 660–673.

Kettner, A., Kumar, L., Antón, I.M., Sasahara, Y., la Fuente, de, M., Pivniouk, V.I., Falet, H., Hartwig, J.H., and Geha, R.S. (2004). WIP regulates signaling via the high affinity receptor for immunoglobulin E in mast cells. J. Exp. Med. *199*, 357–368.

Kettner, A., Pivniouk, V., Kumar, L., Falet, H., Lee, J.-S., Mulligan, R., and Geha, R.S. (2003). Structural requirements of SLP-76 in signaling via the high-affinity immunoglobulin E receptor (Fc epsilon RI) in mast cells. Mol. Cell. Biol. *23*, 2395–2406.

Khan, A.R., Amu, S., Saunders, S.P., Hams, E., Blackshields, G., Leonard, M.O., Weaver, C.T., Sparwasser, T., Sheils, O., and Fallon, P.G. (2015). Ligation of TLR7 on CD19(+) CD1d(hi) B cells suppresses allergic lung inflammation via regulatory T cells. Eur. J. Immunol. *45*, 1842–1854.

Khodoun, M.V., Strait, R., Armstrong, L., Yanase, N., and Finkelman, F.D. (2011). Identification of markers that distinguish IgE- from IgG-mediated anaphylaxis. Proc Natl Acad Sci USA *108*, 12413–12418.

Kim, B.S., Siracusa, M.C., Saenz, S.A., Noti, M., Monticelli, L.A., Sonnenberg, G.F., Hepworth, M.R., Van Voorhees, A.S., Comeau, M.R., and Artis, D. (2013). TSLP elicits IL-33-independent innate lymphoid cell responses to promote skin inflammation. Sci Transl Med *5*, 170ra16–170ra16.

Kim, B.S., Wang, K., Siracusa, M.C., Saenz, S.A., Brestoff, J.R., Monticelli, L.A., Noti, M., Tait Wojno, E.D., Fung, T.C., Kubo, M., et al. (2014). Basophils promote innate lymphoid cell responses in inflamed skin. The Journal of Immunology *193*, 3717–3725.

Kim, C.H., Kunkel, E.J., Boisvert, J., Johnston, B., Campbell, J.J., Genovese, M.C., Greenberg, H.B., and Butcher, E.C. (2001). Bonzo/CXCR6 expression defines type 1-polarized T-cell subsets with extralymphoid tissue homing potential. J. Clin. Invest. *107*, 595–601.

Kim, C.H., Johnston, B., and Butcher, E.C. (2002). Trafficking machinery of NKT cells: shared and differential chemokine receptor expression among V alpha 24(+)V beta 11(+) NKT cell subsets with distinct cytokine-producing capacity. Blood *100*, 11–16.

Kim, H.P., Vance, R.B., Shaheen, N.J., and Dellon, E.S. (2012). The prevalence and diagnostic utility of endoscopic features of eosinophilic esophagitis: a meta-analysis. Clin. Gastroenterol. Hepatol. *10*, 988–96.e5.

Kim, H.-J., Guo, D., and Sant'Angelo, D.B. (2005). Coevolution of TCR-MHC interactions: conserved MHC tertiary structure is not sufficient for interactions with the TCR. Proc. Natl. Acad. Sci. U.S.a. *102*, 7263–7267.

Kinnunen, T., Chamberlain, N., Morbach, H., Choi, J., Kim, S., Craft, J., Mayer, L., Cancrini, C., Passerini, L., Bacchetta, R., et al. (2013). Accumulation of peripheral autoreactive B cells in the absence of functional human regulatory T cells. Blood *121*, 1595–1603.

Kjellman, N.M., Johansson, S.G., and Roth, A. (1976). Serum IgE levels in healthy children quantified by a sandwich technique (PRIST). Clin. Allergy *6*, 51–59.

Kolhatkar, N.S., Brahmandam, A., Thouvenel, C.D., Becker-Herman, S., Jacobs, H.M., Schwartz, M.A., Allenspach, E.J., Khim, S., Panigrahi, A.K., Luning Prak, E.T., et al. (2015). Altered BCR and TLR signals promote enhanced positive selection of autoreactive transitional B cells in Wiskott-Aldrich syndrome. J. Exp. Med. *212*, 1663–1677.

Kopp, M.V., Stenglein, S., Kamin, W., Friedrichs, F., Berg, von, A., Zielen, S., Hamelmann, E., Wahn, U., and Kuehr, J. (2007). Omalizumab (Xolair) in children with seasonal allergic rhinitis: leukotriene release as a potential in vitro parameter to monitor therapeutic effects. Pediatr Allergy Immunol *18*, 523–527.

Koprak, S., Matheravidathu, S., Springer, M., Gould, S., and Dumont, F.J. (2003). Down-regulation of cell surface CXCR6 expression during T cell activation is predominantly mediated by calcineurin. Cell. Immunol. *223*, 1–12.

Kottyan, L.C., Davis, B.P., Sherrill, J.D., Liu, K., Rochman, M., Kaufman, K., Weirauch, M.T., Vaughn, S., Lazaro, S., Rupert, A.M., et al. (2014). Genome-wide association analysis of eosinophilic esophagitis provides insight into the tissue specificity of this allergic disease. Nat. Genet. *46*, 895–900.

Kracker, S., and Radbruch, A. (2004). Immunoglobulin class switching: in vitro induction and analysis. Methods Mol. Biol. *271*, 149–159.

Kraft, S., and Kinet, J.-P. (2007). New developments in FcepsilonRI regulation, function and inhibition. Nat Rev Immunol *7*, 365–378.

Kronenberg, M. (2005). Toward an understanding of NKT cell biology: progress and paradoxes. Annu. Rev. Immunol. *23*, 877–900.

Kumlin, M., Stensvad, F., Larsson, L., Dahlén, B., and Dahlén, S.E. (1995). Validation and application of a new simple strategy for measurements of urinary leukotriene E4 in humans. Clin. Exp. Allergy *25*, 467–479.

Kummeling, I., and Thijs, C. (2008). Reverse causation and confounding-by-indication: do they or do they not explain the association between childhood antibiotic treatment and subsequent development of respiratory illness? Clin. Exp. Allergy *38*, 1249–1251.

Kummeling, I., Stelma, F.F., Dagnelie, P.C., Snijders, B.E.P., Penders, J., Huber, M., van Ree, R., van den Brandt, P.A., and Thijs, C. (2007). Early life exposure to antibiotics and the subsequent development of eczema, wheeze, and allergic sensitization in the first 2 years of life: the KOALA Birth Cohort Study. Pediatrics *119*, e225–e231.

Lack, G., Fox, D., Northstone, K., Golding, J., Avon Longitudinal Study of Parents and Children Study Team (2003). Factors associated with the development of peanut allergy in childhood. N. Engl. J. Med. *348*, 977–985.

Lahl, K., Loddenkemper, C., Drouin, C., Freyer, J., Arnason, J., Eberl, G., Hamann, A., Wagner, H., Huehn, J., and Sparwasser, T. (2007). Selective depletion of Foxp3+ regulatory T cells induces a scurfy-like disease. J. Exp. Med. *204*, 57–63.

Lampasona, V., Passerini, L., Barzaghi, F., Lombardoni, C., Bazzigaluppi, E., Brigatti, C., Bacchetta, R., and Bosi, E. (2013). Autoantibodies to harmonin and villin are diagnostic markers in children with IPEX syndrome. PLoS ONE *8*, e78664.

Lane, P.W. (1972). Two new mutations in linkage group XVI of the house mouse. Flaky tail and varitint-waddler-J. J. Hered. *63*, 135–140.

Lanzi, G., Moratto, D., Vairo, D., Masneri, S., Delmonte, O., Paganini, T., Parolini, S., Tabellini, G., Mazza, C., Savoldi, G., et al. (2012). A novel primary human immunodeficiency due to deficiency in the WASP-interacting protein WIP. J. Exp. Med. *209*, 29–34.

Lathrop, S.K., Bloom, S.M., Rao, S.M., Nutsch, K., Lio, C.-W., Santacruz, N., Peterson, D.A., Stappenbeck, T.S., and Hsieh, C.-S. (2011). Peripheral education of the immune system by colonic commensal microbiota. Nature *478*, 250–254.

Lee, Y.J., Holzapfel, K.L., Zhu, J., Jameson, S.C., and Hogquist, K.A. (2013). Steady-state production of IL-4 modulates immunity in mouse strains and is determined by lineage diversity of iNKT cells. Nat Immunol *14*, 1146–1154.

Leitenberg, D., Boutin, Y., Constant, S., and Bottomly, K. (1998). CD4 regulation of TCR signaling and T cell differentiation following stimulation with peptides of different affinities for the TCR. J. Immunol. *161*, 1194–1203.

León, B., Bradley, J.E., Lund, F.E., Randall, T.D., and Ballesteros-Tato, A. (2014). FoxP3+ regulatory T cells promote influenza-specific Tfh responses by controlling IL-2 availability. Nat Commun *5*, 3495.

Lepus, C.M., Gibson, T.F., Gerber, S.A., Kawikova, I., Szczepanik, M., Hossain, J., Ablamunits, V., Kirkiles-Smith, N., Herold, K.C., Donis, R.O., et al. (2009). Comparison of human fetal liver, umbilical cord blood, and adult blood hematopoietic stem cell engraftment in NOD-scid/gammac-/-, Balb/c-Rag1-/-gammac-/-, and C.B-17-scid/bg immunodeficient mice. Hum. Immunol. *70*, 790–802.

Leung, D.Y.M. (2013). New insights into atopic dermatitis: role of skin barrier and immune dysregulation. Allergol Int *62*, 151–161.

Levine, A.G., Arvey, A., Jin, W., and Rudensky, A.Y. (2014). Continuous requirement for the TCR in regulatory T cell function. Nat Immunol *15*, 1070–1078.

Lexmond, W.S., Pardo, M., Rooney, K., Goettel, J.A., Snapper, S.B., Yen, E.H., Dehlink, E., Nurko, S., and Fiebiger, E. (2013). Elevated levels of leukotriene C4 synthase mRNA distinguish a subpopulation of eosinophilic oesophagitis patients. Clin. Exp. Allergy *43*, 902–913.

Lexmond, W.S., Neves, J.F., Nurko, S., Olszak, T., Exley, M.A., Blumberg, R.S., and Fiebiger, E. (2014). Involvement of the iNKT cell pathway is associated with early-onset eosinophilic esophagitis and response to allergen avoidance therapy. Am J Gastroenterology *109*, 646–657.

Lexmond, W., Mee, der, J.V., Ruiter, F., Platzer, B., Stary, G., Yen, E.H., Dehlink, E., Nurko, S., and Fiebiger, E. (2011). Development and validation of a standardized ELISA for the detection of soluble Fc-epsilon-RI in human serum. Journal of Immunological Methods *373*, 192–199.

Leyva-Castillo, J.M., Hener, P., Michea, P., Karasuyama, H., Chan, S., Soumelis, V., and Li, M. (2013). Skin thymic stromal lymphopoietin initiates Th2 responses through an orchestrated immune cascade. Nat Commun *4*, 2847.

Li, X., and Zheng, Y. (2015). Regulatory T cell identity: formation and maintenance. Trends Immunol.

Liacouras, C.A., Furuta, G.T., Hirano, I., Atkins, D., Attwood, S.E., Bonis, P.A., Burks, A.W., Chehade, M., Collins, M.H., Dellon, E.S., et al. (2011). Eosinophilic esophagitis: updated consensus recommendations for children and adults. J. Allergy Clin. Immunol. *128*, 3–20.e6; quiz21–quiz22.

Liacouras, C.A., Spergel, J.M., Ruchelli, E., Verma, R., Mascarenhas, M., Semeao, E., Flick, J., Kelly, J., Brown-Whitehorn, T., Mamula, P., et al. (2005). Eosinophilic esophagitis: a 10-year experience in 381 children. Clin. Gastroenterol. Hepatol. *3*, 1198–1206.

Liacouras, C.A., Spergel, J., and Gober, L.M. (2014). Eosinophilic esophagitis: clinical presentation in children. Gastroenterol. Clin. North Am. *43*, 219–229.

Lin, W., Truong, N., Grossman, W.J., Haribhai, D., Williams, C.B., Wang, J., Martín, M.G., and Chatila, T.A. (2005). Allergic dysregulation and hyperimmunoglobulinemia E in Foxp3 mutant mice. J. Allergy Clin. Immunol. *116*, 1106–1115.

Linder, S., Nelson, D., Weiss, M., and Aepfelbacher, M. (1999). Wiskott-Aldrich syndrome protein regulates podosomes in primary human macrophages. Proc. Natl. Acad. Sci. U.S.a. *96*, 9648–9653.

Linterman, M.A., Pierson, W., Lee, S.K., Kallies, A., Kawamoto, S., Rayner, T.F., Srivastava, M., Divekar, D.P., Beaton, L., Hogan, J.J., et al. (2011). Foxp3+ follicular regulatory T cells control the germinal center response. Nat. Med. *17*, 975–982.

Lisbonne, M., Diem, S., de Castro Keller, A., Lefort, J., Araujo, L.M., Hachem, P., Fourneau, J.-M., Sidobre, S., Kronenberg, M., Taniguchi, M., et al. (2003). Cutting edge: invariant V alpha 14 NKT cells are required for allergen-induced airway inflammation and hyperreactivity in an experimental asthma model. J. Immunol. *171*, 1637–1641.

Liston, A., Enders, A., and Siggs, O.M. (2008). Unravelling the association of partial T-cell immunodeficiency and immune dysregulation. Nat Rev Immunol *8*, 545–558.

Liu, A.H., Jaramillo, R., Sicherer, S.H., Wood, R.A., Bock, S.A., Burks, A.W., Massing, M., Cohn, R.D., and Zeldin, D.C. (2010). National prevalence and risk factors for food allergy and relationship to asthma: results from the National Health and Nutrition Examination Survey 2005-2006. J. Allergy Clin. Immunol. *126*, 798–806.e13.

Liu, Z., Gerner, M.Y., van Panhuys, N., Levine, A.G., Rudensky, A.Y., and Germain, R.N. (2015). Immune homeostasis enforced by co-localized effector and regulatory T cells. Nature *528*, 225–230.

Locci, M., Draghici, E., Marangoni, F., Bosticardo, M., Catucci, M., Aiuti, A., Cancrini, C., Marodi, L., Espanol, T., Bredius, R.G.M., et al. (2009). The Wiskott-Aldrich syndrome protein is required for iNKT cell maturation and function. J. Exp. Med. *206*, 735–742.

Longo, G., Berti, I., Burks, A.W., Krauss, B., and Barbi, E. (2013). IgE-mediated food allergy in children. Lancet *382*, 1656–1664.

López, S.I., Ciocca, M., Oleastro, M., Cuarterolo, M.L., Rocca, A., de Dávila, M.T.G., Roy, A., Fernández, M.C., Nievas, E., Bosaleh, A., et al. (2011). Autoimmune hepatitis type 2 in a child with IPEX syndrome. Journal of Pediatric Gastroenterology and Nutrition *53*, 690–693.

Lucendo, A.J., Arias, Á., and Tenias, J.M. (2014). Relation between eosinophilic esophagitis and oral immunotherapy for food allergy: a systematic review with meta-analysis. Ann. Allergy Asthma Immunol. *113*, 624–629.

Lucendo, A.J., Arias, Á., González-Cervera, J., Yagüe-Compadre, J.L., Guagnozzi, D., Angueira, T., Jiménez-Contreras, S., González-Castillo, S., Rodríguez-Domíngez, B., De Rezende, L.C., et al. (2013). Empiric 6-food elimination diet induced and maintained prolonged remission in patients

with adult eosinophilic esophagitis: a prospective study on the food cause of the disease. J. Allergy Clin. Immunol. *131*, 797–804.

Lucendo, A.J., De Rezende, L.C., Jiménez-Contreras, S., Yagüe-Compadre, J.L., González-Cervera, J., Mota-Huertas, T., Guagnozzi, D., Angueira, T., González-Castillo, S., and Arias, Á. (2011). Montelukast was inefficient in maintaining steroid-induced remission in adult eosinophilic esophagitis. Dig Dis Sci *56*, 3551–3558.

Luzzati, A.L., and Jacobson, E.B. (1972). Serum immunoglobulin levels in nude mice. Eur. J. Immunol. *2*, 473–474.

Macpherson, A.J., and McCoy, K.D. (2015). Standardised animal models of host microbial mutualism. Mucosal Immunol *8*, 476–486.

Maillard, M.H., Cotta-de-Almeida, V., Takeshima, F., Nguyen, D.D., Michetti, P., Nagler, C., Bhan, A.K., and Snapper, S.B. (2007). The Wiskott-Aldrich syndrome protein is required for the function of CD4(+) CD25(+)Foxp3(+) regulatory T cells. J. Exp. Med. *204*, 381–391.

Male, D., Brostoff, J., Roth, D., and Roitt, I. (2006). Immunology (Mosby Elsevier).

Malinova, D., Fritzsche, M., Nowosad, C.R., Armer, H., Munro, P.M.G., Blundell, M.P., Charras, G., Tolar, P., Bouma, G., and Thrasher, A.J. (2015). WASp-dependent actin cytoskeleton stability at the dendritic cell immunological synapse is required for extensive, functional T cell contacts. J. Leukoc. Biol. jlb.2A0215–050RR.

Mani, M., Venkatasubrahmanyam, S., Sanyal, M., Levy, S., Butte, A., Weinberg, K., and Jahn, T. (2009). Wiskott-Aldrich syndrome protein is an effector of Kit signaling. Blood *114*, 2900–2908.

Marangoni, F., Trifari, S., Scaramuzza, S., Panaroni, C., Martino, S., Notarangelo, L.D., Baz, Z., Metin, A., Cattaneo, F., Villa, A., et al. (2007). WASP regulates suppressor activity of human and murine CD4(+) CD25(+)FOXP3(+) natural regulatory T cells. J. Exp. Med. *204*, 369–380.

Marichal, T., Starkl, P., Reber, L.L., Kalesnikoff, J., Oettgen, H.C., Tsai, M., Metz, M., and Galli, S.J. (2013). A Beneficial Role for Immunoglobulin E in Host Defense against Honeybee Venom. Immunity *39*, 963–975.

Marodon, G., Desjardins, D., Mercey, L., Baillou, C., Parent, P., Manuel, M., Caux, C., Bellier, B., Pasqual, N., and Klatzmann, D. (2009). High diversity of the immune repertoire in humanized NOD.SCID.gamma c-/- mice. Eur. J. Immunol. *39*, 2136–2145.

Marone, G., Albini, F., di Martino, L., Quattrin, S., Poto, S., and Condorelli, M. (1986). The Wiskott-Aldrich syndrome: studies of platelets, basophils and polymorphonuclear leucocytes. Br. J. Haematol. *62*, 737–745.

Martin, H., Reuter, S., Dehzad, N., Heinz, A., Bellinghausen, I., Saloga, J., Haasler, I., Korn, S., Jonuleit, H., Buhl, R., et al. (2012). CD4-mediated regulatory T-cell activation inhibits the development of disease in a humanized mouse model of allergic airway disease. J. Allergy Clin. Immunol. *129*, 521–8–528.e1–7.

Massaad, M.J., Ramesh, N., and Geha, R.S. (2013). Wiskott-Aldrich syndrome: a comprehensive review. Ann. N. Y. Acad. Sci. *1285*, 26–43.

Masterson, J.C., Furuta, G.T., and Lee, J.J. (2011). Update on clinical and immunological features of eosinophilic gastrointestinal diseases. Curr. Opin. Gastroenterol. *27*, 515–522.

Matalon, O., Reicher, B., and Barda-Saad, M. (2013). Wiskott-Aldrich syndrome protein - dynamic regulation of actin homeostasis: from activation through function and signal termination in T lymphocytes. Immunol. Rev. *256*, 10–29.

Mauri, C., and Bosma, A. (2012). Immune regulatory function of B cells. Annu. Rev. Immunol. *30*, 221–241.

Mayer, C.T., Tian, L., Hesse, C., Kuhl, A.A., Swallow, M., Kruse, F., Thiele, M., Gershwin, M.E., Liston, A., and Sparwasser, T. (2014). Anti-CD4 treatment inhibits autoimmunity in scurfy mice through the attenuation of co-stimulatory signals. J. Autoimmun. *50*, 23–32.

McCoy, K.D., Harris, N.L., Diener, P., Hatak, S., Odermatt, B., Hangartner, L., Senn, B.M., Marsland, B.J., Geuking, M.B., Hengartner, H., et al. (2006). Natural IgE production in the absence of MHC Class II cognate help. Immunity *24*, 329–339.

McGowan, E.C., and Keet, C.A. (2013). Prevalence of self-reported food allergy in the National Health and Nutrition Examination Survey (NHANES) 2007-2010. J. Allergy Clin. Immunol. *132*, 1216–1219.e5.

McKenzie, R., Fried, M.W., Sallie, R., Conjeevaram, H., Di Bisceglie, A.M., Park, Y., Savarese, B., Kleiner, D., Tsokos, M., and Luciano, C. (1995). Hepatic failure and lactic acidosis due to fialuridine (FIAU), an investigational nucleoside analogue for chronic hepatitis B. N. Engl. J. Med. *333*, 1099–1105.

