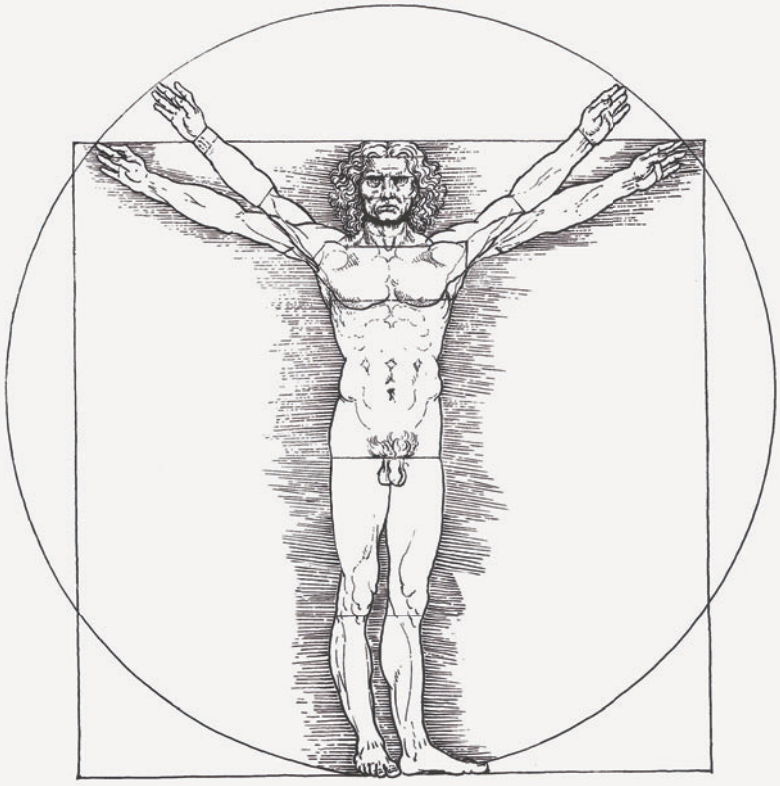
# The B-cell side of Allergy and Chronic Inflammatory Disease: Studies on the source of IgE and IgG4



Jorn J. Heeringa

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J.J. Heeringa


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# The B-cell side of Allergy and Chronic Inflammatory Disease: Studies on the source of IgE and IgG4

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# PART I

## GENERAL INTRODUCTION

# CHAPTER 1

## General introduction

Based on sections of:
**De bron van specifiek IgE: B-geheugencellen en plasmacellen.**
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## Allergy

Allergy is an abnormal response of the immune system against otherwise harmless substances and it is therefore often related to as a hypersensitivity reaction. Allergies can cause considerable symptoms, and in the case of anaphylaxis even death. In the past decades, the prevalence of allergy has dramatically increased, and at present up to 40% of the Western world population is affected by one or more allergies.[1] Based on the underlying immune response, allergic reactions can be divided into four types. The most common type (type I) is mediated by IgE antibodies and is associated with asthma, hay fever, food allergy and atopic dermatitis. Normally, immunoglobulin (Ig)E is involved in the protection against parasites, such as helminths. However in IgE-mediated allergies specific IgE molecules are directed against common allergens of house dust mite, pollen or peanut, and exposure can result in allergic symptoms. In this **General Introduction**, the function of a healthy immune system and the abnormal response in IgE-mediated allergic diseases will be described. In particular the mechanisms which lead to the production of IgE will be highlighted.