Melkus, M.W., Estes, J.D., Padgett-Thomas, A., Gatlin, J., Denton, P.W., Othieno, F.A., Wege, A.K., Haase, A.T., and Garcia, J.V. (2006). Humanized mice mount specific adaptive and innate immune responses to EBV and TSST-1. Nat. Med. *12*, 1316–1322.

Meyer-Bahlburg, A., Becker-Herman, S., Humblet-Baron, S., Khim, S., Weber, M., Bouma, G., Thrasher, A.J., Batista, F.D., and Rawlings, D.J. (2008). Wiskott-Aldrich syndrome protein deficiency in B cells results in impaired peripheral homeostasis. Blood *112*, 4158–4169.

Mikhail, I., and Sampson, H. (2016). Eosinophilic Gastrointestinal Diseases. J Allergy Clin Immunol Pract *4*, 369–370.

Milner, J.D., Fazilleau, N., McHeyzer-Williams, M., and Paul, W. (2010). Cutting edge: lack of high affinity competition for peptide in polyclonal CD4+ responses unmasks IL-4 production. The Journal of Immunology *184*, 6569–6573.

Milner, J.D., Vogel, T.P., Forbes, L., Ma, C.A., Stray-Pedersen, A., Niemela, J.E., Lyons, J.J., Engelhardt, K.R., Zhang, Y., Topcagic, N., et al. (2015). Early-onset lymphoproliferation and autoimmunity caused by germline STAT3 gain-of-function mutations. Blood *125*, 591–599.

Milner, J.D., Ward, J.M., Keane-Myers, A., and Paul, W.E. (2007). Lymphopenic mice reconstituted with limited repertoire T cells develop severe, multiorgan, Th2-associated inflammatory disease. Proc. Natl. Acad. Sci. U.S.a. *104*, 576–581.

Mirotti, L., Florsheim, E., Rundqvist, L., Larsson, G., Spinozzi, F., Leite-de-Moraes, M., Russo, M., and Alcocer, M. (2013). Lipids are required for the development of Brazil nut allergy: the role of mouse and human iNKT cells. Allergy *68*, 74–83.

Mishra, A., Hogan, S.P., Brandt, E.B., and Rothenberg, M.E. (2001). An etiological role for aeroallergens and eosinophils in experimental esophagitis. J. Clin. Invest. *107*, 83–90.

Mishra, A., Wang, M., Pemmaraju, V.R., Collins, M.H., Fulkerson, P.C., Abonia, J.P., Blanchard, C., Putnam, P.E., and Rothenberg, M.E. (2008). Esophageal remodeling develops as a consequence of tissue specific IL-5-induced eosinophilia. Gastroenterology *134*, 204–214.

Molodecky, N.A., Soon, I.S., Rabi, D.M., Ghali, W.A., Ferris, M., Chernoff, G., Benchimol, E.I., Panaccione, R., Ghosh, S., Barkema, H.W., et al. (2012). Increasing incidence and prevalence of the inflammatory bowel diseases with time, based on systematic review. Gastroenterology *142*, 46–54.e42–quize30.

Molofsky, A.B., Savage, A.K., and Locksley, R.M. (2015). Interleukin-33 in Tissue Homeostasis, Injury, and Inflammation. Immunity *42*, 1005–1019.

Mond, J.J., Scher, I., Cossman, J., Kessler, S., Mongini, P.K., Hansen, C., Finkelman, F.D., and Paul, W.E. (1982). Role of the thymus in directing the development of a subset of B lymphocytes. J. Exp. Med. *155*, 924–936.

Moniaga, C.S., Egawa, G., Kawasaki, H., Hara-Chikuma, M., Honda, T., Tanizaki, H., Nakajima, S., Otsuka, A., Matsuoka, H., Kubo, A., et al. (2010). Flaky tail mouse denotes human atopic dermatitis in the steady state and by topical application with Dermatophagoides pteronyssinus extract. Am. J. Pathol. *176*, 2385–2393.

Moon, C., Baldridge, M.T., Wallace, M.A., Burnham, C.-A.D., Virgin, H.W., and Stappenbeck, T.S. (2015). Vertically transmitted faecal IgA levels determine extra-chromosomal phenotypic variation. Nature *521*, 90–93.

Morales-Tirado, V., Sojka, D.K., Katzman, S.D., Lazarski, C.A., Finkelman, F.D., Urban, J.F., and Fowell, D.J. (2010). Critical requirement for the Wiskott-Aldrich syndrome protein in Th2 effector function. Blood *115*, 3498–3507.

Moratto, D., Giliani, S., Bonfim, C., Mazzolari, E., Fischer, A., Ochs, H.D., Cant, A.J., Thrasher, A.J., Cowan, M.J., Albert, M.H., et al. (2011). Long-term outcome and lineage-specific chimerism in 194 patients with Wiskott-Aldrich syndrome treated by hematopoietic cell transplantation in the period 1980-2009: an international collaborative study. Blood *118*, 1675–1684.

Mori, M., Beatty, P.G., Graves, M., Boucher, K.M., and Milford, E.L. (1997). HLA gene and haplotype frequencies in the North American population: the National Marrow Donor Program Donor Registry. Transplantation *64*, 1017–1027.

Morita, H., Arae, K., Unno, H., Toyama, S., Motomura, K., Matsuda, A., Suto, H., Okumura, K., Sudo, K., Takahashi, T., et al. (2015). IL-25 and IL-33 Contribute to Development of Eosinophilic Airway Inflammation in Epicutaneously Antigen-Sensitized Mice. PLoS ONE *10*, e0134226.

Motomura, Y., Morita, H., Moro, K., Nakae, S., Artis, D., Endo, T.A., Kuroki, Y., Ohara, O., Koyasu, S., and Kubo, M. (2014). Basophil-derived interleukin-4 controls the function of natural helper cells, a member of ILC2s, in lung inflammation. Immunity *40*, 758–771.

Moulding, D.A., Record, J., Malinova, D., and Thrasher, A.J. (2013). Actin cytoskeletal defects in immunodeficiency. Immunol. Rev. *256*, 282–299.

Mukai, K., Matsuoka, K., Taya, C., Suzuki, H., Yokozeki, H., Nishioka, K., Hirokawa, K., Etori, M., Yamashita, M., Kubota, T., et al. (2005). Basophils play a critical role in the development of IgE-mediated chronic allergic inflammation independently of T cells and mast cells. Immunity *23*, 191–202.

Mukherjee, S., and Thrasher, A.J. (2013). Gene therapy for PIDs: progress, pitfalls and prospects. Gene *525*, 174–181.

Mulder, D.J., and Justinich, C.J. (2011). Understanding eosinophilic esophagitis: the cellular and molecular mechanisms of an emerging disease. Mucosal Immunol *4*, 139–147.

Murk, W., Risnes, K.R., and Bracken, M.B. (2011). Prenatal or early-life exposure to antibiotics and risk of childhood asthma: a systematic review. Pediatrics *127*, 1125–1138.

Murphy, R.C., and Gijón, M.A. (2007). Biosynthesis and metabolism of leukotrienes. Biochem. J. *405*, 379–395.

Mutalithas, K., Croudace, J., Guillen, C., Siddiqui, S., Thickett, D., Wardlaw, A., Lammas, D., and Brightling, C. (2007). Bronchoalveolar lavage invariant natural killer T cells are not increased in asthma. J. Allergy Clin. Immunol. *119*, 1274–1276.

Nakajima, S., Igyártó, B.Z., Honda, T., Egawa, G., Otsuka, A., Hara-Chikuma, M., Watanabe, N., Ziegler, S.F., Tomura, M., Inaba, K., et al. (2012). Langerhans cells are critical in epicutaneous sensitization with protein antigen via thymic stromal lymphopoietin receptor signaling. J. Allergy Clin. Immunol. *129*, 1048–55.e6.

Nakashima, C., Otsuka, A., Kitoh, A., Honda, T., Egawa, G., Nakajima, S., Nakamizo, S., Arita, M., Kubo, M., Miyachi, Y., et al. (2014). Basophils regulate the recruitment of eosinophils in a murine model of irritant contact dermatitis. J. Allergy Clin. Immunol. *134*, 100–107.

Neustrom, M.R., and Friesen, C. (1999). Treatment of eosinophilic gastroenteritis with montelukast. J. Allergy Clin. Immunol. *104*, 506.

Nguyen, D.D., Maillard, M.H., Cotta-de-Almeida, V., Mizoguchi, E., Klein, C., Fuss, I., Nagler, C., Mizoguchi, A., Bhan, A.K., and Snapper, S.B. (2007). Lymphocyte-dependent and Th2 cytokine-associated colitis in mice deficient in Wiskott-Aldrich syndrome protein. Gastroenterology *133*, 1188–1197.

Nguyen, D.D., Muthupalani, S., Goettel, J.A., Eston, M.A., Mobley, M., Taylor, N.S., McCabe, A., Marin, R., Snapper, S.B., and Fox, J.G. (2013). Colitis and Colon Cancer in WASP-Deficient Mice Require Helicobacter Species. Inflamm Bowel Dis.

Nguyen, D.D., Wurbel, M.-A., Goettel, J.A., Eston, M.A., Ahmed, O.S., Marin, R., Boden, E.K., Villablanca, E.J., Paidassi, H., Ahuja, V., et al. (2012). Wiskott-Aldrich syndrome protein deficiency in innate immune cells leads to mucosal immune dysregulation and colitis in mice. Gastroenterology *143*, 719–729.e2.

Nielsen, J.A., Lager, D.J., Lewin, M., Rendon, G., and Roberts, C.A. (2014). The optimal number of biopsy fragments to establish a morphologic diagnosis of eosinophilic esophagitis. Am J Gastroenterology *109*, 515–520.

Nikolov, N.P., Shimizu, M., Cleland, S., Bailey, D., Aoki, J., Strom, T., Schwartzberg, P.L., Candotti, F., and Siegel, R.M. (2010). Systemic autoimmunity and defective Fas ligand secretion in the absence of the Wiskott-Aldrich syndrome protein. Blood *116*, 740–747.

Niranjan, R., Mavi, P., Rayapudi, M., Dynda, S., and Mishra, A. (2013a). Pathogenic role of mast cells in experimental eosinophilic esophagitis. AJP: Gastrointestinal and Liver Physiology *304*, G1087–G1094.

Niranjan, R., Rayapudi, M., Mishra, A., Dutt, P., Dynda, S., and Mishra, A. (2013b). Pathogenesis of allergen-induced eosinophilic esophagitis is independent of interleukin (IL)-13. Immunol. Cell Biol. *91*, 408–415.

Noel, R.J., Putnam, P.E., and Rothenberg, M.E. (2004). Eosinophilic esophagitis. N. Engl. J. Med. *351*, 940–941.

Norvell, J.M., Venarske, D., and Hummell, D.S. (2007). Eosinophilic esophagitis: an allergist's approach. Ann. Allergy Asthma Immunol. *98*, 207–214; quiz214–quiz217, 238.

Notarangelo, L.D. (2013). Functional T cell immunodeficiencies (with T cells present). Annu. Rev. Immunol. *31*, 195–225.

Noti, M., Kim, B.S., Siracusa, M.C., Rak, G.D., Kubo, M., Moghaddam, A.E., Sattentau, Q.A., Comeau, M.R., Spergel, J.M., and Artis, D. (2014). Exposure to food allergens through inflamed skin promotes intestinal food allergy through the thymic stromal lymphopoietin-basophil axis. J. Allergy Clin. Immunol.

Noti, M., Wojno, E.D.T., Kim, B.S., Siracusa, M.C., Giacomin, P.R., Nair, M.G., Benitez, A.J., Ruymann, K.R., Muir, A.B., Hill, D.A., et al. (2013). Thymic stromal lymphopoietin-elicited basophil responses promote eosinophilic esophagitis. Nat. Med. *19*, 1005–1013.

Novak, N., and Bieber, T. (2003). Allergic and nonallergic forms of atopic diseases. J. Allergy Clin. Immunol. *112*, 252–262.

Noval Rivas, M., Burton, O.T., Wise, P., Charbonnier, L.-M., Georgiev, P., Oettgen, H.C., Rachid, R., and Chatila, T.A. (2015). Regulatory T cell reprogramming toward a Th2-cell-like lineage impairs oral tolerance and promotes food allergy. Immunity *42*, 512–523.

Noval Rivas, M., Burton, O.T., Wise, P., Zhang, Y.-Q., Hobson, S.A., Garcia-Lloret, M., Chehoud, C., Kuczynski, J., Desantis, T., Warrington, J., et al. (2013). A microbiota signature associated with experimental food allergy promotes allergic sensitization and anaphylaxis. J. Allergy Clin. Immunol. *131*, 201–212.

Nurieva, R.I., Duong, J., Kishikawa, H., Dianzani, U., Rojo, J.M., Ho, I.C., Flavell, R.A., and Dong, C. (2003). Transcriptional regulation of th2 differentiation by inducible costimulator. Immunity *18*, 801–811.

Nurieva, R.I., Podd, A., Chen, Y., Alekseev, A.M., Yu, M., Qi, X., Huang, H., Wen, R., Wang, J., Li, H.S., et al. (2012). STAT5 protein negatively regulates T follicular helper (Tfh) cell generation and function. Journal of Biological Chemistry *287*, 11234–11239.

O'Connell, A.E., Volpi, S., Dobbs, K., Fiorini, C., Tsitsikov, E., de Boer, H., Barlan, I.B., Despotovic, J.M., Espinosa-Rosales, F.J., Hanson, I.C., et al. (2014). Next generation sequencing reveals skewing of the T and B cell receptor repertoires in patients with wiskott-Aldrich syndrome. Front Immunol *5*, 340.

Oh, J., Freeman, A.F., NISC Comparative Sequencing Program, Park, M., Sokolic, R., Candotti, F., Holland, S.M., Segre, J.A., and Kong, H.H. (2013). The altered landscape of the human skin microbiome in patients with primary immunodeficiencies. Genome Res. *23*, 2103–2114.

Ohkura, N., Kitagawa, Y., and Sakaguchi, S. (2013). Development and maintenance of regulatory T cells. Immunity *38*, 414–423.

Ohnmacht, C., Schwartz, C., Panzer, M., Schiedewitz, I., Naumann, R., and Voehringer, D. (2010). Basophils orchestrate chronic allergic dermatitis and protective immunity against helminths. Immunity *33*, 364–374.

Oliphant, C.J., Hwang, Y.Y., Walker, J.A., Salimi, M., Wong, S.H., Brewer, J.M., Englezakis, A., Barlow, J.L., Hams, E., Scanlon, S.T., et al. (2014). MHCII-Mediated Dialog between Group 2 Innate Lymphoid Cells and CD4(+) T Cells Potentiates Type 2 Immunity and Promotes Parasitic Helminth Expulsion. Immunity *41*, 283–295.

Olszak, T., An, D., Zeissig, S., Vera, M.P., Richter, J., Franke, A., Glickman, J.N., Siebert, R., Baron, R.M., Kasper, D.L., et al. (2012). Microbial exposure during early life has persistent effects on natural killer T cell function. Science *336*, 489–493.

Onbasi, K., Sin, A.Z., Doganavsargil, B., Onder, G.F., Bor, S., and Sebik, F. (2005). Eosinophil infiltration of the oesophageal mucosa in patients with pollen allergy during the season. Clin. Exp. Allergy *35*, 1423–1431.

Osborne, N.J., Koplin, J.J., Martin, P.E., Gurrin, L.C., Lowe, A.J., Matheson, M.C., Ponsonby, A.-L., Wake, M., Tang, M.L.K., Dharmage, S.C., et al. (2011). Prevalence of challenge-proven IgE-mediated food allergy using population-based sampling and predetermined challenge criteria in infants. J. Allergy Clin. Immunol. *127*, 668–76.e1–2.

Oxenius, A., Bachmann, M.F., Zinkernagel, R.M., and Hengartner, H. (1998). Virus-specific MHC-class II-restricted TCR-transgenic mice: effects on humoral and cellular immune responses after viral infection. Eur. J. Immunol. *28*, 390–400.

Oyoshi, M.K. (2015). Recent research advances in eosinophilic esophagitis. Curr. Opin. Pediatr. *27*, 741–747.

Oyoshi, M.K., Beaupré, J., Venturelli, N., Lewis, C.N., Iwakura, Y., and Geha, R.S. (2015). Filaggrin deficiency promotes the dissemination of cutaneously inoculated vaccinia virus. J. Allergy Clin. Immunol. *135*, 1511–8.e1516.

Oyoshi, M.K., ElKhal, A., Scott, J.E., Wurbel, M.-A., Hornick, J.L., Campbell, J.J., and Geha, R.S. (2011). Epicutaneous challenge of orally immunized mice redirects antigen-specific gut-homing T cells to the skin. J. Clin. Invest. *121*, 2210–2220.

Oyoshi, M.K., He, R., Kanaoka, Y., ElKhal, A., Kawamoto, S., Lewis, C.N., Austen, K.F., and Geha, R.S. (2012). Eosinophil-derived leukotriene C4 signals via type 2 cysteinyl leukotriene receptor to promote skin fibrosis in a mouse model of atopic dermatitis. Proc Natl Acad Sci USA *109*, 4992–4997.

Oyoshi, M.K., He, R., Kumar, L., Yoon, J., and Geha, R.S. (2009a). Cellular and molecular mechanisms in atopic dermatitis. Adv. Immunol. *102*, 135–226.

Oyoshi, M.K., Larson, R.P., Ziegler, S.F., and Geha, R.S. (2010). Mechanical injury polarizes skin dendritic cells to elicit a T(H)2 response by inducing cutaneous thymic stromal lymphopoietin expression. J. Allergy Clin. Immunol. *126*, 976–84–984.e1–5.

Oyoshi, M.K., Murphy, G.F., and Geha, R.S. (2009b). Filaggrin-deficient mice exhibit TH17-dominated skin inflammation and permissiveness to epicutaneous sensitization with protein antigen. J. Allergy Clin. Immunol. *124*, 485–93–493.e1.

Oyoshi, M.K., Oettgen, H.C., Chatila, T.A., Geha, R.S., and Bryce, P.J. (2014). Food allergy: Insights into etiology, prevention, and treatment provided by murine models. J. Allergy Clin. Immunol. *133*, 309–317.

Ozcan, E., Notarangelo, L.D., and Geha, R.S. (2008). Primary immune deficiencies with aberrant IgE production. J. Allergy Clin. Immunol. *122*, 1054–62–quiz1063–4.

Palanichamy, A., Barnard, J., Zheng, B., Owen, T., Quach, T., Wei, C., Looney, R.J., Sanz, I., and Anolik, J.H. (2009). Novel human transitional B cell populations revealed by B cell depletion therapy. The Journal of Immunology *182*, 5982–5993.

Palm, N.W., Rosenstein, R.K., and Medzhitov, R. (2012). Allergic host defences. Nature *484*, 465–472.

Palmer, C.N.A., Irvine, A.D., Terron-Kwiatkowski, A., Zhao, Y., Liao, H., Lee, S.P., Goudie, D.R., Sandilands, A., Campbell, L.E., Smith, F.J.D., et al. (2006). Common loss-of-function variants of the epidermal barrier protein filaggrin are a major predisposing factor for atopic dermatitis. Nat. Genet. *38*, 441–446.

Papadopoulou, A., Koletzko, S., Heuschkel, R., Dias, J.A., Allen, K.J., Murch, S.H., Chong, S., Gottrand, F., Husby, S., Lionetti, P., et al. (2014). Management guidelines of eosinophilic esophagitis in childhood. Journal of Pediatric Gastroenterology and Nutrition *58*, 107–118.

Parolini, O., Ressmann, G., Haas, O.A., Pawlowsky, J., Gadner, H., Knapp, W., and Holter, W. (1998). X-linked Wiskott-Aldrich syndrome in a girl. N. Engl. J. Med. *338*, 291–295.

Pecaric-Petkovic, T., Didichenko, S.A., Kaempfer, S., Spiegl, N., and Dahinden, C.A. (2009). Human basophils and eosinophils are the direct target leukocytes of the novel IL-1 family member IL-33. Blood *113*, 1526–1534.

Penrose, J.F. (1999). LTC4 synthase. Enzymology, biochemistry, and molecular characterization. Clin Rev Allergy Immunol *17*, 133–152.

Perkin, M.R., Logan, K., Tseng, A., Raji, B., Ayis, S., Peacock, J., Brough, H., Marrs, T., Radulovic, S., Craven, J., et al. (2016). Randomized Trial of Introduction of Allergenic Foods in Breast-Fed Infants. N. Engl. J. Med.

Pfaffl, M.W. (2001). A new mathematical model for relative quantification in real-time RT-PCR. Nucleic Acids Res. *29*, e45.

Pivniouk, V.I., Snapper, S.B., Kettner, A., Alenius, H., Laouini, D., Falet, H., Hartwig, J., Alt, F.W., and Geha, R.S. (2003). Impaired signaling via the high-affinity IgE receptor in Wiskott-Aldrich syndrome protein-deficient mast cells. Int. Immunol. *15*, 1431–1440.

Platts-Mills, T.A.E. (2015). The allergy epidemics: 1870-2010. J. Allergy Clin. Immunol. *136*, 3–13.