## The human immune system

All humans are continuously exposed to micro-organisms such as bacteria, viruses, fungi and parasites. To protect itself against the harmful effects of these microorganisms, the human body has developed a physical and chemical barrier (skin, mucosa) and the innate and the adaptive responses of the immune system. The cells lining the skin and the mucosal surfaces of the lung and gut are the first to come into contact with micro-organisms. These epithelial cells are tightly connected together to form a physical barrier making it difficult for micro-organisms and substances to penetrate.[2] An additional mechanical barrier is provided by coughing and sneezing and by peristaltic movement of the bowel, which expels micro-organisms and irritants.[3] Next to that, epithelial cells produce peptides with antimicrobial properties to provide a chemical barrier which prevents the attachment and proliferation of micro-organisms.[4] Furthermore, the skin and the mucosal surfaces of the gut and lung are populated with commensal bacteria, as a whole called the microbiota, which are typically harmless for the human body and prevent colonization of pathogenic micro-organisms.[5] Still, sometimes pathogens pass these barriers, and come into contact with cells of the immune system.

## Innate immune responses

After the penetration of a pathogen, the nonspecific innate arm of the immune system will get activated, which induces a fast response, but which has a limited capacity. Still innate immune cells are able to act against a variety of microorganisms. Typical for cells of the innate immune system is their direct activation and immediate effector functions. These cells are mostly located in tissues with contact to the outside world.[6] There are several cells of the innate immune system, i.e. natural killer (NK) cells, granulocytes (neutrophils,

eosinophils, and basophils), mast cells and monocytes, and each responds differently against micro-organisms. A cellular response is elicited by viruses and intracellular bacteria. If cells get infected, peptides of these microorganisms can be degraded internally in the cells and get displayed externally by the major histocompatibility complex class I (MHC-I) molecule, thereby forming a complex which can be recognized by immunocompetent T-lymphocytes. NK cells continuously scan cells for the presence of MHC-I and if expression lacks, induce cell death. Extracellular bacteria elicit a humoral immune response, in which soluble factors of the immune system, such as complement and antibodies, attach to bacteria. This facilitates recognition of bacteria by phagocytes (mainly monocytes and neutrophils). Subsequently these cells can locally release chemokines and pro-inflammatory cytokines, which induce the dilatation of blood vessels and thereby facilitate the recruitment of other cells of the immune system.[9] Mediators released by these cells cause the features of inflammation, including redness, swelling and pain or itch. Monocytes migrate into tissue, and differentiate into macrophages which together with neutrophils can take up pathogens or cell debris by phagocytosis and subsequently degrade these fragments internally in endosomal structures, leading to the clearance of unwanted intruding microbes.[10] Since parasites are often too big for phagocytosis a specific IgE-mediated immune response is triggered, in which the IgE antibody recognizes epitopes of the parasite and thereby activates eosinophils and mast cells. Upon activation, these cells release mediators which induce mucus production and itch, thereby facilitating parasite clearance. Some of these effects are also a feature of an allergic reaction. This can be explained by the fact that these responses share a common immunological response.

The innate immune system is not capable to develop any memory function for pathogens, but the adaptive immune system can. To activate the adaptive immune system, antigen presenting cells (APCs) phagocytose micro-organisms, process these internally and display small peptides on their cell surface via a specific receptor (MHC- II).[6] Dendritic cells are the predominant APCs and are therefore regarded as the bridging cells between innate and adaptive immunity. Another important cell type in the activation of the adaptive arm of the immune system are innate lymphoid cells. These cells lack antigen specific receptors, but can influence the type of adaptive immune response through the production of specific cytokines.[7]

### Adaptive immune responses

Adaptive immune responses are generated by B and T-lymphocytes, which have the unique property of memory formation against previously encountered pathogens. Both cell types originate from hematopoietic stem cells in the bone marrow (BM). While subsequent B-lymphopoiesis occurs in parallel with innate cell development in BM, early lymphoid progenitors also seed the thymus, where these commit to the T-cell lineage and develop into mature CD4+ or CD8+ T cells. Throughout differentiation in the bone marrow or thymus,

B- and T-cells each undergo multiple steps, leading to the formation of a large pool of cells with a unique B-cell or T-cell receptor.[8] When fully matured, these cells migrate to secondary peripheral organs where upon recognition of a micro-organism (or parts of a micro-organism presented by APCs) they can differentiate into effector cells or into memory cells. T-cells include cytotoxic T-cells (CD8+), which are involved in the direct killing of infected cells, and T-helper (Th) cells (CD4+), which aid other immune cells in the immune response. Cytotoxic T-cells are activated through MHC-I-mediated peptide presentation and thus any infected cell can induce a cytotoxic T-cell response. T-helper cells only recognize peptides presented through MHC-II. In contrast to MHC-I, MHC-II is only expressed by immune cells, and in particular APCs.

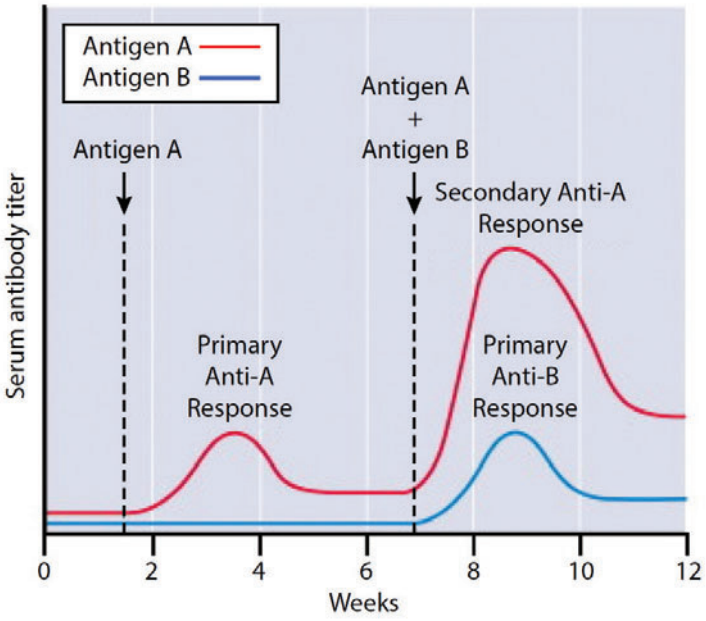


**Figure 1 Memory function adaptive immunity.** After a primary infection with antigen A, the immune system (i.e B-cells) produces antibodies against the encountered antigen (in the figure depicted as a small peak termed primary anti-A response). When the antigen is cleared from the body, serum antibodies against antigen A (red line) remain present. When the body then encounters the same antigen again, the immune system induces a faster and stronger response against the antigen, which can be seen as a higher serum antibody titer. This principle is called the memory function of the adaptive immune system.

Effector B-cells, also called plasma cells, produce antibodies which are the soluble counterparts of the B-cell receptor.[9] The main actions of antibodies are: 1) neutralization of microorganisms by blocking parts of the surface of a bacteria or virus; 2) agglutination, in which antibodies form complexes between microbes which are then attractive

for phagocytosis and immune cell activation and 3) complement activation in which complement can bind to antibodies and thereby lead to cell lysis.[8] Antibodies exist in five different classes: IgM, IgD, IgG, IgA and IgE. Initially, early in the immune response, plasma cells produce IgM, which is important for the primary immunity. However, during an immune response, B-cells can switch to the production of one of the other antibody classes. Each of the five Ig classes has unique effector functions and can lead to a different immune response. This often results in the most appropriate response against the encountered microorganism. Memory B- and T-cells do not have direct effector functions but, upon a secondary encounter with the same antigen, can differentiate faster into effector cells resulting in a quicker and stronger immune response, which is the hallmark of immunological memory (Figure 1).[10, 11]