Platzer, B., Baker, K., Vera, M.P., Singer, K., Panduro, M., Lexmond, W.S., Turner, D., Vargas, S.O., Kinet, J.P., Maurer, D., et al. (2015a). Dendritic cell-bound IgE functions to restrain allergic inflammation at mucosal sites. Mucosal Immunol *8*, 516–532.

Platzer, B., Stout, M., and Fiebiger, E. (2015b). Functions of dendritic-cell-bound IgE in allergy. Mol. Immunol. *68*, 116–119.

Prasad, G.A., Alexander, J.A., Schleck, C.D., Zinsmeister, A.R., Smyrk, T.C., Elias, R.M., Locke, G.R., and Talley, N.J. (2009). Epidemiology of eosinophilic esophagitis over three decades in Olmsted County, Minnesota. Clin. Gastroenterol. Hepatol. *7*, 1055–1061.

Prete, F., Catucci, M., Labrada, M., Gobessi, S., Castiello, M.C., Bonomi, E., Aiuti, A., Vermi, W., Cancrini, C., Metin, A., et al. (2013). Wiskott-Aldrich syndrome protein-mediated actin dynamics control type-I interferon production in plasmacytoid dendritic cells. J. Exp. Med. *210*, 355–374.

Profet, M. (1991). The function of allergy: immunological defense against toxins. Q Rev Biol *66*, 23–62.

Puck, J.M., and Candotti, F. (2006). Lessons from the Wiskott-Aldrich syndrome. N. Engl. J. Med. *355*, 1759–1761.

Pulecio, J., Tagliani, E., Scholer, A., Prete, F., Fetler, L., Burrone, O.R., and Benvenuti, F. (2008). Expression of Wiskott-Aldrich syndrome protein in dendritic cells regulates synapse formation and activation of naive CD8+ T cells. The Journal of Immunology *181*, 1135–1142.

Pulendran, B., and Artis, D. (2012). New paradigms in type 2 immunity. Science *337*, 431–435.

Rabinovitch, N. (2007). Urinary leukotriene E4. Immunology and Allergy Clinics of North America *27*, 651–664; vii.

Radano, M.C., Yuan, Q., Katz, A., Fleming, J.T., Kubala, S., Shreffler, W., and Keet, C.A. (2014). Cesarean section and antibiotic use found to be associated with eosinophilic esophagitis. J Allergy Clin Immunol Pract *2*, 475–477.e1.

Rajavelu, P., Rayapudi, M., Moffitt, M., Mishra, A., and Mishra, A. (2012). Significance of para-esophageal lymph nodes in food or aeroallergen-induced iNKT cell-mediated experimental eosinophilic esophagitis. AJP: Gastrointestinal and Liver Physiology *302*, G645–G654.

Rajesh, D., Zhou, Y., Jankowska-Gan, E., Roenneburg, D.A., Dart, M.L., Torrealba, J., and Burlingham, W.J. (2010). Th1 and Th17 immunocompetence in humanized NOD/SCID/IL2rgammanull mice. Hum. Immunol. *71*, 551–559.

Ram, G., Lee, J., Ott, M., Brown-Whitehorn, T.F., Cianferoni, A., Shuker, M., Wang, M.-L., Verma, R., Liacouras, C.A., and Spergel, J.M. (2015). Seasonal exacerbation of esophageal eosinophilia in children with eosinophilic esophagitis and allergic rhinitis. Ann. Allergy Asthma Immunol. *115*, 224–228.e1.

Ramsdell, F., and Ziegler, S.F. (2014). FOXP3 and scurfy: how it all began. Nat Rev Immunol *14*, 343–349.

Randall, K.L., Lambe, T., Johnson, A.L., Johnson, A., Treanor, B., Kucharska, E., Domaschenz, H., Whittle, B., Tze, L.E., Enders, A., et al. (2009). Dock8 mutations cripple B cell immunological synapses, germinal centers and long-lived antibody production. Nat Immunol *10*, 1283–1291.

Rao, A., Kamani, N., Filipovich, A., Lee, S.M., Davies, S.M., Dalal, J., and Shenoy, S. (2007). Successful bone marrow transplantation for IPEX syndrome after reduced-intensity conditioning. Blood *109*, 383–385.

Reber, L.L., Marichal, T., and Galli, S.J. (2012). New models for analyzing mast cell functions in vivo. Trends Immunol. *33*, 613–625.

Recher, M., Burns, S.O., la Fuente, de, M.A., Volpi, S., Dahlberg, C., Walter, J.E., Moffitt, K., Mathew, D., Honke, N., Lang, P.A., et al. (2012). B cell-intrinsic deficiency of the Wiskott-Aldrich syndrome

protein (WASp) causes severe abnormalities of the peripheral B-cell compartment in mice. Blood *119*, 2819–2828.

Rengan, R., Ochs, H.D., Sweet, L.I., Keil, M.L., Gunning, W.T., Lachant, N.A., Boxer, L.A., and Omann, G.M. (2000). Actin cytoskeletal function is spared, but apoptosis is increased, in WAS patient hematopoietic cells. Blood *95*, 1283–1292.

Richet, C. (1913). Nobel Lecture.

Rocha, R., Vitor, A.B., Trindade, E., Lima, R., Tavares, M., Lopes, J., and Dias, J.A. (2011). Omalizumab in the treatment of eosinophilic esophagitis and food allergy. Eur. J. Pediatr. *170*, 1471–1474.

Rongvaux, A., Willinger, T., Martinek, J., Strowig, T., Gearty, S.V., Teichmann, L.L., Saito, Y., Marches, F., Halene, S., Palucka, A.K., et al. (2014). Development and function of human innate immune cells in a humanized mouse model. Nat. Biotechnol. *32*, 364–372.

Rothenberg, M.E. (2009). Biology and treatment of eosinophilic esophagitis. Gastroenterology *137*, 1238–1249.

Rothenberg, M.E. (2015). Molecular, genetic, and cellular bases for treating eosinophilic esophagitis. Gastroenterology *148*, 1143–1157.

Rothenberg, M.E., Spergel, J.M., Sherrill, J.D., Annaiah, K., Martin, L.J., Cianferoni, A., Gober, L., Kim, C., Glessner, J., Frackelton, E., et al. (2010). Common variants at 5q22 associate with pediatric eosinophilic esophagitis. Nat. Genet. *42*, 289–291.

Rothenberg, M.E., Wen, T., Greenberg, A., Alpan, O., Enav, B., Hirano, I., Nadeau, K., Kaiser, S., Peters, T., Perez, A., et al. (2015). Intravenous anti-IL-13 mAb QAX576 for the treatment of eosinophilic esophagitis. J. Allergy Clin. Immunol. *135*, 500–507.

Roy-Ghanta, S., Larosa, D.F., and Katzka, D.A. (2008). Atopic characteristics of adult patients with eosinophilic esophagitis. Clin. Gastroenterol. Hepatol. *6*, 531–535.

Ruitenberg, E.J., and Elgersma, A. (1976). Absence of intestinal mast cell response in congenitally athymic mice during Trichinella spiralis infection. Nature *264*, 258–260.

Russell, S.L., Gold, M.J., Hartmann, M., Willing, B.P., Thorson, L., Wlodarska, M., Gill, N., Blanchet, M.-R., Mohn, W.W., McNagny, K.M., et al. (2012). Early life antibiotic-driven changes in microbiota enhance susceptibility to allergic asthma. EMBO Rep. *13*, 440–447.

Sage, P.T., Francisco, L.M., Carman, C.V., and Sharpe, A.H. (2012). The receptor PD-1 controls follicular regulatory T cells in the lymph nodes and blood. Nat Immunol.

Sakaguchi, S., Fukuma, K., Kuribayashi, K., and Masuda, T. (1985). Organ-specific autoimmune diseases induced in mice by elimination of T cell subset. I. Evidence for the active participation of T cells in natural self-tolerance; deficit of a T cell subset as a possible cause of autoimmune disease. J. Exp. Med. *161*, 72–87.

Salimi, M., Barlow, J.L., Saunders, S.P., Xue, L., Gutowska-Owsiak, D., Wang, X., Huang, L.-C., Johnson, D., Scanlon, S.T., McKenzie, A.N.J., et al. (2013). A role for IL-25 and IL-33-driven type-2 innate lymphoid cells in atopic dermatitis. J. Exp. Med. *210*, 2939–2950.

Sallmann, E., Reininger, B., Brandt, S., Duschek, N., Hoflehner, E., Garner-Spitzer, E., Platzer, B., Dehlink, E., Hammer, M., Holcmann, M., et al. (2011). High-affinity IgE receptors on dendritic cells exacerbate Th2-dependent inflammation. The Journal of Immunology *187*, 164–171.

Salo, P.M., Arbes, S.J., Jaramillo, R., Calatroni, A., Weir, C.H., Sever, M.L., Hoppin, J.A., Rose, K.M., Liu, A.H., Gergen, P.J., et al. (2014). Prevalence of allergic sensitization in the United States: Results from the National Health and Nutrition Examination Survey (NHANES) 2005-2006. J. Allergy Clin. Immunol.

Sampson, H.A., Bock, S.A., James, J., Nadeau, K., Oppenheimer, J., Sicherer, S.H., Simon, R.A., Vickery, B.P., Wood, R., Randolph, C., et al. (2014). Food allergy: A practice parameter update-2014. J. Allergy Clin. Immunol. *134*, 1016–1025.e1043.

Sassi, A., Lazaroski, S., Wu, G., Haslam, S.M., Fliegauf, M., Mellouli, F., Patiroglu, T., Unal, E., Ozdemir, M.A., Jouhadi, Z., et al. (2014). Hypomorphic homozygous mutations in phosphoglucomutase 3 (PGM3) impair immunity and increase serum IgE levels. J. Allergy Clin. Immunol. *133*, 1410–9–1419.e1–13.

Savinko, T., Matikainen, S., Saarialho-Kere, U., Lehto, M., Wang, G., Lehtimäki, S., Karisola, P., Reunala, T., Wolff, H., Lauerma, A., et al. (2012). IL-33 and ST2 in atopic dermatitis: expression profiles and modulation by triggering factors. J. Invest. Dermatol. *132*, 1392–1400.

Sawaguchi, M., Tanaka, S., Nakatani, Y., Harada, Y., Mukai, K., Matsunaga, Y., Ishiwata, K., Oboki, K., Kambayashi, T., Watanabe, N., et al. (2012). Role of mast cells and basophils in IgE responses and in allergic airway hyperresponsiveness. The Journal of Immunology *188*, 1809–1818.

Scanlon, S.T., Thomas, S.Y., Ferreira, C.M., Bai, L., Krausz, T., Savage, P.B., and Bendelac, A. (2011). Airborne lipid antigens mobilize resident intravascular NKT cells to induce allergic airway inflammation. J. Exp. Med. *208*, 2113–2124.

Scharschmidt, T.C., Man, M.-Q., Hatano, Y., Crumrine, D., Gunathilake, R., Sundberg, J.P., Silva, K.A., Mauro, T.M., Hupe, M., Cho, S., et al. (2009). Filaggrin deficiency confers a paracellular barrier abnormality that reduces inflammatory thresholds to irritants and haptens. J. Allergy Clin. Immunol. *124*, 496–506–506.e1–6.

Schmitz, J., Owyang, A., Oldham, E., Song, Y., Murphy, E., McClanahan, T.K., Zurawski, G., Moshrefi, M., Qin, J., Li, X., et al. (2005). IL-33, an interleukin-1-like cytokine that signals via the IL-1 receptor-related protein ST2 and induces T helper type 2-associated cytokines. Immunity *23*, 479–490.

Schneider, E., Petit-Bertron, A.-F., Bricard, R., Levasseur, M., Ramadan, A., Girard, J.-P., Herbelin, A., and Dy, M. (2009). IL-33 activates unprimed murine basophils directly in vitro and induces their in vivo expansion indirectly by promoting hematopoietic growth factor production. The Journal of Immunology *183*, 3591–3597.

Schoepfer, A.M., Simon, D., and Straumann, A. (2011). Eosinophilic oesophagitis: latest intelligence. Clin. Exp. Allergy *41*, 630–639.

Schubert, L.A., Jeffery, E., Zhang, Y., Ramsdell, F., and Ziegler, S.F. (2001). Scurfin (FOXP3) acts as a repressor of transcription and regulates T cell activation. J. Biol. Chem. *276*, 37672–37679.

Seok, J., Warren, H.S., Cuenca, A.G., Mindrinos, M.N., Baker, H.V., Xu, W., Richards, D.R., McDonald-Smith, G.P., Gao, H., Hennessy, L., et al. (2013). Genomic responses in mouse models poorly mimic human inflammatory diseases. Proc. Natl. Acad. Sci. U.S.a. *110*, 3507–3512.

Shah, A., Kagalwalla, A.F., Gonsalves, N., Melin-Aldana, H., Li, B.U.K., and Hirano, I. (2009). Histopathologic variability in children with eosinophilic esophagitis. Am J Gastroenterology *104*, 716–721.

Shaw, S.Y., Blanchard, J.F., and Bernstein, C.N. (2010). Association between the use of antibiotics in the first year of life and pediatric inflammatory bowel disease. Am J Gastroenterology *105*, 2687–2692.

Sherrill, J.D., Gao, P.-S., Stucke, E.M., Blanchard, C., Collins, M.H., Putnam, P.E., Franciosi, J.P., Kushner, J.P., Abonia, J.P., Assa'ad, A.H., et al. (2010). Variants of thymic stromal lymphopoietin and its receptor associate with eosinophilic esophagitis. J. Allergy Clin. Immunol. *126*, 160–5.e163.

Shimizu, M., Matsuda, A., Yanagisawa, K., Hirota, T., Akahoshi, M., Inomata, N., Ebe, K., Tanaka, K., Sugiura, H., Nakashima, K., et al. (2005). Functional SNPs in the distal promoter of the ST2 gene are associated with atopic dermatitis. Hum. Mol. Genet. *14*, 2919–2927.

Shultz, L.D., Brehm, M.A., Garcia-Martinez, J.V., and Greiner, D.L. (2012). Humanized mice for immune system investigation: progress, promise and challenges. Nat Rev Immunol *12*, 786–798.

Sicherer, S.H., and Sampson, H.A. (2010). Food allergy. J. Allergy Clin. Immunol. *125*, S116–S125.

Sicherer, S.H., and Sampson, H.A. (2014). Food allergy: Epidemiology, pathogenesis, diagnosis, and treatment. J. Allergy Clin. Immunol. *133*, 291–307–quiz308.

Siggs, O.M., Miosge, L.A., Yates, A.L., Kucharska, E.M., Sheahan, D., Brdicka, T., Weiss, A., Liston, A., and Goodnow, C.C. (2007). Opposing functions of the T cell receptor kinase ZAP-70 in immunity and tolerance differentially titrate in response to nucleotide substitutions. Immunity *27*, 912–926.

Simon, D., Cianferoni, A., Spergel, J.M., Aceves, S., Holbreich, M., Venter, C., Rothenberg, M.E., Terreehorst, I., Muraro, A., Lucendo, A.J., et al. (2016). Eosinophilic esophagitis is characterized by a non-IgE-mediated food hypersensitivity. Allergy *71*, 611–620.

Simon, D., Radonjic-Hösli, S., Straumann, A., Yousefi, S., and Simon, H.-U. (2015). Active eosinophilic esophagitis is characterized by epithelial barrier defects and eosinophil extracellular trap formation. Allergy *70*, 443–452.

Simon, K.L., Anderson, S.M., Garabedian, E.K., Moratto, D., Sokolic, R.A., and Candotti, F. (2014). Molecular and phenotypic abnormalities of B lymphocytes in patients with Wiskott-Aldrich syndrome. J. Allergy Clin. Immunol. *133*, 896–9.e4.

Sims, T.N., Soos, T.J., Xenias, H.S., Dubin-Thaler, B., Hofman, J.M., Waite, J.C., Cameron, T.O., Thomas, V.K., Varma, R., Wiggins, C.H., et al. (2007). Opposing effects of PKCtheta and WASp on symmetry breaking and relocation of the immunological synapse. Cell *129*, 773–785.

Siracusa, M.C., Kim, B.S., Spergel, J.M., and Artis, D. (2013). Basophils and allergic inflammation. J. Allergy Clin. Immunol. *132*, 789–801.

Siracusa, M.C., Saenz, S.A., Hill, D.A., Kim, B.S., Headley, M.B., Doering, T.A., Wherry, E.J., Jessup, H.K., Siegel, L.A., Kambayashi, T., et al. (2011). TSLP promotes interleukin-3-independent basophil haematopoiesis and type 2 inflammation. Nature *477*, 229–233.

Skarnes, W.C., Rosen, B., West, A.P., Koutsourakis, M., Bushell, W., Iyer, V., Mujica, A.O., Thomas, M., Harrow, J., Cox, T., et al. (2011). A conditional knockout resource for the genome-wide study of mouse gene function. Nature *474*, 337–342.

Sleiman, P.M.A., Wang, M.-L., Cianferoni, A., Aceves, S., Gonsalves, N., Nadeau, K., Bredenoord, A.J., Furuta, G.T., Spergel, J.M., and Hakonarson, H. (2014). GWAS identifies four novel eosinophilic esophagitis loci. Nat Commun *5*, 5593.

Snapper, S.B., and Rosen, F.S. (1999). The Wiskott-Aldrich syndrome protein (WASP): roles in signaling and cytoskeletal organization. Annu. Rev. Immunol. *17*, 905–929.

Snapper, S.B., Rosen, F.S., Mizoguchi, E., Cohen, P., Khan, W., Liu, C.H., Hagemann, T.L., Kwan, S.P., Ferrini, R., Davidson, L., et al. (1998). Wiskott-Aldrich syndrome protein-deficient mice reveal a role for WASP in T but not B cell activation. Immunity *9*, 81–91.

Snapper, S.B., Meelu, P., Nguyen, D., Stockton, B.M., Bozza, P., Alt, F.W., Rosen, F.S., Andrian, von, U.H., and Klein, C. (2005). WASP deficiency leads to global defects of directed leukocyte migration in vitro and in vivo. J. Leukoc. Biol. *77*, 993–998.

Sommers, C.L., Park, C.-S., Lee, J., Feng, C., Fuller, C.L., Grinberg, A., Hildebrand, J.A., Lacaná, E., Menon, R.K., Shores, E.W., et al. (2002). A LAT mutation that inhibits T cell development yet induces lymphoproliferation. Science *296*, 2040–2043.

Spergel, J.M. (2005). Eosinophilic oesophagitis and pollen. Clin. Exp. Allergy *35*, 1421–1422.

Spergel, J.M., Mizoguchi, E., Brewer, J.P., Martin, T.R., Bhan, A.K., and Geha, R.S. (1998). Epicutaneous sensitization with protein antigen induces localized allergic dermatitis and hyperresponsiveness to methacholine after single exposure to aerosolized antigen in mice. J. Clin. Invest. *101*, 1614–1622.

Spergel, J.M. (2010). New genetic links in eosinophilic esophagitis. Genome Med *2*, 60.

Spergel, J.M., Andrews, T., Brown-Whitehorn, T.F., Beausoleil, J.L., and Liacouras, C.A. (2005). Treatment of eosinophilic esophagitis with specific food elimination diet directed by a combination of skin prick and patch tests. Ann. Allergy Asthma Immunol. *95*, 336–343.

Spergel, J.M., Brown-Whitehorn, T.F., Beausoleil, J.L., Franciosi, J., Shuker, M., Verma, R., and Liacouras, C.A. (2009). 14 years of eosinophilic esophagitis: clinical features and prognosis. Journal of Pediatric Gastroenterology and Nutrition *48*, 30–36.

Spergel, J.M., Brown-Whitehorn, T.F., Cianferoni, A., Shuker, M., Wang, M.-L., Verma, R., and Liacouras, C.A. (2012). Identification of causative foods in children with eosinophilic esophagitis treated with an elimination diet. J. Allergy Clin. Immunol. *130*, 461–7.e465.

Spits, H., Artis, D., Colonna, M., Diefenbach, A., Di Santo, J.P., Eberl, G., Koyasu, S., Locksley, R.M., McKenzie, A.N.J., Mebius, R.E., et al. (2013). Innate lymphoid cells--a proposal for uniform nomenclature. Nat Rev Immunol *13*, 145–149.

Steckelbroeck, S., Ballmer-Weber, B.K., and Vieths, S. (2008). Potential, pitfalls, and prospects of food allergy diagnostics with recombinant allergens or synthetic sequential epitopes. J. Allergy Clin. Immunol. *121*, 1323–1330.

Stefka, A.T., Feehley, T., Tripathi, P., Qiu, J., McCoy, K., Mazmanian, S.K., Tjota, M.Y., Seo, G.-Y., Cao, S., Theriault, B.R., et al. (2014). Commensal bacteria protect against food allergen sensitization. Proc Natl Acad Sci USA.

Stein, M.L., Collins, M.H., Villanueva, J.M., Kushner, J.P., Putnam, P.E., Buckmeier, B.K., Filipovich, A.H., Assa'ad, A.H., and Rothenberg, M.E. (2006). Anti-IL-5 (mepolizumab) therapy for eosinophilic esophagitis. J. Allergy Clin. Immunol. *118*, 1312–1319.