## GENERATION OF ANTIGEN RECEPTOR DIVERSITY IN LYMPHOCYTE DEVELOPMENT

### Antigen-independent B-cell differentiation

B-cell development starts in the bone marrow where B-cell precursors are continuously produced from hematopoietic stem cells via stepwise differentiation. During these differentiation steps, B-cells generate a functional B-cell receptor (BCR) which is composed of two identical heavy chains (IgH) and two identical light chains (either Igκ or Igλ) which together form a functional membrane Immunoglobulin (Ig) molecule (Figure 2).[12, 13] Both the heavy chains and the light chains are composed of variable domains and constant domains. An Ig molecule has two antigen-binding domains, each composed of one variable region from the heavy- and one from the light chain. The variable domains are the domains which directly interact with the future antigen. The constant domains from the heavy chains determine the effector function of the soluble Ig.[14] Whereas the constant domains are germline encoded, rearrangements in the genomic DNA encoding heavy- and light chains are needed to form functional variable domains. Separate Variable (V), Diversity (D) and Joining (J) genes in the *IGH*, *IGK* and *IGL* loci rearrange to encode a functional protein in a process termed V(D)J recombination (Figure 2).[15-17] The process of V(D)J recombination starts in the heavy chain locus, where first DH to JH rearrangement is initiated, followed by rearrangement of a VH gene to the previously formed DJH element. This VDJ exon will be transcribed and spliced to the exons encoding the constant region, and if this is in-frame, it will encode the BCR receptor (Figure 2). When a functional heavy chain protein is generated, rearrangements in the *IGK* or *IGL* loci are initiated between V and J genes.[18] Once an Ig light chain protein is formed that can pair with the IgH protein, the cell will undergo positive and negative selection processes to ensure the generation of a functional BCR which does not recognize self-antigen. Thereby it finally develops into a functional immature B-cell.[19] The human Ig loci contain many different V, D and J genes and as a result of the random nature

of V(D)J recombination, this yields a large pool of B-cells with a unique receptor for each cell. Together, this forms a broad repertoire of antigen receptors which have the potential to specifically recognize a wide range of pathogens.[20]

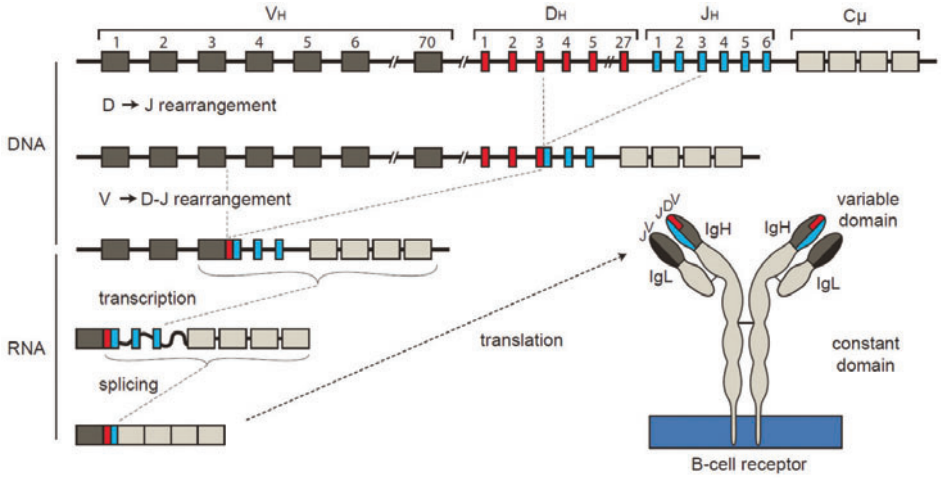


**Figure 2. V(D)J recombination.** V(D)J recombination commences in the *IGH* locus, where first a $D_H$ to $J_H$ rearrangement occurs, followed by complete $V_H$ to $DJ_H$ rearrangement to form a functional exon. The VDJ exon is transcribed and spliced to the *IGHM* exons. After a functional IgH protein is generated, similar rearrangements are initiated in the *IGK* or *IGL* loci between one V and one J gene segment. Together these can result in the formation of a functional B-cell receptor.