Straumann, A., Aceves, S.S., Blanchard, C., Collins, M.H., Furuta, G.T., Hirano, I., Schoepfer, A.M., Simon, D., and Simon, H.-U. (2012). Pediatric and adult eosinophilic esophagitis: similarities and differences. Allergy *67*, 477–490.

Straumann, A., Bauer, M., Fischer, B., Blaser, K., and Simon, H.-U. (2001). Idiopathic eosinophilic esophagitis is associated with a T(H)2-type allergic inflammatory response. J. Allergy Clin. Immunol. *108*, 954–961.

Straumann, A. (2009). Clinical evaluation of the adult who has eosinophilic esophagitis. Immunology and Allergy Clinics of North America *29*, 11–18, vii.

Strober, W., Fuss, I.J., and Blumberg, R.S. (2002). The immunology of mucosal models of inflammation. Annu. Rev. Immunol. *20*, 495–549.

Stumphy, J., Al-Zubeidi, D., Guerin, L., Mitros, F., and Rahhal, R. (2011). Observations on use of montelukast in pediatric eosinophilic esophagitis: insights for the future. Dis. Esophagus *24*, 229–234.

Suntharalingam, G., Perry, M.R., Ward, S., Brett, S.J., Castello-Cortes, A., Brunner, M.D., and Panoskaltsis, N. (2006). Cytokine storm in a phase 1 trial of the anti-CD28 monoclonal antibody TGN1412. N. Engl. J. Med. *355*, 1018–1028.

Suscovich, T.J., Perdue, N.R., and Campbell, D.J. (2012). Type-1 immunity drives early lethality in scurfy mice. Eur. J. Immunol. *42*, 2305–2310.

Suzukawa, M., Iikura, M., Koketsu, R., Nagase, H., Tamura, C., Komiya, A., Nakae, S., Matsushima, K., Ohta, K., Yamamoto, K., et al. (2008). An IL-1 cytokine member, IL-33, induces human basophil activation via its ST2 receptor. The Journal of Immunology *181*, 5981–5989.

Tai, X., Van Laethem, F., Pobezinsky, L., Guinter, T., Sharrow, S.O., Adams, A., Granger, L., Kruhlak, M., Lindsten, T., Thompson, C.B., et al. (2012). Basis of CTLA-4 function in regulatory and conventional CD4(+) T cells. Blood *119*, 5155–5163.

Talay, O., Yan, D., Brightbill, H.D., Straney, E.E.M., Zhou, M., Ladi, E., Lee, W.P., Egen, J.G., Austin, C.D., Xu, M., et al. (2012). IgE(+) memory B cells and plasma cells generated through a germinal-center pathway. Nat Immunol.

Tamagawa-Mineoka, R., Okuzawa, Y., Masuda, K., and Katoh, N. (2014). Increased serum levels of interleukin 33 in patients with atopic dermatitis. J. Am. Acad. Dermatol. *70*, 882–888.

Tanaka, S., Saito, Y., Kunisawa, J., Kurashima, Y., Wake, T., Suzuki, N., Shultz, L.D., Kiyono, H., and Ishikawa, F. (2012). Development of mature and functional human myeloid subsets in hematopoietic stem cell-engrafted NOD/SCID/IL2r**γ** KO mice. The Journal of Immunology *188*, 6145–6155.

Tang, H., Cao, W., Kasturi, S.P., Ravindran, R., Nakaya, H.I., Kundu, K., Murthy, N., Kepler, T.B., Malissen, B., and Pulendran, B. (2010). The T helper type 2 response to cysteine proteases requires dendritic cell-basophil cooperation via ROS-mediated signaling. Nat Immunol *11*, 608–617.

Taylor, M.D., Sadhukhan, S., Kottangada, P., Ramgopal, A., Sarkar, K., D'Silva, S., Selvakumar, A., Candotti, F., and Vyas, Y.M. (2010). Nuclear role of WASp in the pathogenesis of dysregulated TH1 immunity in human Wiskott-Aldrich syndrome. Sci Transl Med *2*, 37ra44.

Thomas, S.Y., Chyung, Y.H., and Luster, A.D. (2010). Natural killer T cells are not the predominant T cell in asthma and likely modulate, not cause, asthma. J. Allergy Clin. Immunol. *125*, 980–984.

Thomas, S.Y., Lilly, C.M., and Luster, A.D. (2006). Invariant natural killer T cells in bronchial asthma. N. Engl. J. Med. *354*, 2613–6–authorreply2613–6.

Thrasher, A.J., and Burns, S.O. (2010). WASP: a key immunological multitasker. Nat Rev Immunol *10*, 182–192.

Tindemans, I., Serafini, N., Di Santo, J.P., and Hendriks, R.W. (2014). GATA-3 function in innate and adaptive immunity. Immunity *41*, 191–206.

Toit, Du, G., Roberts, G., Sayre, P.H., Bahnson, H.T., Radulovic, S., Santos, A.F., Brough, H.A., Phippard, D., Basting, M., Feeney, M., et al. (2015). Randomized trial of peanut consumption in infants at risk for peanut allergy. N. Engl. J. Med. *372*, 803–813.

Toit, Du, G., Sayre, P.H., Roberts, G., Sever, M.L., Lawson, K., Bahnson, H.T., Brough, H.A., Santos, A.F., Harris, K.M., Radulovic, S., et al. (2016). Effect of Avoidance on Peanut Allergy after Early Peanut Consumption. N. Engl. J. Med.

Tordesillas, L., Goswami, R., Benedé, S., Grishina, G., Dunkin, D., Järvinen, K.M., Maleki, S.J., Sampson, H.A., and Berin, M.C. (2014). Skin exposure promotes a Th2-dependent sensitization to peanut allergens. J. Clin. Invest. *124*, 4965–4975.

Traggiai, E., Chicha, L., Mazzucchelli, L., Bronz, L., Piffaretti, J.-C., Lanzavecchia, A., and Manz, M.G. (2004). Development of a human adaptive immune system in cord blood cell-transplanted mice. Science *304*, 104–107.

Trifari, S., Scaramuzza, S., Catucci, M., Ponzoni, M., Mollica, L., Chiesa, R., Cattaneo, F., Lafouresse, F., Calvez, R., Vermi, W., et al. (2010). Revertant T lymphocytes in a patient with Wiskott-Aldrich syndrome: analysis of function and distribution in lymphoid organs. J. Allergy Clin. Immunol. *125*, 439–448.e8.

Trifari, S., Sitia, G., Aiuti, A., Scaramuzza, S., Marangoni, F., Guidotti, L.G., Martino, S., Saracco, P., Notarangelo, L.D., Roncarolo, M.-G., et al. (2006). Defective Th1 cytokine gene transcription in CD4+ and CD8+ T cells from Wiskott-Aldrich syndrome patients. J. Immunol. *177*, 7451–7461.

Tsuboi, S., and Meerloo, J. (2007). Wiskott-Aldrich syndrome protein is a key regulator of the phagocytic cup formation in macrophages. J. Biol. Chem. *282*, 34194–34203.

Tsuda, M., Torgerson, T.R., Selmi, C., Gambineri, E., Carneiro-Sampaio, M., Mannurita, S.C., Leung, P.S.C., Norman, G.L., and Gershwin, M.E. (2010). The spectrum of autoantibodies in IPEX syndrome is broad and includes anti-mitochondrial autoantibodies. J. Autoimmun. *35*, 265–268.

Tsujimura, Y., Obata, K., Mukai, K., Shindou, H., Yoshida, M., Nishikado, H., Kawano, Y., Minegishi, Y., Shimizu, T., and Karasuyama, H. (2008). Basophils play a pivotal role in immunoglobulin-G-mediated but not immunoglobulin-E-mediated systemic anaphylaxis. Immunity *28*, 581–589.

Tuano, K.S., Orange, J.S., Sullivan, K., Cunningham-Rundles, C., Bonilla, F.A., and Davis, C.M. (2014). Food allergy in patients with primary immunodeficiency diseases: Prevalence within the US Immunodeficiency Network (USIDNET). J. Allergy Clin. Immunol.

Tubo, N.J., and Jenkins, M.K. (2014). TCR signal quantity and quality in CD4+ T cell differentiation. Trends Immunol. *35*, 591–596.

Tubo, N.J., Pagán, A.J., Taylor, J.J., Nelson, R.W., Linehan, J.L., Ertelt, J.M., Huseby, E.S., Way, S.S., and Jenkins, M.K. (2013). Single naive CD4+ T cells from a diverse repertoire produce different effector cell types during infection. Cell *153*, 785–796.

Ulges, A., Klein, M., Reuter, S., Gerlitzki, B., Hoffmann, M., Grebe, N., Staudt, V., Stergiou, N., Bohn, T., Brühl, T.-J., et al. (2015). Protein kinase CK2 enables regulatory T cells to suppress excessive TH2 responses in vivo. Nat Immunol *16*, 267–275.

Umetsu, D.T., and DeKruyff, R.H. (2010). Natural killer T cells are important in the pathogenesis of asthma: the many pathways to asthma. J. Allergy Clin. Immunol. *125*, 975–979.

Unsinger, J., McDonough, J.S., Shultz, L.D., Ferguson, T.A., and Hotchkiss, R.S. (2009). Sepsis-induced human lymphocyte apoptosis and cytokine production in “humanized” mice. J. Leukoc. Biol. *86*, 219–227.

Vahl, J.C., Drees, C., Heger, K., Heink, S., Fischer, J.C., Nedjic, J., Ohkura, N., Morikawa, H., Poeck, H., Schallenberg, S., et al. (2014). Continuous T cell receptor signals maintain a functional regulatory T cell pool. Immunity *41*, 722–736.

Van Gramberg, J.L., de Veer, M.J., O’Hehir, R.E., Meeusen, E.N.T., and Bischof, R.J. (2013). Use of animal models to investigate major allergens associated with food allergy. J Allergy (Cairo) *2013*, 635695.

van Panhuys, N., Klauschen, F., and Germain, R.N. (2014). T-Cell-Receptor-Dependent Signal Intensity Dominantly Controls CD4(+) T Cell Polarization In Vivo. Immunity *41*, 63–74.

van Rhijn, B.D., van Ree, R., Versteeg, S.A., Vlieg-Boerstra, B.J., Sprikkelman, A.B., Terreehorst, I., Smout, A.J.P.M., and Bredenoord, A.J. (2013). Birch pollen sensitization with cross-reactivity to food allergens predominates in adults with eosinophilic esophagitis. Allergy *68*, 1475–1481.

Vanderhoof, J.A., Young, R.J., Hanner, T.L., and Kettlehut, B. (2003). Montelukast: use in pediatric patients with eosinophilic gastrointestinal disease. Journal of Pediatric Gastroenterology and Nutrition *36*, 293–294.

Vicario, M., Blanchard, C., Stringer, K.F., Collins, M.H., Mingler, M.K., Ahrens, A., Putnam, P.E., Abonia, J.P., Santos, J., and Rothenberg, M.E. (2010). Local B cells and IgE production in the oesophageal mucosa in eosinophilic oesophagitis. Gut *59*, 12–20.

Vijayanand, P., Seumois, G., Pickard, C., Powell, R.M., Angco, G., Sammut, D., Gadola, S.D., Friedmann, P.S., and Djukanovic, R. (2007). Invariant natural killer T cells in asthma and chronic obstructive pulmonary disease. N. Engl. J. Med. *356*, 1410–1422.

Voehringer, D. (2013). Protective and pathological roles of mast cells and basophils. Nat Rev Immunol *13*, 362–375.

Volovitz, B., Osur, S.L., Bernstein, J.M., and Ogra, P.L. (1988). Leukotriene C4 release in upper respiratory mucosa during natural exposure to ragweed in ragweed-sensitive children. J. Allergy Clin. Immunol. *82*, 414–418.

Vudattu, N.K., Waldron-Lynch, F., Truman, L.A., Deng, S., Preston-Hurlburt, P., Torres, R., Raycroft, M.T., Mamula, M.J., and Herold, K.C. (2014). Humanized mice as a model for aberrant responses in human T cell immunotherapy. The Journal of Immunology *193*, 587–596.

Wada, T., Schurman, S.H., Otsu, M., Garabedian, E.K., Ochs, H.D., Nelson, D.L., and Candotti, F. (2001). Somatic mosaicism in Wiskott--Aldrich syndrome suggests in vivo reversion by a DNA slippage mechanism. Proc. Natl. Acad. Sci. U.S.a. *98*, 8697–8702.

Wada, T., Schurman, S.H., Garabedian, E.K., Yachie, A., and Candotti, F. (2005). Analysis of T-cell repertoire diversity in Wiskott-Aldrich syndrome. Blood *106*, 3895–3897.

Wada, T., Ishiwata, K., Koseki, H., Ishikura, T., Ugajin, T., Ohnuma, N., Obata, K., Ishikawa, R., Yoshikawa, S., Mukai, K., et al. (2010). Selective ablation of basophils in mice reveals their nonredundant role in acquired immunity against ticks. J. Clin. Invest. *120*, 2867–2875.

Wang, J., and Sampson, H.A. (2011). Food allergy. J. Clin. Invest. *121*, 827–835.

Watanabe, Y., Takahashi, T., Okajima, A., Shiokawa, M., Ishii, N., Katano, I., Ito, R., Ito, M., Minegishi, M., Minegishi, N., et al. (2009). The analysis of the functions of human B and T cells in humanized NOD/shi-scid/gammac(null) (NOG) mice (hu-HSC NOG mice). Int. Immunol. *21*, 843–858.

Weidinger, S., Illig, T., Baurecht, H., Irvine, A.D., Rodriguez, E., Diaz-Lacava, A., Klopp, N., Wagenpfeil, S., Zhao, Y., Liao, H., et al. (2006). Loss-of-function variations within the filaggrin gene predispose for atopic dermatitis with allergic sensitizations. J. Allergy Clin. Immunol. *118*, 214–219.

Weigmann, B., Schughart, N., Wiebe, C., Sudowe, S., Lehr, H.A., Jonuleit, H., Vogel, L., Becker, C., Neurath, M.F., Grabbe, S., et al. (2012). Allergen-induced IgE-dependent gut inflammation in a human PBMC-engrafted murine model of allergy. J. Allergy Clin. Immunol. *129*, 1126–1135.

Weiss, J.M., Bilate, A.M., Gobert, M., Ding, Y., Curotto de Lafaille, M.A., Parkhurst, C.N., Xiong, H., Dolpady, J., Frey, A.B., Ruocco, M.G., et al. (2012). Neuropilin 1 is expressed on thymus-derived natural regulatory T cells, but not mucosa-generated induced Foxp3+ T reg cells. J. Exp. Med. *209*, 1723–42–S1.

Wen, T., Dellon, E.S., Moawad, F.J., Furuta, G.T., Aceves, S.S., and Rothenberg, M.E. (2015). Transcriptome analysis of proton pump inhibitor-responsive esophageal eosinophilia reveals proton pump inhibitor-reversible allergic inflammation. J. Allergy Clin. Immunol. *135*, 187–197.

Wen, T., Stucke, E.M., Grotjan, T.M., Kemme, K.A., Abonia, J.P., Putnam, P.E., Franciosi, J.P., Garza, J.M., Kaul, A., King, E.C., et al. (2013). Molecular diagnosis of eosinophilic esophagitis by gene expression profiling. Gastroenterology *145*, 1289–1299.

Wengler, G., Gorlin, J.B., Williamson, J.M., Rosen, F.S., and Bing, D.H. (1995). Nonrandom inactivation of the X chromosome in early lineage hematopoietic cells in carriers of Wiskott-Aldrich syndrome. Blood *85*, 2471–2477.

Westerberg, L., Greicius, G., Snapper, S.B., Aspenström, P., and Severinson, E. (2001). Cdc42, Rac1, and the Wiskott-Aldrich syndrome protein are involved in the cytoskeletal regulation of B lymphocytes. Blood *98*, 1086–1094.

Westerberg, L.S., Dahlberg, C., Baptista, M., Moran, C.J., Detre, C., Keszei, M., Eston, M.A., Alt, F.W., Terhorst, C., Notarangelo, L.D., et al. (2012). Wiskott-Aldrich syndrome protein (WASP) and N-WASP are critical for peripheral B-cell development and function. Blood *119*, 3966–3974.

Westerberg, L.S., la Fuente, de, M.A., Wermeling, F., Ochs, H.D., Karlsson, M.C.I., Snapper, S.B., and Notarangelo, L.D. (2008). WASP confers selective advantage for specific hematopoietic cell populations and serves a unique role in marginal zone B-cell homeostasis and function. Blood *112*, 4139–4147.

Westerberg, L., Larsson, M., Hardy, S.J., Fernández, C., Thrasher, A.J., and Severinson, E. (2005). Wiskott-Aldrich syndrome protein deficiency leads to reduced B-cell adhesion, migration, and homing, and a delayed humoral immune response. Blood *105*, 1144–1152.

Westerberg, L., Wallin, R.P.A., Greicius, G., Ljunggren, H.-G., and Severinson, E. (2003). Efficient antigen presentation of soluble, but not particulate, antigen in the absence of Wiskott-Aldrich syndrome protein. Immunology *109*, 384–391.

Wing, K., Onishi, Y., Prieto-Martin, P., Yamaguchi, T., Miyara, M., Fehervari, Z., Nomura, T., and Sakaguchi, S. (2008). CTLA-4 control over Foxp3+ regulatory T cell function. Science *322*, 271–275.

Wingender, G., Rogers, P., Batzer, G., Lee, M.S., Bai, D., Pei, B., Khurana, A., Kronenberg, M., and Horner, A.A. (2011). Invariant NKT cells are required for airway inflammation induced by environmental antigens. J. Exp. Med. *208*, 1151–1162.

Wirtz, S., and Neurath, M.F. (2007). Mouse models of inflammatory bowel disease. Adv. Drug Deliv. Rev. *59*, 1073–1083.

Wollenberg, I., Agua-Doce, A., Hernández, A., Almeida, C., Oliveira, V.G., Faro, J., and Graca, L. (2011). Regulation of the germinal center reaction by Foxp3+ follicular regulatory T cells. The Journal of Immunology *187*, 4553–4560.

Worbs, T., Bode, U., Yan, S., Hoffmann, M.W., Hintzen, G., Bernhardt, G., Förster, R., and Pabst, O. (2006). Oral tolerance originates in the intestinal immune system and relies on antigen carriage by dendritic cells. J. Exp. Med. *203*, 519–527.

Wu, J., Liu, D., Tu, W., Song, W., and Zhao, X. (2015). T-cell receptor diversity is selectively skewed in T-cell populations of patients with Wiskott-Aldrich syndrome. J. Allergy Clin. Immunol. *135*, 209–216.e8.

Wu, L.C., and Zarrin, A.A. (2014). The production and regulation of IgE by the immune system. Nat Rev Immunol *14*, 247–259.

Wynn, T.A. (2015). Type 2 cytokines: mechanisms and therapeutic strategies. Nat Rev Immunol *15*, 271–282.

Xu, D., Nishimura, T., Nishimura, S., Zhang, H., Zheng, M., Guo, Y.-Y., Masek, M., Michie, S.A., Glenn, J., and Peltz, G. (2014a). Fialuridine induces acute liver failure in chimeric TK-NOG mice: a model for detecting hepatic drug toxicity prior to human testing. PLoS Med. *11*, e1001628.

Xu, H., Wang, X., Lackner, A.A., and Veazey, R.S. (2014b). PD-1(HIGH) Follicular CD4 T Helper Cell Subsets Residing in Lymph Node Germinal Centers Correlate with B Cell Maturation and IgG Production in Rhesus Macaques. Front Immunol *5*, 85.

Yadav, M., Louvet, C., Davini, D., Gardner, J.M., Martinez-Llordella, M., Bailey-Bucktrout, S., Anthony, B.A., Sverdrup, F.M., Head, R., Kuster, D.J., et al. (2012). Neuropilin-1 distinguishes natural and inducible regulatory T cells among regulatory T cell subsets in vivo. J. Exp. Med. *209*, 1713–22–S1–19.

Yang, Z., Sullivan, B.M., and Allen, C.D.C. (2012). Fluorescent in vivo detection reveals that IgE(+) B cells are restrained by an intrinsic cell fate predisposition. Immunity *36*, 857–872.

Yen, E.H., Hornick, J.L., Dehlink, E., Dokter, M., Baker, A., Fiebiger, E., and Nurko, S. (2010). Comparative analysis of Fcγ RI expression patterns in patients with eosinophilic and reflux esophagitis. Journal of Pediatric Gastroenterology and Nutrition *51*, 584–592.

Yong, P.F., Freeman, A.F., Engelhardt, K.R., Holland, S., Puck, J.M., and Grimbacher, B. (2012). An update on the hyper-IgE syndromes. Arthritis Res. Ther. *14*, 228.

Yu, F., Sharma, S., Edwards, J., Feigenbaum, L., and Zhu, J. (2015). Dynamic expression of transcription factors T-bet and GATA-3 by regulatory T cells maintains immunotolerance. Nat Immunol *16*, 197–206.

Zhang, J., Shehabeldin, A., da Cruz, L.A., Butler, J., Somani, A.K., McGavin, M., Kozieradzki, I., Santos, dos, A.O., Nagy, A., Grinstein, S., et al. (1999). Antigen receptor-induced activation and cytoskeletal rearrangement are impaired in Wiskott-Aldrich syndrome protein-deficient lymphocytes. J. Exp. Med. *190*, 1329–1342.

Zhang, Q., Davis, J.C., Lamborn, I.T., Freeman, A.F., Jing, H., Favreau, A.J., Matthews, H.F., Davis, J., Turner, M.L., Uzel, G., et al. (2009). Combined immunodeficiency associated with DOCK8 mutations. N. Engl. J. Med. *361*, 2046–2055.

Zhang, Y., Yu, X., Ichikawa, M., Lyons, J.J., Datta, S., Lamborn, I.T., Jing, H., Kim, E.S., Biancalana, M., Wolfe, L.A., et al. (2014). Autosomal recessive phosphoglucomutase 3 (PGM3) mutations link glycosylation defects to atopy, immune deficiency, autoimmunity, and neurocognitive impairment. J. Allergy Clin. Immunol. *133*, 1400–9–1409.e1–5.

Zhao, D.-M., Thornton, A.M., DiPaolo, R.J., and Shevach, E.M. (2006). Activated CD4+CD25+ T cells selectively kill B lymphocytes. Blood *107*, 3925–3932.

Zheng, J., Liu, Y., Liu, Y., Liu, M., Xiang, Z., Lam, K.-T., Lewis, D.B., Lau, Y.-L., and Tu, W. (2013). Human CD8+ regulatory T cells inhibit GVHD and preserve general immunity in humanized mice. Sci Transl Med *5*, 168ra9–168ra9.

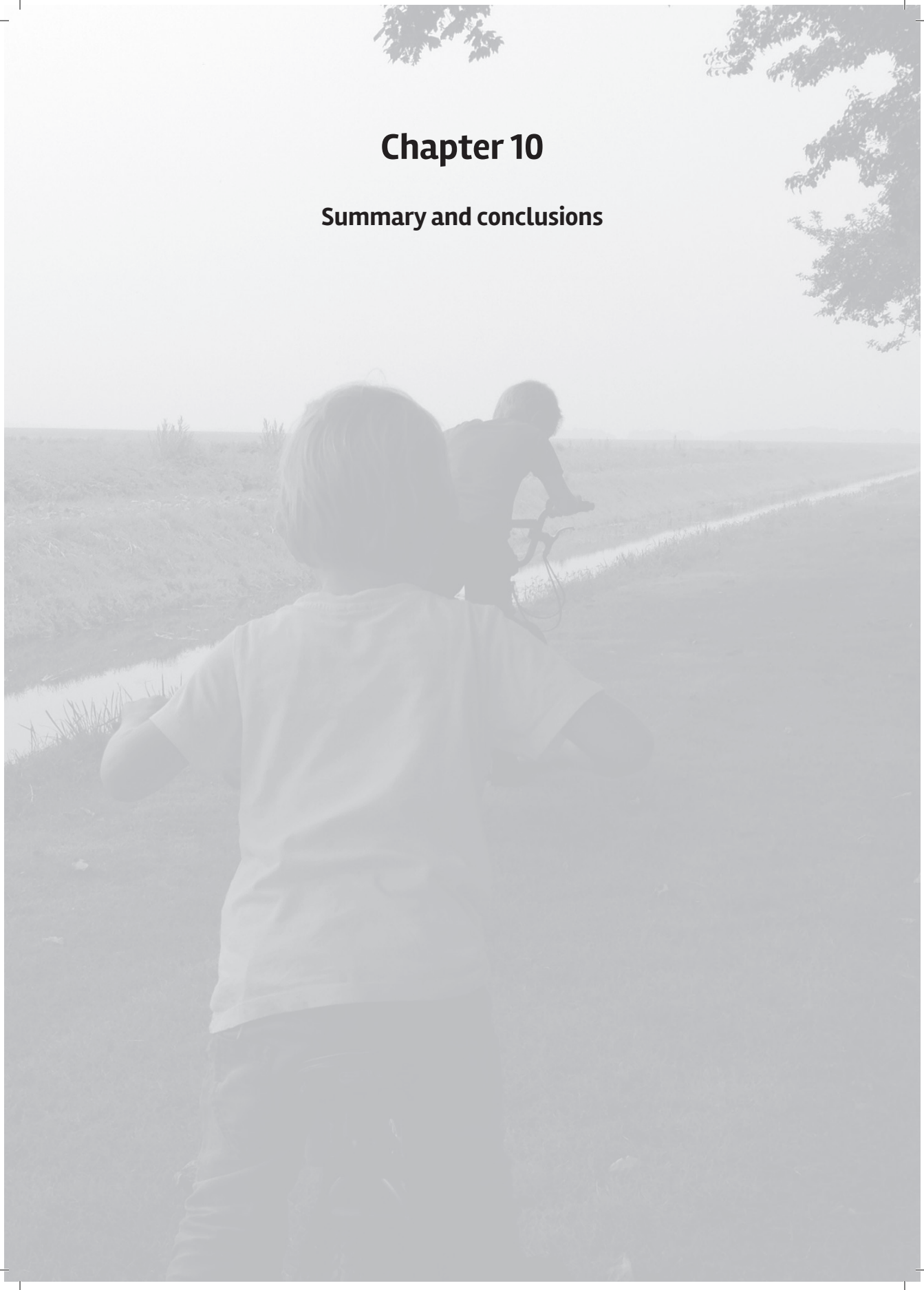
Zheng, Y., Josefowicz, S., Chaudhry, A., Peng, X.P., Forbush, K., and Rudensky, A.Y. (2010). Role of conserved non-coding DNA elements in the Foxp3 gene in regulatory T-cell fate. Nature *463*, 808–812.

Ziegler, S.F. (2012). Thymic stromal lymphopoietin and allergic disease. J. Allergy Clin. Immunol. *130*, 845–852.

Zikherman, J., Parameswaran, R., and Weiss, A. (2012). Endogenous antigen tunes the responsiveness of naive B cells but not T cells. Nature *489*, 160–164.

Chapter 10

Summary and conclusions



The experiments described in the chapters of this thesis pertain to type 2 inflammatory immune responses within the gastrointestinal tract. When directed against innocuous antigens present in the lumen of the intestine, these immune reactions can result in a variety of symptoms in the host that give rise to the clinical phenotype of food allergic and eosinophilic gastrointestinal diseases. **The aim of this work has been to enhance our insight into the immunological mechanisms that are responsible for the induction (i.e. the *origin*) of Th2-mediated inflammation**. In light of this overarching theme, we describe two lines of investigation with their own specific aims that divide the thesis into a first and second part. Although working towards the same unifying goal, the two approaches have different starting points within the from-bench-to-bedside-and-back paradigm of translational medicine. In **Part 1**, we focus primarily on the pediatric patient that suffers from an established case of type 2 intestinal inflammation. **By studying tissue biopsies of affected intestine, we aim to better diagnose these children and to characterize patient subsets that differ according to their immunological inflammatory profile. Within the inflammatory profiles of these patients, we look for clues that could help elucidate the pathogenesis of aberrant type 2 immunity in the intestinal tract.** The work described in **Part 2**, in contrast, originated at the lab bench, where **we assessed whether a mouse model based on a rare human primary immunodeficiency could be used to study the spontaneous induction of food allergic responses in the intestinal tract.** Following their characterization as a *bona fide* model of food allergy, **we use these animals to test how different types of immune cells contribute to the origin of aberrant type 2 inflammation**. By subsequently investigating whether our findings in the mouse also apply to human patients suffering from the same immunological disorder, we have attempted to translate our observations from the bench back to the bedside.

Chapter 1 provides a general introduction of Th2-mediated intestinal inflammation and gives an outline of the aims and most important results of the thesis. It can be read as a stand-alone summary of the work and afterwards the reader will have sufficient information to successfully navigate between the individual parts and chapters. Instead of providing a comprehensive summary of the research findings, the **focus of chapter 1 is to illustrate the cohesion between the different papers in this thesis**. To avoid redundancy, the summary that is presented in this final chapter will follow the more conventional, chapter-by-chapter-style discussion and highlight the key research findings of each paper as to serve as an additional navigational aid for the interested reader.

Eosinophilic esophagitis results from a type 2 inflammatory process of the upper gastrointestinal tract. In the absence of systemic biomarkers that accurately predict the presence or absence of disease, proper patient characterization relies on quantification (i.e. a manual count by a pathologist) of eosinophils in tissue biopsies obtained via upper

gastrointestinal endoscopy. Since multiple conditions are associated with esophageal eosinophilia, this diagnostic approach suffers from poor specificity. The aim of the work described in **Chapter 2** is **to test whether mRNA expression data obtained via high-throughput gene expression analysis could aid in establishing a diagnosis of eosinophilic esophagitis (EoE) in children with inflammation of the esophagus**. To this end, we have studied gene expression data from 196 pediatric patients that were enrolled in a cohort study at Boston Children's Hospital. Study biopsies were obtained from these children at time of inclusion and they were subsequently followed for a minimum of two years, which allowed for confirmation of the original clinical-histopathological diagnosis. **Using a mathematical model that takes into account the expression of a combination of 10 EoE-specific genes, we were able to identify EoE patients from children with gastroesophageal reflux disease (GERD) or normal controls with up to 94% sensitivity**. These findings led us to **conclude that mRNA profiling of esophageal tissue is an accurate diagnostic strategy for detecting EoE that could help stratify these patients at time of their first diagnostic endoscopy**.

In **Chapter 3**, we have used gene expression data from esophageal tissue of EoE and control patients **to assess whether mRNA expression of the enzyme leukotriene C_4 synthase (LTC_4S) could serve as an EoE-specific marker**. Leukotrienes are inflammatory mediators that contribute to type 2 inflammatory response in tissue and leukotriene receptor antagonists have a firm place in the maintenance treatment of asthma. Although we did identify an overall, statistically significant increase in expression of *LTC4S* in the esophagus of EoE patients versus controls, this difference was mainly caused by a **subgroup of approximately 30% of EoE patients with *LTC4S* mRNA expression levels >2 standard deviations above the mean**. Further characterization of this subpopulation revealed that children belonging to this subgroup had equivalent degrees of gastrointestinal symptoms and tissue eosinophilia, as well as a similar appearance of the esophagus at endoscopy as the 70% of EoE patients with normal *LTC4S* expression levels. However, **serum IgE levels and co-occurrence of food allergies were higher in the *LTC4S*^{High} patient population**. This heterogeneity at the tissue level within the EoE patient population thus appears to be directly related to the epidemiological observation that IgE sensitization to food antigens occurs in only a subset of EoE patients. It is likely that the identification of such subpopulations may not only have relevance for our understanding of the pathogenesis of EoE, but also for the optimal medical management of patients because different subpopulations may show different responses to any given therapeutic modality.

What determines whether children develop an intestinal type 2 inflammatory process such as EoE? Over the last decade, advances in high-throughput sequencing of species-specific 16s ribosomal RNA have implicated the composition of our commensal microbial floras

(the microbiome) at mucosal surfaces as a critical determinant in health and disease. One mechanism through which signals from bacteria can affect immune responses in the host has been uncovered following the observation that germ-free mice showed an increased susceptibility to experimental asthma and colitis. The absence of commensal microbes in lung and colon resulted in mucosal upregulation of CXCL16 and chemotaxis of invariant chain natural killer T cells (iNKT cells), conferring an increased susceptibility to mucosal inflammation. For **Chapter 4**, **we have studied our EoE patient cohort for evidence of involvement of the CXCL16-iNKT cell axis to determine whether a similar mechanism could be operative in human patients with immune-mediated intestinal diseases.** We observed that ***CXCL16* gene expression was upregulated in children with EoE** when compared to those with normal tissue histology or reflux esophagitis. Similarly, we found **increased gene expression of Vα24 (comprising the invariant TCR) and Vα24-to-CD3ε ratio, as well as CD1d in EoE patients**. Involvement of the CXCL16-iNKT-CD1d axis was **especially pronounced in our youngest patients (<6 years of age)** and an **increased CXCL16-iNKT cell signature was associated with a more pronounced food allergic phenotype**, as implicated by increased prevalence of a positive food allergen test (48% vs. 25%, p=0.008) and elevated levels of *LTC4S*, our previously identified (**Chapter 3**) marker for the food allergic subset of EoE patients. In these early-onset EoE patients, **treatment with elimination diet resulted in normalization of the CXCL16-iNKT-CD1d axis**, demonstrating that iNKT cell involvement in EoE is amenable to dietary interventions that aim to eliminate common food allergens. Furthermore, **using logistic regression analysis on clinical patient data, we found a positive association between antibiotics use in the first year of life and the occurrence of EoE**. Combined, our results led us **to propose a model for the pathophysiology of (early-onset) EoE** that starts with insufficient immune imprinting by environmental microorganisms (1) resulting in upregulation of CXCL16 and chemotaxis of iNKT cells into the esophagus (2). Increased numbers of iNKT cells can become pathologically activated via CD1d-associated lipid antigens derived from food, aeroallergens, microorganisms, and/or potentially self-antigens associated with the esophagus (3). Activated iNKT cells, subsequently, act as an early source of IL-4, IL-5, and IL-13, which can facilitate Th2-mediated allergic sensitization and induction of IgE responses that further promote and sustain inflammation (4). **This model would help explain how environmental factors (in this case changes in signals derived from commensal bacteria) can predispose to type 2 intestinal inflammation and allergic sensitization to food antigens**.

The model presented at the end of Chapter 4 proposes a mechanism by which type 2 intestinal inflammation and allergic sensitization to food antigens may originate locally, within the predisposed esophagus. **Chapter 5** describes the work that originated in the lab of dr. Michiko Oyoshi at the Division of Immunology at Boston Children's Hospital, who asked

whether allergic sensitization via the skin could affect eosinophilic inflammation in the esophagus. This hypothesis followed from evidence that demonstrates that sensitization to allergens can result from exposure to environmental antigen through an impaired skin barrier. From a pathophysiological point of view, cutaneous allergic sensitization is an attractive mechanism because it helps explain two important clinical phenomena: 1. why severe allergic reactions have been described following a first time ingestion of a food antigen (e.g. peanut), and 2. why atopic dermatitis is typically the earliest (<1 year of age) symptom within the atopic march. **Chapter 5** demonstrates that **epicutaneous sensitization to ovalbumin promotes eosinophilia and increased expression of type 2 cytokines in the esophagus of mice that are subsequently challenged with intranasal ovalbumin.** Animals with mutations in the gene encoding for the keratin-associated skin protein filaggrin, which in humans can give rise to severe forms of eczema, were particularly susceptible to skin-mediated allergic sensitization and accumulation of eosinophils in the esophagus. Mechanistically, **accumulation of eosinophils in the esophagus could be abrogated by disruption of IL-33-mediated signals,** and **was dependent on basophils, as basophil-deficient mice were less susceptible to esophageal eosinophilia**. The observation that **pediatric EoE patients have increased expression of the IL-33 receptor (*ST2*) in the esophagus** suggests that the IL-33/ST2 axis is also operative in human patients and that blockade of the IL-33 pathway might prove a novel therapeutic target in treatment of EoE.

The experimental strategy employed in **Chapter 5** follows an investigator-controlled temporal organization of immunological events. At the age of 4-6 weeks, mice are epicutaneously sensitized over the course of 7 days, followed by a series of three intranasal allergen challenges at day 9, 11 and 13 (**Figure 5.1, page 95**). Similarly, a commonly used model of food allergy that we encounter in **Chapter 8** (**Figure 8.2, page 191**) relies on 3 intraperitoneal sensitization of adjuvant-coupled allergen at day 0, 7 and 14 followed by repetitive oral challenge from day 21 to 39. The mechanistic advances of the results from these investigations notwithstanding, it is clear that the study of factors that affect disease pathogenesis could be hampered by such temporal restrictions and the assumptions that these models require. Ideally, we would study the onset of disease in the absence of any bias that may be related to the timing of experimental interventions. In **Part 2** of this thesis, **we investigate strategies to promote homology of animal models in the study of type 2 intestinal inflammation**.

As delineated in the introductory chapter of this thesis, one possible approach to increasing the translational value of experiments in murine models is to make use of mice that have been reconstituted with human immune cells. Most easily, this can be achieved by isolation of peripheral blood mononuclear cells from a human donor followed by transfer of these cells to a murine immunodeficient recipient. However, human T cells in the isolate will recognize

the antigenic environment in the murine host as non-self, resulting in severe and lethal graft-versus-host disease that has the potential to confound the study of any downstream immune response of interest. This problem could be bypassed by reconstituting human adaptive immune cells from hematopoietic stem cells within the murine recipient, which will result in generation of T cells that have been educated against murine MHCII molecules and that will therefore be less prone to give rise to autoimmunity. Unfortunately, reconstitution of T lymphocytes in this setting has typically been poor, perhaps because of the stringency of positive selection in the thymus. **Chapter 6** described the work that was led by our close collaborator dr. Jeremy Goettel, who **hypothesized that improved reconstitution of human T cells could be obtained if these cells are educated against human instead of murine MHC class II molecules**. This was achieved following a transgenic approach in which MHCII-deficient mice were made to express the human HLA-DR1*0101 transgene (NSGAb^{o}DR1) under the control of the murine MHCII promoter. Although there were no gross differences between the number of lymphocytes reconstituted in this novel system compared with the murine MHCII or MHCII$^{-/-}$ controls, **NSGAb^{o}DR1 animals displayed improved delayed type hypersensitivity reactions, a richer TCR repertoire, and improved B cell maturation and class switch recombination.** Furthermore, when **mice were reconstituted with hematopoietic stem cells from an IPEX patient** (an immune disorder caused by an absence of FOXP3^{+} Tregs that results in severe autoimmunity), **NSGAb^{o}DR1 but not murine MHCII or MHCII$^{-/-}$ controls succumbed to lethal autoimmune responses and auto-antibody production**, illustrating that the pathophysiology of the disease was to a large extent phenocopied in this novel system.

IPEX patients (and their murine *scurfy* equivalent) are deficient in FOXP3^{+} Tregs and, consequently, suffer from unrestrained immune activation directed against foreign or self-antigens. In addition to proliferation of Th1 and Th17 cells, the ensuing immune responses also contain a Th2 component, which gives rise to increased levels of serum IgE and allergic tissue inflammation. Besides the IPEX syndrome, dysregulated Th2 responses, atopy and elevated IgE levels occur in a variety of primary human immunodeficiencies. In **Chapters 7-9**, we have investigated patients and mice deficient in the Wiskott-Aldrich syndrome protein (WASP) as an example of one such disorder, with the **overarching hypothesis that unraveling the pathogenesis of Th2 immunity in the setting of a rare genetic immunodeficiency might shed light on the origin of type 2 intestinal inflammation**. Although it has long been known that patients with Wiskott-Aldrich syndrome (WAS) spontaneously develop increased IgE levels, the question to which degree this is associated with an increased burden of food allergies has never been systematically studied. In **Chapter 7**, we demonstrate that **patients with mutations in *WAS* are at increased risk of developing clinically relevant food allergy**. Similarly, we observed **IgE with specificity against chow antigens in the serum of *Was*$^{-/-}$ mice**, and **these animals developed expansion of mast cells in the**

small intestine. By using a Cre-lox recombination system that allowed for cell-specific knock-out of WASP, we found that **deletion of WASP confined to FOXP3$^+$ Tregs was not only sufficient for the allergic sensitization and intestinal mast cell expansion, but that this allergic phenotype was even aggravated when compared to complete *Was$^{-/-}$* mice.** In strong contrast to IPEX syndrome, where immune proliferation is a mix of Th1, Th2 and Th17 responses, **we observed *in vivo* that loss of WASP from FOXP3$^+$ Tregs only interferes with their ability to restrain Th2-mediated immune responses, whereas Th1 or Th17 suppression remains intact in the absence of WASP.** Mechanistically, we believe that this might be related to the fact that **WASP-deficient FOXP3$^+$ Tregs in mice and human patients assume a Th2-like phenotype which includes the increased expression of Th2-type transcription factors ICOS and GATA3 that occurred in the absence of IL4 signaling in *Was$^{-/-}$/Il4$^{-/-}$* animals** and therefore appears to be a cell-intrinsic feature resulting from loss of WASP rather than a circumstantial finding that occurs in the setting of food allergy.

The study of food allergy in mice is hampered by a lack of models that faithfully recapitulate the process of spontaneous allergic sensitization as it occurs in human patients. Commonly used isomorphic murine models of food allergy rely on sensitization via co-administration of adjuvant together with a model allergen, and are therefore primarily suitable for the study of allergic responses in an already sensitized host. However, the study of the pathogenesis of allergic sensitization itself requires a homologous experimental model, i.e. one that mimics both induction and effector phases of human food allergies. Having identified spontaneously occurring IgE responses to chow antigens in *Was$^{-/-}$* mice, we **hypothesized that these animals might serve as a novel experimental model of food allergy that would bypass the need for sensitization via adjuvant**. Indeed, in **Chapter 8** we showed that **following ingestion of ovalbumin, WASP-deficient mice develop OVA-specific IgE antibodies.** In comparison with an established, adjuvant-based model of food allergy, **spontaneously induced OVA-IgE in *Was$^{-/-}$* animals was equally effective in mediating mast cell degranulation following OVA exposure *in vitro***, demonstrating that the process of affinity maturation in WASP-deficient hosts is of sufficient quality to result in high affinity IgE antibodies with specificity for the model antigen. **Following in vivo oral challenge, spontaneously sensitized *Was$^{-/-}$* mice developed allergic diarrhea to a comparable degree as adjuvant-sensitized experimental animals**, demonstrating that allergic effector responses are also recapitulated in this novel experimental model. By generating WASP-deficient mice that were also deficient in the high-affinity IgE receptor FcεRI, **we observed that the process of spontaneous allergic sensitization in these animals does not require IgE-FcεRI mediated signals**. **We conclude that *Was$^{-/-}$* mice are a useful adjunct to the experimental arsenal for mechanistic studies into the pathogenesis of food allergy**.

Chapter 9, the final part of this thesis, was written as both a synthesis of the data presented in Chapters 6, 7 and 8, as well as a platform to incorporate additional findings and experiments that had not yet been described in the previous manuscripts. The section is built around the observation from **Chapter 7** that ***Was*$^{-/-}$ and *Was*$^{fl/fl}$*Foxp3*-Cre mice display a significantly different susceptibility to food allergic disease**, with the latter genotype being more severely affected. Following arguments from both a thorough review of existing WAS literature and additional experimental data, **we systematically investigate how the absence of WASP from a variety of immune compartments could potentially contribute to this differential disease susceptibility**. We suggest that loss of WASP from antigen presenting cells will affect their ability of 1. phagocytosis and antigen uptake, 2. tissue homing and migration, 3. immune synapse formation and T cell activation and 4. interpretation of environmental stimuli resulting in both pro and anti allergic effects on disease pathogenesis. We furthermore **show that WASP-deficiency in the B cell compartment results in lower levels of food-specific IgM in mice and human patients** and argue that this could reduce the overall humoral anti-food response. Data from other groups that have used B-cell specific WASP-deficient animals (*Was*$^{fl/fl}$*Mb1*-Cre) have demonstrated a reduced number of total B lymphocytes in the spleen and an altered distribution of B cell subsets compared to WT counterparts, demonstrating a role of WASP in the development, differentiation and survival of B cells. In addition, we have reviewed evidence for WASP-dependent functions in mobility and chemotaxis, activation and isotype switching, variation of the BCR repertoire and function of regulatory B cells. Generation of WASP-deficient IgE reporter mice is suggested as a worthwhile endeavor to shed additional insight in the question of the origin of food-specific IgE in *Was*$^{-/-}$ mice. Data from our own experiments suggest that **WASP-deficiency in B cells may contribute to their altered distribution throughout the GALT and indicate that aberrant germinal center responses also occur in a WASP-competent B cell compartment in the presence of WASP-deficient Tregs**. Furthermore, we have identified aberrant follicular helper T cell (T_{FH}) responses in *Was*$^{-/-}$ animals, but go on to show that an **increased T_{FH} compartment is dispensable for aberrant germinal center formation and allergic sensitization**. Based on recently published experiments, **we hypothesize that low levels of circulating IL-2 in systemic *Was* deficiency underlie increased T_{FH} differentiation**, and this may be related to a decrease the formation of tissue resident Th2 cells.

In **chapter 7**, we argued that WASP deficiency in effector T cells is an important determinant in the differential disease susceptibility between *Was*$^{-/-}$ and *Was*$^{fl/fl}$*Foxp3*-Cre mice because Th2 cells produce reduced levels of IL-4 in the absence of WASP. Indeed, following *ex vivo* stimulation, **we found significantly lower IL-4 production by CD4^{+} T cells isolated from MLNs of *Was*$^{-/-}$ mice when compared to *Was*$^{fl/fl}$*Foxp3*-Cre animals**, although this appeared to result from diminished Th2 differentiation rather than secretory defects in individual Th2 cells. Reduced T cell activation due to aberrant formation of immune synapses with food-antigen presenting mononuclear phagocytes provides an additional

explanation for a dampened food allergic response in *Was*$^{-/-}$ mice. In addition, alterations in the TCR repertoire may affect the availability of food antigen-specific T cells in the same way as we hypothesized to occur for B cells, which could reduce the likelihood of antigen-specific proliferation upon encountering food antigens.

In comparing the allergic phenotype of *Was*$^{-/-}$ and *Was*$^{fl/fl}$*Foxp3*-Cre mice, we have assumed FOXP3^{+} Tregs to be the one constant determinant between the animals. However, in **Chapter 9** we also provide a number of arguments against this assumption. First, constitutional versus conditional WASP deletion from Tregs may affect the thymic selection process. Second, **WASP-deficient dendritic cells may alter peripheral Treg maintenance,** for which we found evidence in the **expression levels of vitamin A metabolizing enzymes RALDH1 (*Aldh1a1*) and RALDH2 (*Aldh1a2*)** that play an important role in the function of tolerogenic CD103^{+} dendritic cells. Third, differences in composition of the microflora between *Was*$^{-/-}$ and *Was*$^{fl/fl}$*Foxp3*-Cre mice could potentially underlie differences in the degree of Treg induction. Fourth, alterations in the Breg compartment may result in altered recruitment of Tregs, and, lastly, alterations in baseline IL-2 levels may affect Treg homeostasis between the strains as a fifth argument against an equal Treg compartment between the two strains. These arguments are consecutively discussed in Chapter 10, in combination with a more elaborate discussion of the hypothesis put forth in the discussion of Chapter 7 in which aberrant TCR signaling in the absence of WASP is linked to a Th2-type differentiation. A plan for follow-up studies is suggested to substantiate these hypotheses.

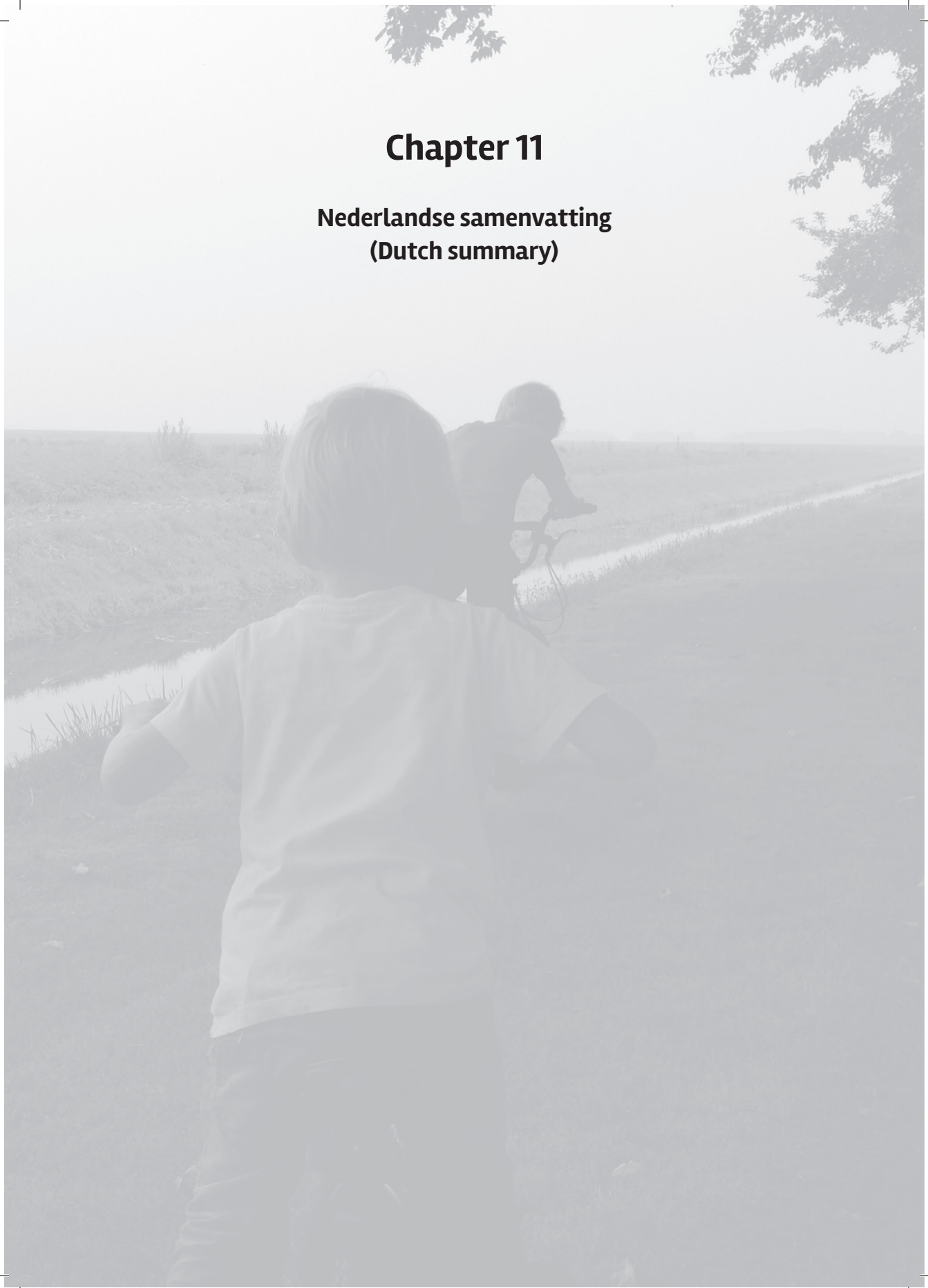
The difference in WASP status of IgE effector cells (i.e. mast cells and basophils) is another factor that could contribute to the differential disease susceptibility between *Was*$^{-/-}$ and *Was*$^{fl/fl}$*Foxp3*-Cre mice that is examined in **Chapter 9**. For mast cells, the effect of loss of WASP was already examined in **Chapter 8**, where we identified reduced production of Th2 cytokines IL-4 and IL-13 following IgE-mediated cell activation. With regards to basophils, **we found that in the absence of adaptive immunity in *Rag2*$^{-/-}$ mice, WASP-deficient basophils were reduced compared to WT basophils, which suggests that loss of WASP is associated with reduced basophil survival**. This could be related to the finding that basophil-specific expression of *Mcpt8* was found to be higher in the intestine of *Was*$^{fl/fl}$*Foxp3*-Cre mice than in *Was*$^{-/-}$ counterparts. However, **following oral antigen challenge, we observed rapid depletion of basophils from the systemic circulation**, and, in combination with the susceptibility of *Was*$^{-/-}$ to IgG-mediated anaphylaxis as observed in **Chapter 8**, we believe that allergen-specific activation of basophils occurs relatively unperturbed in *Was*$^{-/-}$ hosts.
Lastly, we **have interrogated whether changes in the microflora could account for the difference in disease severity between *Was*$^{-/-}$ and *Was*$^{fl/fl}$*Foxp3*-Cre mice**. Using high throughput genetic sequencing strategies, we assessed the relative abundance of species-specific 16s ribosomal RNA sequences in stool samples of co-housed WT, *Was*$^{-/-}$ and *Was*$^{fl/fl}$*Foxp3*-Cre mice at the age of 6 weeks. **We observed that a the *family* level, the composition of the microbiome is significantly different between mice with different genotypes**,

with relatively increased abundances of both *Prevotellaceae* and *Bacteroidaceae* in *Was*$^{fl/fl}$*Foxp3*-Cre mice in comparison with co-housed WT and *Was*$^{-/-}$ counterparts. Overall, **food allergy associated with WASP-deficiency in mice was associated with a microbiota signature that differs from co-housed WT control mice**. For one OTU belonging to a member of the *Rikanellaceae* family, we found that its relative abundance correlated positively and significantly with serum levels of MCPT1 in two independent analyses of *Was*$^{fl/fl}$*Foxp3*-Cre as well as *Was*$^{-/-}$ animals, **allowing speculation about a disease-promoting role for this bacterium**. These findings thus illustrate how alterations in the microflora could also contribute to differential disease activity between *Was*$^{-/-}$ and *Was*$^{fl/fl}$*Foxp3*-Cre animals.

Two important findings remained that are not directly related to loss of WASP from a single immune compartment, but are nevertheless discussed in **Chapter 9**. First, prospective follow-up of cohorts of *Was*$^{-/-}$ and *Was*$^{fl/fl}$*Foxp3*-Cre mice revealed that whereas MCPT1 levels stayed consistently elevated in *Was*$^{fl/fl}$*Foxp3*-Cre animals between 2 and 5 months of age, there was a significant decline over time in levels of this serum marker in *Was*$^{-/-}$ mice. Food allergy in these latter animals may thus be **susceptible to spontaneous resolution as is commonly seen in pediatric patients**. Second, we made the curious observation that **heterozygous females with one *Was*^{fl} and one *Was*^{wt} allele in combination with the *Foxp3*-Cre transgene develop increased serum MCPT1 levels, but only when the *Was*^{fl} gene was inherited from a *Was*$^{fl/fl}$*Foxp3*-Cre mother and not when it was obtained from a *Was*^{fl}*Foxp3*-Cre father**. The tentative conclusions that can be drawn from this result is, first, that **WASP-deficient FOXP3^{+} Tregs can cause allergic disease in the presence of WT counterparts** and may therefore play a pro-inflammatory role in disease pathogenesis. Second, **the genetic susceptibility to food allergy that results from loss of WASP in FOXP3^{+} Tregs is influenced by non-genetic maternal factors**, and in heterozygous females, these factors (e.g. composition of breast milk or the maternal microbiome) can make the difference between overt type 2 intestinal inflammation and intestinal homeostasis. It is obvious that **these conclusions greatly increase the complexity of the mechanism of intestinal type 2 inflammation that results from loss of WASP in Tregs, but they also emphasize the translational value of a model of WASP-deficiency in Tregs in study of allergic disease**.

Chapter 11

Nederlandse samenvatting (Dutch summary)



Het werk dat beschreven staat in dit proefschrift is gericht op het ontrafelen van de pathogenese van type 2 ontstekingsprocessen in het maag-darmkanaal. Type 2 inflammatie is een evolutionair oude tak van ons immuunsysteem die naast een beschermende functie tegen parasieten ook een cruciaal onderdeel vormt van verschillende homeostatische processen zoals het behoud van de integriteit van de epitheelcelbarrière, thermoregulatie, en controle van wondgenezing en stofwisseling (Palm et al., 2012; Pulendran en Artis, 2012; Wynn, 2015). Deze functies worden verricht door een samenspel van leukocyten uit zowel het aangeboren (aspecifieke) en verworven (adaptieve) afweersysteem, waarbij een centrale organiserende rol gespeeld wordt door type 2 helper T (Th2) cellen. Middels de productie van cytokines zoals IL-4, IL-5 en IL-13 sturen deze lymfocyten op antigeen-specifieke wijze type 2 ontsteking in de darmmucosa aan. Hieronder valt ook de hulp aan B cellen bij hun productie van immuunglobuline E (IgE), een antistof die zich via de IgE receptor (FcεRI) kan binden op het oppervlak van mestcellen en basofiele granulocyten. Via IgE:FcεRI interacties kunnen deze IgE effector cellen bij een volgend antigeencontact reageren met snelle productie van ontstekingsmediatoren zoals histamine en proteasen (Burton en Oettgen, 2011; Gould and Sutton, 2008; Kraft en Kinet, 2007). Het vrijkomen van deze eiwitten leidt tot symptomen van een allergische reactie zoals lokale vasodilatatie, oedeem en jeuk. Bij ernstige systemische effecten spreken we van een IgE-gemedieerde anafylactische shock, gekenmerkt door luchtwegoedeem en ernstige hypotensie, hetgeen in een tijdsbestek van minuten tot een levensbedreigende situatie kan leiden in allergische patiënten.

Gezien de hevigheid en het potentiele gevaar van zulke immuunreacties is het niet verwonderlijk dat er meerdere mechanismen zijn ontstaan die type 2 immuniteit onder fysiologische omstandigheden in toom houden (Wynn, 2015). Onder onze moderne leefomstandigheden blijken dergelijke mechanismen echter steeds vaker tekort te schieten, waardoor allergische ziekten die gekenmerkt worden door aberrante type 2 inflammatie zijn gestegen in prevalentie. Een belangrijk voorbeeld is de vrij recente stijging in het aantal patiënten dat lijdt aan voedselallergie, maar deze trend werd in de vorige decaden reeds voorafgegaan door een toename in incidentie van astma, eczeem en hooikoorts. De oorzaak van dit falen ligt verscholen in gen-omgevingsinteracties en wordt tot op heden onvolledig begrepen. Het **overkoepelend doel van dit proefschrift is om bij te dragen aan ons begrip van de pathofysiologie van type 2 inflammatie in het maag-darmkanaal**, waarbij we twee onafhankelijke onderzoeksstrategieën hebben gevolgd die zijn beschreven in Deel 1 en Deel 2. In **Deel 1** proberen we om tot **nieuw inzicht te komen in de pathogenese van allergische darmontsteking middels de studie van een cohort van patiënten die lijden aan eosinofiele oesofagitis**, een type 2 ontstekingsproces van de slokdarm. Met behulp van een combinatie van klinische parameters en genexpressiedata in slokdarmbiopten tonen we aan dat er aanzienlijke immunologische heterogeniteit bestaat binnen deze patiëntengroep. In **Deel 2** benaderen we het probleem van aberrante type 2 inflammatie via **het ontwikkelen van een nieuw muismodel dat spontaan voedselallergie ontwikkelt en waarmee**

derhalve de bijdrage van verschillende immunologische cellulaire componenten kan worden onderzocht. In beide delen hebben we voortdurend gestreefd naar translationele waarde van de experimenten. Multifactoriële, immuun-gemedieerde ziekten in darmmucosa zijn gedefinieerd in patiënten, maar worden veelal bestudeerd in muizen. Daar waar gebruik gemaakt wordt van muismodellen hebben we ook gekeken naar de mate van overlap met de menselijke situatie en deze vergelijkingen vormen een belangrijk onderdeel van het werk in dit proefschrift (**Deel 1, Hoofdstuk 5; Deel 2, Hoofdstuk 6-9**).

11.1 Introductie Deel 1: Eosinofiele oesofagitis

Eosinofiele oesofagitis (EoE) maakt onderdeel uit van de eosinofiele gastro-intestinale syndromen, die gekenmerkt worden door infiltratie van eosinofiele granulocyten (eosinofielen) in de maag-darm mucosa. De pathogenese bestaat uit een complex samenspel van genetische predispositie, allergeen blootstelling en Th2-gemedieerde immuunactivatie. De huidige prevalentie ligt rond 0.5-1/1000 personen in Westerse samenlevingen en de totale incidentie neemt toe in Amerika, Australië en Europese landen (Dellon, 2014; Furuta en Katzka, 2015). De ziekte manifesteert zich veelal op de kinderleeftijd met symptomen die kunnen variëren van failure-to-thrive en voedingsproblemen in zuigelingen en peuters, tot buikpijn, spugen, dysfagie en passageproblemen en voedselimpactie in oudere kinderen en volwassenen (Assa'ad et al., 2007; Brown-Whitehorn en Liacouras, 2007; Straumann et al., 2012). Aangezien de differentiaal diagnose van deze symptomen breed is komt diagnostische delay regelmatig voor (Hruz et al., 2011; Schoepfer et al., 2011). De gouden standaard voor een diagnose van EoE is histopathologische evaluatie van slokdarmbiopten, verkregen uit endoscopische onderzoek, waarin tenminste 15 eosinofielen per HPF aanwezig moeten zijn. Deze bevinding is op zichzelf echter verre van pathognomonisch en diagnostische onzekerheid kan de start van therapie in de praktijk nog verder vertragen (Liacouras et al., 2011; Straumann, 2009). Behandeling geschiedt met ingestie van steroïdenpreparaten of met een voedingsinterventie die gericht is op het verwijderen van veelvoorkomende allergenen uit het dieet (Arias en Lucendo, 2014; Arias et al., 2014; Gonsalves et al., 2012; Lucendo et al., 2013). EoE is echter een chronische ziekte, die na staken van de behandeling terugkeert en op termijn leiden tot weefselveranderingen die kunnen resulteren in strictuurvorming en slokdarmvernauwing. De aandoening is geassocieerd met een verminderde kwaliteit van leven met persisterend symptomen 15 jaar na eerste presentatie (DeBrosse et al., 2011).

Immunologisch gezien bestaat EoE uit een Th2-gemedieerd, allergeenafhankelijk ontstekingsproces van de slokdarm. Comorbiditeit met andere allergische ziekten is hoog en varieert van 42-93% bij kinderen en 28-86% van de volwassen patiënten populatie (Liacouras et al., 2005; 2014; Roy-Ghanta et al., 2008; Spergel et al., 2005). Een groot deel van patiënten heeft voedingsantigeen-specifiek IgE, maar ondanks de duidelijk associatie tussen EoE en

een atopische constitutie worden in 50% van de gevallen normale IgE waarden gevonden, waaruit volgt dat eosinofiele ontsteking grotendeels onafhankelijk lijkt te zijn van IgE sensibilisatie (Simon et al., 2016). Dit verklaart ook waarom de resultaten van behandeling met het monoklonale anti-IgE antilichaam omalizumab teleurstellend zijn gebleken in zowel kinderen als volwassenen met EoE (Clayton et al., 2014; Rocha et al., 2011). Een alternatieve hypothese stelt dat de antigen-specificiteit van de ziekte met name gemedieerd wordt door antilichamen van de IgG4 klasse, dat eveneens in hogere waarden gevonden worden in EoE patiënten vergeleken met een controlegroep (Clayton et al., 2014). Net als IgE is IgG4 een IL-4-afhankelijk (dus type 2) antilichaam (Aalberse et al., 2016), en mogelijk bestaat er *in vivo* competitie tussen antigeen-specifieke IgE en IgG4 moleculen voor binding van een beperkte hoeveelheid allergeen. Dit fenomeen wordt in verband gebracht met het ontstaan van orale tolerantie bij immuuntherapie (Akdis en Akdis, 2014b), en het is in dit opzicht noemenswaardig dat er meerdere gevallen zijn beschreven waarin patiënten EoE ontwikkelden na het starten van desensibilisatie protocollen middels orale immuuntherapie (Lucendo et al., 2014).

Onafhankelijk van serologisch onderzoek in de individuele patiënt blijkt het Th2 karakter van EoE verder uit genexpressiedata in de aangedane slokdarm (Blanchard et al., 2006a; 2006b; Furuta et al., 2007; Noti et al., 2013), uit de dominante rol van IL-13 in de pathogenese van de aandoening in experimenteel onderzoek (Blanchard et al., 2010; Davis en Rothenberg, 2016), en uit infiltratie van de slokdarm met mestcellen en basofiele granulocyten naast eosinofielen (Abonia et al., 2010; Noti et al., 2013). Volledig inzicht in het samenspel tussen allergische ziekten en een atopische constitutie enerzijds en de evolutie van de pathofysiologie van EoE anderzijds behoeft nog uitgebreid aanvullend onderzoek. In **Hoofdstuk 5** wordt een van de factoren in dit samenspel verder onderzocht en demonstreren we dat allergische sensibilisatie door een beschadigde huid zoals die gezien wordt bij atopische dermatitis het ontstaan van EoE beïnvloedt via IL-33 en basofielen. Deze bevinding koppelt EoE aan eczeem op jonge leeftijd en suggereert dat EoE, net als astma en hooikoorts, een station zou kunnen zijn in wat wel de atopische mars genoemd wordt.

11.2 Rationale en onderzoeksvragen behorend bij Deel 1

In tegenstelling tot astma kan EoE alleen gediagnosticeerd worden middels de microscopische beoordeling van weefselbiopten na afname via gastroscopie. Dankzij deze diagnostische eis zijn er de laatste jaren op verschillende plekken in Noord-Amerika grote cohorten gevormd waarin klinische patiëntkenmerken gecombineerd kunnen worden met histologische en genexpressie data in de aangedane slokdarm teneinde beter inzicht te krijgen in het pathofysiologisch substraat van de ziekte (Dellon et al., 2012; 2013; Blanchard et al., 2011; Wen et al., 2015; 2013). Het patiëntencohort van het Boston Children's Hospital dat we beschrijven in de **Hoofdstukken 2**, **3**, **4** en **5** van dit proefschrift is hier een voorbeeld

van. Dit translationele onderzoeksproject is gericht op een drietal doelen: (1) de noodzaak voor herhaaldelijk endoscopisch onderzoek bij patiënten verminderen middels identificatie van minder invasieve biomarkers; (2) een beter begrip van de pathogenese van EoE en (3) de diagnostische waarde vergroten van oesofagiale biopten.

In werk dat niet in dit proefschrift is geïncludeerd hebben we met betrekking tot het eerste onderzoeksdoel gekeken of een oplosbare vorm van de IgE oppervlakte receptor FcεRI gebruikt kan worden om EoE patiënten te identificeren (Lexmond et al., 2011). Daarnaast hebben we onderzocht of waarden van leukotrieën E4 in urine (**Hoofdstuk 3**) of de oplosbare IL-33 receptor in serum (**hoofdstuk 5**) onderscheid maken tussen kinderen met en zonder EoE. Andere groepen hebben gekeken naar combinaties van cytokines in bloed (Blanchard et al., 2011) of hebben getracht de diagnose te stellen op grond van materiaal verkregen via een doorgeslikte capsule aan een sonde (Furuta et al., 2013). Ondanks deze pogingen is er tot op heden geen klinisch alternatief voor gastroscopie in de diagnose van EoE.

Slokdarmbiopten van patiënten vormen een waardevolle bron bij het ontrafelen van de pathogenese van EoE. In 2006 werd voor het eerst beschreven dat EoE gepaard gaat met een ziekte-specifiek transcriptoom, wat wil zeggen dat er zich bij EoE patiënten in de oesophagus veranderingen voordoen in de mRNA expressie van verschillende (inflammatoire) genen (Blanchard et al., 2006b). In **Hoofdstuk 2** demonstreren we dat een rekenkundig model gebaseerd op de expressie van een tiental van dit soort genen in staat is om met een sensitiviteit tot 94% patiënten met EoE te onderscheiden van kinderen met gastro-oesofageale refluxziekte of die met normale slokdarmhistologie. De hoge sensitiviteit van dit model is te verklaren door het feit dat het gebruik maakt van genen die tot expressie gebracht worden in de context van eosinofiele inflammatie en die derhalve gezien kunnen worden als passend bij een 'final common pathway'. In het oorspronkelijke transcriptoom onderzoek werd reeds gevonden dat de meest karakteristieke veranderingen (verhoogde expressie van eotaxin-3 en periostin) optreden bij EoE patiënten ongeacht IgE sensibilisatie tegen voedselallergenen (Blanchard et al., 2006a; 2007). Zoals we hiervoor reeds bespraken is EoE echter een heterogene ziekte wat betreft klinische presentatie, leeftijd van ontstaan, en relatie tot het al dan niet bestaan van andere allergische ziekten. Derhalve vroegen we ons af of het EoE transcriptoom niet ook subtielere verschillen behelst die samenhangen met verschillende uitingsvormen van de aandoening. Deze hypothese vormt het uitgangspunt van **Hoofdstuk 3**, waar we een subpopulatie (ongeveer 30%) van EoE patiënten definiëren die gekenmerkt wordt door verhoogde mRNA expressie van het enzym leukotrieën C4 synthase (LTC4S) dat uit arachidonzuur leukotrieën C4 genereert, een ontstekingsfactor betrokken bij allergische weefselresponsen. EoE patiënten met lokaal verhoogde *LTC4S* transcriptie hadden vaker IgE-gemedieerde voedselallergieën en vormden daarmee dus een subgroep die op grond van zowel functionele als klinische eigenschappen kan worden gedefinieerd. Noemenswaardig is dat klinische trials met de leukotrieën receptor antagonist montelukast in EoE patiënten gemengde resultaten hebben gerapporteerd. Hoewel een aantal patiënten

verbetering vertoonde was de werkzaamheid in de totale patiëntengroep onvoldoende. Een aantrekkelijke hypothese die voortkomt uit de resultaten beschreven in **Hoofdstuk 3** is dat alleen patiënten met verhoogde *LTC4S* expressie baat zullen hebben van medicamenteuze interventie in het leukotrieën metabolisme.

Naast dit werk gericht op het verbeteren van het diagnostisch proces bij EoE hebben we in **Hoofdstuk 4 en 5** de biopten van ons EoE patiëntencohort gebruikt voor pathofysiologisch-georiënteerde vraagstellingen. Zoals het geval is voor andere allergische ziekten is de incidentie van EoE snel stijgende in Westerse samenlevingen, wat betekent dat omgevingsfactoren een doorslaggevende rol spelen bij het ontstaan van deze aandoening (Davis en Rothenberg, 2016; Furuta en Katzka, 2015). Een belangrijke factor die de laatste tien jaar veel wetenschappelijke aandacht heeft gekregen is de samenstelling van onze microflora – het geheel van micro-organismen op en in ons lichaam – waarbij moleculaire signalen van bacteriën een grote invloed blijken te hebben op de homeostase van onderdelen van het immuunsysteem in de mucosa (zie ook **Hoofdstuk 7 en 9**). Een type leukocyt waarvoor zo'n verband in sterke mate is aangetoond is de *invariant chain natural killer T* (iNKT) cel, die in verhoogde aantallen voorkomt in de darm en longen van muizen die opgroeien zonder bacteriële flora (zogenaamde germ-free muizen) en deze dieren daarmee gevoelig maakt voor colitis en allergisch astma (Olszak et al., 2012). In **Hoofdstuk 4** beschrijven we dat iNKT cellen voorkomen in de slokdarm van patiënten met EoE. Hun betrokkenheid bleek het grootst in kinderen die op jonge leeftijd EoE ontwikkelden (voor hun zesde levensjaar) en bij wie we eveneens aanwijzingen vonden voor IgE-sensibilisatie tegen voedselallergenen en verhoogde *LTC4S* expressie. Verwijdering van allergenen uit het dieet van onze patiënten ging gepaard met normaliseren van de iNKT-cel as. We vonden verder dat EoE is geassocieerd met het gebruik van antibiotica op jonge leeftijd, wat inmiddels ook door andere groepen is gesuggereerd (Jensen et al., 2013; Radano et al., 2014), en in combinatie met het vaststellen van een ziekte-specifiek microbioom (Harris et al., 2015) kunnen deze bevindingen in de toekomst mogelijk leiden tot preventieve strategieën.

Als ook eerder al benadrukt moet EoE beschouwd worden als een complexe, multifactoriële aandoening, en een verstoorde bacteriële kolonisatie en iNKT cel homeostase alleen is niet voldoende om alle aspecten van de ziekte te verklaren. Zo wordt polymorfisme in de genen *TSLP* of *IL33* in verhoogde mate gevonden bij EoE patiënten (Kottyan et al., 2014; Rothenberg et al., 2010; Sherrill et al., 2010; Spergel, 2010) en komt de ziekte vaak voor in de context van andere allergische aandoeningen. In **Hoofdstuk 5** beschrijven we dat allergische sensibilisatie via de huid van muizen met dermatitis gevolgd door intranasale allergeen blootstelling leidt tot eosinofiele ontsteking van de slokdarm via verhoogde expressie van IL-33 en TSLP. Hierbij is een kritieke rol weggelegd voor basofiele granulocyten, want muizen zonder basofielen vertoonden minder eosinofielen in de oesophagus na allergeen stimulatie. Daarnaast vonden we in de slokdarm van kinderen met EoE verhoogde expressie van de IL-33 receptor. De bevindingen in dit hoofdstuk ondersteunen de theorie dat atopische

dermatitis (allergisch eczeem), vaak de eerste uiting van atopie in de atopische mars, direct kan bijdragen aan de manifestatie van allergische ziekten in het latere leven.

11.3 Introductie Deel 2: proefdiermodellen in de studie van type 2 inflammatie van het maag–darmkanaal

Hoofdstuk 5 maakt gebruik van een experimentele strategie die erop gericht is om allergische sensibilisatie in muizen te bewerkstelligen via allergeen blootstelling door een mechanisch beschadigde huid. In dit protocol werden dieren op de leeftijd van 4-6 weken gedurende 7 dagen blootgesteld aan allergeen-bevattende pleisters, gevolgd door intranasale allergeen toediening op dagen 9, 11 en 13. Een vergelijkbaar protocol dat we zullen tegenkomen in **Hoofdstuk 8** is gebaseerd op de allergische sensibilisatie van proefdieren via intraperitoneale injectie met aan adjuvans gekoppeld allergeen op dag 0, 7 en 14 en daaropvolgende intragastrische provocaties van dag 21 tot 39. Hoewel dergelijke strategieën ons inzicht in de pathofysiologie van allergische ziekten ruimschoots vergroot hebben moeten we ons ook bewust zijn van de beperkingen die deze modellen met zich meebrengen en van de aannames die verscholen liggen in de timing van de immunologische interventies. Immers, het proces van allergische sensibilisatie zelf bevindt zich in deze experimenten volledig onder controle van de onderzoeker en is gebaseerd op relatief onfysiologische prikkels zoals het mechanisch beschadigen van de geschoren huid met plakband, of injectie met aan aluminium hydroxide gekoppeld ovalbumine. Deze experimenten gaan hiermee in hun opzet voorbij aan het feit dat de kritieke invloed van omgevingsfactoren op atopie heel goed zou kunnen liggen in het verhogen van de gevoeligheid voor IgE sensibilisatie. Om te komen tot mechanistisch inzicht in de vraag hoe omgevingsfactoren bijdragen aan de verhoogde incidentie van allergische ziekten zouden we juist het proces van allergische sensibilisatie zelf moeten bestuderen. Een ideaal experiment om een causaal verband aan te tonen dient de mate van sensibilisatie te vergelijken binnen twee groepen muizen die alleen van elkaar verschillen in de blootstelling aan de ene onderzochte omgevingsfactor. Natuurlijk zijn zulke studies alleen uitvoerbaar als allergische sensibilisatie daadwerkelijk optreedt in proefdieren op een wijze die min of meer overeenkomt met de pathofysiologie van de aandoening in patiënten. Voor de studie van voedselallergie zit hier een probleem. Wild-type muizen zijn zeer resistent voor aberrante IgE reacties tegen voedselantigenen dankzij het principe van orale tolerantie, waarmee het samenspel van immuun mechanismen bedoeld wordt dat ongewilde responsen tegen (onschadelijke) antigenen in het lumen van de darm dient te voorkomen. De robuustheid van orale tolerantie zorgt ervoor dat de inductie van type 2 inflammatie onder normale omstandigheden alleen bestudeerd kan worden in muizen na het uitvoeren van immunologische manipulatie met adjuvans, waarvan hierboven al een voorbeeld werd geschetst, of – zo mogelijk nog minder fysiologisch – na passieve immunisatie

met allergeen specifieke IgE antilichamen. Zulke diermodellen, die de effector fase van de ziekte benaderen maar niet de totstandkoming ervan, worden 'isomorf' genoemd en staan daarmee in tegenstelling tot 'homologe' modellen, die zowel effector als inductiefase weergeven op een manier die overeenkomt met de bij mensen voorkomende aandoening (Oyoshi et al., 2014). **In Deel 2 van dit proefschrift richten we ons op strategieën om de homologie van diermodellen van type 2 inflammatie te vergroten.**

11.4 Rationale en onderzoeksvragen behorend bij Deel 2

Een van de mogelijke strategieën om de translationele waarde van dierexperimenten te vergroten is om gebruik te maken van muizen met (componenten van) een menselijk immuunsysteem (zogenaamde gehumaniseerde muizen, zie **Hoofdstuk 1**). Dit kan op vrij eenvoudige wijze worden bewerkstelligd door geïsoleerde leukocyten uit bloed van patiënten of vrijwilligers te transplanteren naar muizen die zelf niet beschikken over een adaptief immuunsysteem en derhalve geen afweerrespons kunnen opwekken tegen de lichaamsvreemde, menselijke immuuncellen. De omgekeerde respons, dat wil zeggen het herkennen van de muis als 'lichaamsvreemd' door de getransplanteerde lymfocyten, is echter wel intact, en de ernstige graft-versus-host reacties die het gevolg zijn leggen grote beperkingen op aan de conclusies die uit dergelijke studies kunnen worden getrokken. Dit graft-versus-host probleem kan worden voorkomen door in dezelfde immunodeficiënte muizen immuun reconstitutie te laten plaatsvinden uit menselijke hemopoietische (CD34-positieve) stamcellen. De T-lymfocyten die zich op deze manier ontwikkelen ondergaan hun negatieve selectie immers via interacties met muizen MHC klasse II in plaats van menselijk MHC II, waardoor het risico op auto-immuniteit sterk wordt teruggebracht. Helaas is de opbrengst van T cellen in dit systeem laag en blijven adaptieve immuunreacties zwak (Baenziger et al., 2006). Reconstitutie van lymfocyten kan weliswaar worden verbeterd door transplantatie van foetaal beenmerg, lever en thymus weefsel samen met CD34-positieve hemopoietische stamcellen, maar dit gaat gepaard met hoge kosten en ethische bezwaren en maakt het tevens onmogelijk om patiëntmateriaal te bestuderen (Shultz et al., 2012). In **Hoofdstuk 6** testen we de hypothese dat adaptieve immuniteit in gehumaniseerde muizen kan worden verbeterd door stamcelreconstitutie te laten plaatsvinden in immunodeficiënte muizen die in plaats van muizen MHC II een menselijk MHC II allel (DR1) tot expressie brengen. We demonstreren dat deze strategie leidt tot een groter T cel receptor repertoire en betere B cel rijping, met daarbij sterkere adaptieve immuunreacties en hogere antistof concentraties (inclusief IgE) in bloed. Transplantatie van stamcellen verkregen van een donor met het immune dysregulation, polyendocrinopathy, enteropathy, X-linked (IPEX) syndroom, gekenmerkt door afwezigheid van $FOXP3^+$ regulatore T cellen door mutatie van transcriptiefactor *FOXP3*, resulteerde in dodelijke auto-immuniteit in $DR1^+$ muizen maar niet

in muizen zonder menselijk DR1. **Hoofdstuk 6** vormt hiermee de eerste beschrijving van een menselijke immuunpathologie die op deze manier wordt gekopieerd in een gehumaniseerd muismodel.

Naast ernstige auto-immuniteit leiden patiënten met het IPEX syndroom aan voedselallergieën, eczeem en verhoogd serum IgE (Akdis en Akdis, 2009; Lin et al., 2005; Suscovich et al., 2012). Deze symptomen zijn het gevolg van de ontregelde Th2 immuun activatie die optreedt in afwezigheid van $FOXP3^+$ regulatore T (Treg) cellen. Verhoogd serum IgE en atopie wordt verder gezien in een aantal primaire immuundeficiënties. Een welbekend voorbeeld van een dergelijke aandoening met hoog serum IgE is het syndroom van Wiskott-Aldrich (WAS), wat veroorzaakt wordt door een mutatie van het gelijknamige gen *WAS* op het X-chromosoom. Het Wiskott-Aldrich syndroom eiwit (WASP) wordt in alle hemopoietische cellen tot expressie gebracht en is een regulator van het actine cytoskelet die ervoor zorgt dat een groot aantal externe stimuli gepaard kan gaan met morfologische en functionele veranderingen in de cel (Snapper en Rosen, 1999; Thrasher en Burns, 2010). Inmiddels zijn meer dan 100 unieke 'loss-of-function' mutaties in *WAS* beschreven waardoor sprake is van een klinisch zeer heterogene patiëntengroep. Jongens met de meest ernstige mutaties presenteren zich in het eerste levensjaar met ernstig eczeem, trombocytopenie, auto-immuniteit en recidiverende infecties, die levensbedreigend kunnen verlopen tenzij patiënten stamceltransplantatie of gentherapie ondergaan. Andere *WAS* mutaties leiden tot X-linked trombocytopenie (XLT), een klinisch syndroom dat veel milder verloopt en ook zonder stamceltransplantatie een uitstekende prognose kent (Albert et al., 2011). Hoewel de relatie tussen mutaties in *WAS* en verhoogd serum IgE al in 1968 voor het eerst werd beschreven is het sindsdien onduidelijk gebleven wat in deze patiënten aanleiding geeft tot verhoogde productie van IgE. Daarnaast is de antigeenspecificiteit van IgE onbekend, en weten we niet wat de consequenties zijn van het verhoogde serum IgE op het bestaan van allergische aandoeningen in patiënten met *WAS* mutaties. WASP is een evolutionair geconserveerd eiwit en ook in muizen wordt op het X-chromosoom een ortholoog van het menselijk *WAS* gen gevonden (genaamd *Was*). Sinds 1998 bestaan WASP-deficiënte ($Was^{-/-}$) muizen, waarin een groot aantal van de immunologische karakteristieken van Wiskott-Aldrich syndroom tot uiting komen (Snapper et al., 1998). Net als voor WAS patiënten geldt ook voor deze muizen dat er geen systematische studie is verricht naar het voorkomen en het mechanisme van aberrante IgE responsen. In **Hoofdstukken 7**, **8** en **9** bestuderen we IgE-gemedieerde immuun responsen in WASP-deficiënte patiënten en muizen. De overkoepelende hypothese bij deze experimenten is dat verhoogd serum IgE specificiteit zou kunnen hebben voor voedselantigenen en dat $Was^{-/-}$ muizen in dat geval gebruikt zouden kunnen worden als een nieuw, mogelijk meer homoloog proefdiermodel voor de studie van voedselallergie.

In **Hoofdstuk 7** beschrijven we dat voedselallergie voorkomt bij 20% van patiënten met mutaties in *WAS*, een significant hogere prevalentie dan in de algehele bevolking. $Was^{-/-}$

muizen hebben net als WAS patiënten verhoogde waarden van serum IgE. Deze IgE antistoffen tonen specificiteit voor voedselantigenen die voorkomen in het dieet van de proefdieren, waaronder tarwe en soja. Aangezien tarwe en soja belangrijke voedselallergenen zijn bij patiënten met voedselallergie vormt deze bevinding een belangrijk argument voor homologie van het model. In **Hoofdstuk 8** wordt onze hypothese uitgewerkt en worden *Was*$^{-/-}$ muizen verder gekarakteriseerd als proefdiermodel voor voedselallergie door ze te vergelijken met een bestaand en veelgebruikt model dat gebaseerd is op sensibilisatie via intraperitoneale injectie van ovalbumine (OVA) in combinatie met adjuvans. In *Was*$^{-/-}$ dieren leidt orale blootstelling aan OVA tot OVA-specifieke IgE productie, en kan de inductie van voedsel-specifiek IgE dus bestudeerd worden zonder immuunmanipulatie met adjuvans. Aangezien een homoloog model naast de inductiefase ook de effectorfase van IgE-gemedieerde voedselallergie moet omvatten, bestuderen we tevens het optreden van allergisch diarree na provocatie met OVA in spontaan gesensibiliseerde *Was*$^{-/-}$ muizen. Dit werd gezien in ongeveer 70% van de dieren, hetgeen overeen kwam met muizen uit het adjuvans-gemedieerde model. Na intraperitoneale injectie met een lage dosis OVA trad in *Was*$^{-/-}$ muizen een anafylactische reactie op gekenmerkt door een snelle daling van de lichaamstemperatuur, die in >50% van de proefdieren een dodelijke afloop kende binnen een uur na provocatie.

Nadat we WASP-deficiënte muizen hadden geïdentificeerd als nieuw proefdiermodel voor de studie van voedselallergie waren we erop gericht om de pathogenese van deze spontane type 2 inflammatie in het maag-darm kanaal te verhelderen. Zoals in **Hoofdstuk 7** wordt beschreven treden spontane IgE reacties tegen voedselallergenen ook op in *Was*$^{-/-}$ muizen gehouden onder germ-free condities, hoewel de humorale immuunrespons in muizen mét en zonder darmflora wel van elkaar verschilde in de verhouding tussen immuunglobuline isotypes. Met behulp van genetische strategieën die het mogelijk maken om te komen tot cel-type-specifieke deletie van WASP tonen we aan dat verlies van WASP uit FOXP3^{+} Tregs voldoende is voor het spontaan ontstaan van voedselallergie en type 2 intestinale inflammatie. Waar Th1 en Th17 responsen niet versterkt voorkomen in *Was*$^{-/-}$ muizen blijken WASP-deficiënte Tregs niet in staat om Th2 proliferatie en activatie te onderdrukken. Dit gaat gepaard met verhoogde expressie van Th2 transcriptiefactor GATA3 in Tregs, zowel in muizen als in patiënten met mutaties in *WAS*. De mate van GATA3 expressie in Tregs hangt in patiënten af van de ernst van de *WAS* mutatie. GATA3 waarden waren lager in patiënten met XLT dan in die met ergere loss-of-functie mutaties, wat suggestief is voor een dosis-respons relatie. De bevinding van een Th2 fenotype van FOXP3^{+} Tregs past binnen een recente studie waarbij dergelijke Tregs ook gevonden worden in jonge kinderen met koemelkallergie buiten de context van WASP deficiëntie (Noval Rivas et al., 2015). Dit vormt een extra argument voor relevante overlap tussen WAS-geassocieerde en veelvoorkomende voedselallergie en pleit daarmee voor homologie van het proefdiermodel.

Zoals weergegeven in **Figuur 7.4 (pagina 164)** resulteert FOXP3^{+} Treg-specifieke deletie van WASP in een versterkt allergisch fenotype met ernstigere type 2 inflammatie in het maag-

darm kanaal ten opzichte van *Was*$^{-/-}$ muizen. Deze observatie vormt het uitgangspunt voor **Hoofdstuk 9**, dat geschreven is met als doel een synthese van de data uit **Hoofdstukken 6**, **7** en **8** te presenteren met een inkadering in de bestaande WAS literatuur. De overkoepelende hypothese hierbij is dat verschillen in het allergisch fenotype tussen *Was*$^{-/-}$ muizen en muizen met FOXP3^{+} Treg-specifieke deletie van WASP het gevolg zijn van WASP-afhankelijke functies in immuuncompartimenten anders dan FOXP3^{+} Tregs (**Tabel 9.1, pagina 241**). Drie argumenten rechtvaardigen een nadere analyse van deze verschillen: 1. De analyse van de rol van WASP in verschillende immuuncompartimenten kan bijdragen aan een beter begrip van voedselallergie in WAS patiënten, in wie WASP ontbreekt in alle hemopoietische lijnen; 2. Groter inzicht in de pathofysiologie van WAS-geassocieerde voedselallergie is relevant voor onze kennis over IgE-gemedieerde reacties tegen voedselantigenen in het algemeen; en 3. Beter begrip van de verschillen tussen beide muizen leidt tot een beter gedefinieerd proefdiermodel voor de studie van voedselallergie in de toekomst. In **Hoofdstuk 9** demonstreren we dat WASP-gemedieerde functies in antigeen presenterende cellen, B cellen, effector T cellen, regulatore T cellen en IgE effector cellen allemaal een vermoedelijke pro- of antiallergische rol spelen in de pathofysiologie van spontane voedselallergie. Verder demonstreren we in **Hoofdstuk 9** preliminaire resultaten waaruit blijkt dat het microbioom en maternale factoren een invloed hebben op WAS-geassocieerde voedselallergie en dat WASP-deficiënte Tregs ziekte kunnen veroorzaken in muizen waar WASP ontbreekt in slechts de helft van de Tregs. Het hoofdstuk sluit af met samenvattende conclusies en suggesties voor verder onderzoek.

Appendices

Acknowledgements

If these are the first words you read in this work, please be advised that a critical introduction of the *dramatis personae* took place in the preface of this thesis. Those who have not yet done so are therefore encouraged to read that particular section in conjunction with the following paragraphs, lest vital contributions of a number of people go unnoticed or underappreciated. Even with this disclaimer, however, I still fear that the following list will never be exhaustive, for I have been extremely fortunate along the way and I have a great many people to thank for that.

The Italian scholar Umberto Eco argues that it is "in bad taste to acknowledge your thesis adviser", for "if he has helped you, he has simply done his job" [5]. I disagree. There are many ways to do a job, and while every PhD student can reasonably expect his adviser to provide professional and scientific counseling and supervision, it is nowhere specified that an academic promotor should also open his home and family to his or her student, return e-mails within the hour, or grant that student 'carte blanche' to schedule meetings with his secretary (Sabrina, thank you for all your help!). To have not one but two of such advisers seems to reflect a disproportional degree of luck. Edda and Edmond, you have always treated me as one of your top priorities; thank you both for all your guidance and support during these years.

And while having two great advisers might seem like already stretching it, I pushed my luck even further and found a third one in dr. Scott Snapper. Scott, while the just mentioned *carte blanche* certainly did not materialize with your secretary (Juanita, thank you for always trying!), let it be clear that this says nothing about your level of interest in my work and in my person, but instead everything about your success and ambition. You have made me feel incredibly welcome within your group, and your passion and enthusiasm for science is inspiring and contagious. The knowledge and insight obtained during our meetings will no doubt serve me greatly during the rest of my career.

Prof. dr. Richard Grand, today I humbly stand before you as the next PhD graduate in a tradition that you have kept going for literally as long as I have lived. You have also trained Edmond – it dawned on me that this renders me your scientific grandchild (or Grandchild if you will) – and if all acknowledgements addressed to you over these years were to be bundled, I have no doubt it would in length compare to a hefty thesis chapter. You always were extremely generous with your time for me, wrote letters of support for me, and got me

5 Umberto Eco. How to write a thesis. MIT Press 2015. Translated from the Italian, *Come si fa una tesi di laurea: le materie umanistische* 1977/2012.

in touch with other people in your infinitely vast, and indeed world wide, web. Dear Dick, thank you for everything. I am incredibly honored that you have made the long journey from Boston to be present here today.

Prof. dr. Hankje Escher, dr. Janneke Samsom, and prof. dr. Frits Koning, thank you for taking part in the review committee of my thesis. I am excited about the prospect of collaborating with you in the future. Prof. dr. Edward Nieuwenhuis, prof. dr. Roy Gerth van Wijk and prof. dr. Arjan Lankester, thank you for your presence here today and for what will no doubt turn out to be your insightful and critical questions during the defense.

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And then, at last, dr. Jeremy Goettel, my closest friend and collaborator of all. Jeremy, where shall I begin? Do I start by stating that this thesis would have looked nothing like this if we had not gotten along as well as we have? Or that you have single-handedly taught me 50% of the methods that I have used throughout these pages? Or that hardly a day has gone by in which we did not talk for at least an hour? Maybe it suffices to say that you are by far the most generous person that I have ever met and that I have cherished each and every one of the 16 *oz.* cans that we enjoyed to fuel our scientific and philosophical musings. I am extremely happy that you are now starting your own lab at Vanderbilt and I hope that our collaboration and friendship will flourish until deep into our retirement. Thank you for everything; I am honored that you are here today as my paranimf.

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The somewhat overused quote from the first paragraph of Leo Tolstoy's *Anna Karenina* states that "happy families are all alike; every unhappy family is unhappy in it's own way"[6]. This is not true. I come from a very happy family, but one that certainly has its very own way of being happy. My sisters Anne and Trudie, thank you both for all the love and support over the years. Trudie, it has been a pleasure to see you move with such focus to where you always wanted to be: happily married, a healthy child, and a job as a general practitioner whose patients never want to see a different doctor again. Anne, I like to think that our healthy dose of sibling rivalry has bettered us both. Let our mutual paranimfism be the crown on that relationship! My parents Géke and Theo, it was around the time that you asked me if it had not, slowly, started to become time that I would come pick up my belongings from your attic – stored up there in cardboard boxes ever since my departure for Australia four years earlier – that I broke the news that 'actually', I was thinking about leaving to the US 'for a couple of years'. A much heard question in those early days was whether this was not too harsh or cruel on my parents, to move away again after having been to Australia and Curaçao for already quite some time. Does it not say everything about the way you raised us that without consulting either of you I could respond to those people with a convinced 'no, it's not, they would not have it any other way'. It is not easy to describe what makes you such great parents. In her thesis, Anne has called it a safety net, which – out of pride (see above) – I certainly will not plagiarize here, but with which I do agree. I have also thought about referring to studies from Michael Meaney at McGill University who showed that epigenetic modifications occur in rats that receive a lot of parental love and warmth after birth, and that these DNA changes protect the rats against stress and disease later in life. However, I have decided that I will just leave it at the following conclusion: it will be very hard to live up to the example you have set. Thank you.

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6 Leo Tolstoy. Anna Karenina. Translated from Russian by Constance Garnett. Barnes & Noble Classics, New York 2003.

PhD portfolio

Candidate:

Name PhD student:	Willem Schulto Lexmond
PhD period:	April 2011 – November 2015
Promotor:	prof. dr. Edmond H.H.M. Rings
Co-promotor:	dr. Edda Fiebiger

Courses, Seminars and Conferences:

2011 – 2015	Harvard Immunology Program; Wednesday Seminar Series (weekly, 1 hr)
2011 – 2014	Center for the Study of IBD, annual symposium and workshop, Massachusetts General Hospital (annually, 2 days)
Jun 2012	Introduction to Translational Medicine; Harvard Catalyst; Harvard Medical School; Boston, MA (80 hrs)
2012 – 2014	Digestive Disease Week, American Gastroenterological Association (annually, 5 days)
2012 – 2013	Certificate in Applied Biostatistics; Harvard Catalyst; Harvard Medical School; Boston, MA (160 hrs)
Mar 2013	Leadership Strategies for the Researcher; Harvard Catalyst; Harvard Medical School; Boston, MA (15 hrs)
Mar 2013	Genetic Literacy; Harvard Catalyst; Brigham and Women's Hospital; Boston, MA (8 hrs)
2014 – 2015	Journal Club on Mucosal Immunology; Boston Children's Hospital (monthly, 1 hr)
2015	International Congress of Mucosal Immunology, Berlin, Society for Mucosal Immunology (5 days)

Scientific Presentations:

International

2012 Leukotriene C4 Synthase mRNA Levels are Upregulated in Esophageal Biopsies of Patients with Eosinophilic Esophagitis. Poster presentation. American Gastroenterological Association. Digestive Disease Week, San Diego, 20 May 2012.

2014 Deletion of Wiskott-Aldrich syndrome protein in regulatory T cells results in spontaneous allergic sensitization to food. Poster presentation. American Gastroenterological Association. Digestive Disease Week, Chicago, 6 May 2014. *Award: Poster of Distinction*

2014 Digital mRNA Profiling of Esophageal Tissue Biopsies as a Novel Diagnostic Approach to Eosinophilic Esophagitis. Voordracht plenaire sessie "Celiac/EoE/Allergic Enteropathy". North American Society for Pediatric Gastroenterology, Hepatology and Nutrition. Annual Meeting, Atlanta, 25 October. *Award: NASPGHAN/APFED Prize for Eosinophilic Disorders*

2015 Deletion of Wiskott Aldrich Syndrome Protein in Regulatory T Cells Results in Regulatory T Cells Results in Spontaneous Allergic Sensitization to Food Antigens. Oral presentation. Society for Mucosal Immunology. 17th International Congress of Mucosal Immunology (ICMI 2015), Berlin, 16 July. *Award: Young Investigator Travel Award*.

National / institutional

2013 Involvement of the iNKT cell pathway is associated with early-onset eosinophilic esophagitis and response to allergen avoidance therapy. Brigham and Women's Hospital and Dana Farber Cancer Institute. Boston, 27 September 2013.

2013 Leukotriene C4 Synthase mRNA Levels are Upregulated in Esophageal Biopsies of Patients with Eosinophilic Esophagitis. Poster presentation. Judah Folkman Research Day, Children's Hospital Boston, 15 May 2013.

2016 $FOXP3^{+}$ regulatory T cells require Wiskott-Aldrich Syndrome protein to restrain Th2-mediated food allergy. Oral presentation. Sophia Research Day, Erasmus Medical Center, 14 April 2016.

2016 iNKT cellen, het microbioom, en de gevoeligheid voor overgevoeligheid. Oral presentation (Dutch). SOWIESO dag kindergeneeskunde. 15 September 2016.

Teaching Activities:

2011-2015 Laboratory training and supervision of medical students from various Dutch MD programs and the Harvard Immunology Summer Program.

Grant Awards:

2014 – 2015 Mead Johnson Nutrition. Research fellowship. Nutritional intervention as a therapeutic strategy to modulate onset and severity of gastrointestinal allergic inflammation.

2011 – 2012 Ter Meulen Fund, Royal Dutch Academy of Sciences. Personal stipend for scientific resarch in the field of pediatrics.

2011 – 2012 Banning-De Jong Fund, Prins Bernhard Cultural Fund. Personal stipend for PhD training at Harvard Medical School in Boston.

List of publications

Included in this thesis:

2013 **Lexmond WS**, Pardo M, Rooney K, Goettel JA, Snapper SB, Yen EH, Dehlink E, Nurko S, Fiebiger E. Elevated levels of leukotriene C4 synthase mRNA distinguish a subpopulation of eosinophilic oesophagitis patients. Clin Exp Allergy. 2013 Aug;43(8):902-13.

2014 **Lexmond WS**, Neves JF, Nurko S, Olszak T, Exley MA, Blumberg RS, Fiebiger E. Involvement of the iNKT cell pathway is associated with early-onset eosinophilic esophagitis and response to allergen avoidance therapy. Am J Gastroenterol. 2014 May;109(5):646-57.

2015 **Lexmond WS**, Hu L, Pardo M, Heinz N, Rooney K, LaRosa J, Dehlink E, Fiebiger E, Nurko S. Accuracy of digital mRNA profiling of oesophageal biopsies as a novel diagnostic approach to eosinophilic oesophagitis. Clin Exp Allergy. 2015 Aug;45(8):1317-27.

2015 Goettel JA, Biswas S, **Lexmond WS**, Yeste A, Passerini L, Patel B, Yang S, Sun J, Ouahed J, Shouval DS, McCann KJ, Horwitz BH, Mathis D, Milford EL, Notarangelo LD, Roncarolo MG, Fiebiger E, Marasco WA, Bacchetta R, Quintana FJ, Pai SY, Klein C, Muise AM, Snapper SB. Fatal autoimmunity in mice reconstituted with human hematopoietic stem cells encoding defective FOXP3. Blood. 2015 Jun 18;125(25):3886-95.

2016 Venturelli N, **Lexmond WS**, Ohsaki A, Nurko S, Karasuyama H, Fiebiger E, Oyoshi MK. Allergic skin sensitization promotes eosinophilic esophagitis through the IL-33-basophil axis in mice. J Allergy Clin Immunol. 2016 Nov;138(5):1367-1380.e5

2016 **Lexmond WS***, Goettel JA*, Lyons JJ*, Jacobse J, Deken MM, Lawrence MG, DiMaggio TH, Kotlarz D, Garabedian E, Sackstein P, Nelson CC, Jones N, Stone KD, Candotti F, Rings EH, Thrasher AJ, Milner JD, Snapper SB, Fiebiger E. FOXP3+ Tregs require WASP to restrain Th2-mediated food allergy. J Clin Invest. 2016 Oct 3;126(10):4030-4044. *co-first authors.

2017 **Lexmond WS**, Goettel JA, Sallis BF, McCann K, Rings EHHM, Jensen-Jarolim E, Nurko S, Snapper SB, Fiebiger E. Spontaneous food allergy in Was-/- mice occurs independent of FcεRI-mediated mast cell activation. Allergy. 2017 Jun 10. doi: 10.1111/all.13219. [Epub ahead of print]

Not included in this thesis:

2011 **Lexmond WS***, Van der Mee J*, Ruiter F, Platzer B, Stary G, Yen EH, Dehlink E, Nurko S, Fiebiger E. Development and validation of a standardized ELISA for the detection of soluble Fc-epsilon-RI in human serum. J Immunol Methods. 2011 Oct 28;373(1-2):192-9. *co-first authors.

2014 Sturm E*, **Lexmond WS***, Verkade HJ. Pediatric acute liver failure: variations in referral timing are associated with disease subtypes. Eur J Pediatr. 2015 Feb;174(2):169-75. *co-first authors.

2014 Platzer B, Baker K, Vera MP, Singer K, Panduro M, **Lexmond WS**, Turner D, Vargas SO, Kinet JP, Maurer D, Baron RM, Blumberg RS, Fiebiger E. Dendritic cell-bound IgE functions to restrain allergic inflammation at mucosal sites. Mucosal Immunol. 2015 May;8(3):516-32.

2015 **Lexmond WS**, Van Dael CM, Scheenstra R, Goorhuis JF, Sieders E, Verkade HJ, Van Rheenen PF, Kömhoff M. Experience with Molecular Adsorbents Recirculation System Treatment in Twenty Children Listed for High Urgency Liver Transplantation. Liver Transpl. 2015 Mar;21(3):369-80.

2015 **Lexmond WS**, Rufo PA, Fiebiger E, Lencer WI. Electrophysiological Studies into the Safety of the Anti-diarrheal Drug Clotrimazole during Oral Rehydration Therapy. PLoS Negl Trop Dis. 2015 Sep 25;9(9).

2016 Mudde AC, **Lexmond WS,** Blumberg RS, Nurko S, Fiebiger E. Eosinophilic esophagitis: published evidences for disease subtypes, indications for patient subpopulations, and how to translate patient observations to murine experimental models. World Allergy Organ J. 2016 Jul 15;9:23

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About the author

Willem Schulto Lexmond was born on the 8th of July 1984 in his parental home in Renkum, Gelderland as the oldest child in what would turn out to be a three-sibling family (a valuable lesson on coalition formation, so reasoned his father). He spent a considerable part of a carefree childhood outside and, on more than one occasion, was not allowed access into the house until a garden hose rinsing had been performed to remove the dirt that inevitably resulted from his ecological investigations (e.g. into the natural habitat of frogs), the performance of ornithological necropsies, or from studies confirming that Archimedes' principle also holds true for the combination of bricks and sewage water. In later childhood, such microbial hyper-exposure stayed confined to school holidays, during which efforts on his uncle's organic farm not only taught him important lessons about the value of money, but also firmly installed the conviction that an intellectual profession was to be preferred over manual labor.

In 2002, he graduated from the Gymnasium at the Marnix College in Ede (Gelderland) and, having identified medicine as an occupation that would allow a combination of scientific work with a more practical set of skills (certainly a safer choice than paleontology, which he still considers his most innate interest), enrolled in the Medical School program at the University of Groningen. He passed his propaedeutic (2003), doctoral (2006) and medical (2009) examinations all with distinction *cum laude,* and was involved in a number of extracurricular activities that included membership of the board of the medical student society Panacea. A research internship at the University of Sydney in 2006 (mentors prof. dr. Chris Gips and dr. Alex Bishop) awakened both his scientific interest and his sense of wanderlust, the latter being indulged even further during a year of clinical rotations in Willemstad, Curaçao in 2007-2008. Following a final, clinical elective at the division of pediatric gastroenterology at the Beatrix Children's Hospital in Groningen, he graduated from medical school in March 2009 and stayed on as an intern at the department of pediatrics and later at the division of pediatric nephrology.

From April 2011 till November 2015, he lived and worked in Boston to work on the PhD thesis you find before you (mentors prof. dr. Edmond Rings and dr. Edda Fiebiger). Upon his return in The Netherlands, he started his residency training in pediatrics at the Sophia Children's Hospital of the Erasmus Medical Center in Rotterdam under prof. dr. Matthijs de Hoog, and the Elisabeth-Twee Steden Hospital in Tilburg (clinical instructor dr. Charlie Obihara). He aspires to a career in academic pediatrics that combines clinical care with basic and translational scientific work. Willem lives in Tilburg with Sandy and their daughter Lore.