## Antigen-dependent B-cell maturation

Upon generation of a functional BCR, precursor B cells migrate from the bone marrow to the periphery as transitional B cells. Here they mature into naive mature B cells, which can respond to antigen. The B-cell response to antigen depends on the binding strength with the encountered antigen and on costimulatory signals, mostly provided by T-helper cells.[21] Typically this response takes place in a germinal center, a highly organized structure within lymph nodes. During this response B cells undergo extensive proliferation accompanied by affinity maturation (Figure 3).[22] In this process, the rearranged exons encoding the variable domains of Ig genes are intensely targeted by random mutations, a process called somatic hypermutation (SHM). The enzyme 'activation induced cytidine deaminase' (AID) induces these mutations which lead to antibody diversity.[23] Whereas mutations in the framework regions (FR) that encode the structural element are unfavourable, mutations in the complementarity determining regions (CDRs: the sites involved in antigen recognition) can result in increased affinity for the encountered antigen. Such mutations provide the B cell with a selective advantage for presenting antigen to T-helper cells and receiving co-stimulation, and are thus positively selected to undergo clonal proliferation.[23, 24] As a result,

the infection with a micro-organism leads to production of antibodies specifically aimed against the microbe.

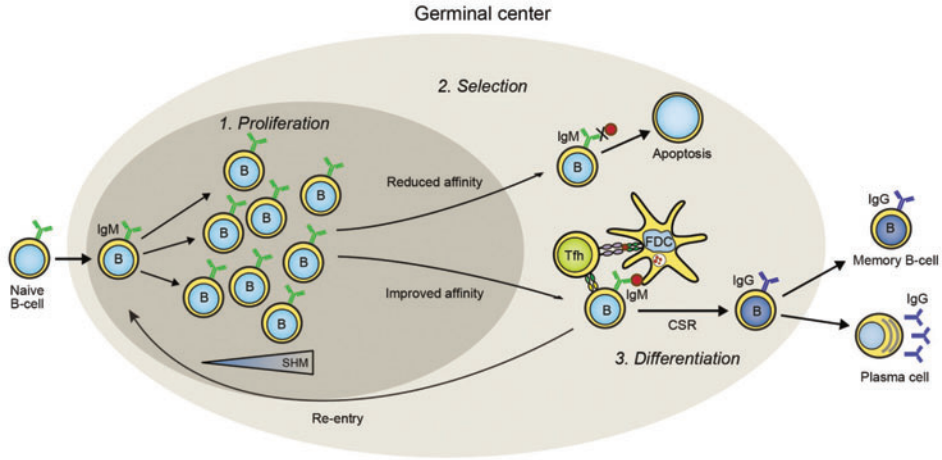


**Figure 3. Germinal center reaction.** Naive B-cells (expressing IgM) circulate through the bloodstream and lymph vessels. In lymph nodes naive B-cells have an increased chance to encounter antigen, after which they can get activated. The cells then proliferate and somatic hypermutation (SHM) occurs in the genes encoding the B-cell receptor. Since SHM is a random process, it can either lead to reduced affinity of the B-cell for the encountered antigen, resulting in lack off co-stimulation and apoptosis. Alternatively, it can also result in increased affinity, after which the cells have improved ability to present the antigen to follicular helper (Tfh) T-cells. Tfh cells can then stimulate naïve B-cells to differentiate and to undergo class switch recombination (CSR). In this process, B-cells undergo an isotype switch, for example from IgM to IgG. Moreover, B-cells can either differentiate into memory B-cells, which recirculate throughout the body, or differentiate into plasma cells, which produce soluble immunoglobulins.

## B-cell effector response

After V(D)J-recombination in the bone marrow, the antigen-binding variable domain is by default spliced to the constant μ or δ regions (Cμ or Cδ), resulting in the expression of IgM or IgD respectively. To increase the efficiency of the immune response to specific antigens, the constant domain of the heavy chain of the BCR has different isotypes, namely IgG (with the 4 different subclasses IgG1, IgG2, IgG3 and IgG4), IgA (with subclasses IgA1 and IgA2) and IgE. All isotypes have different properties resulting in different effector functions. In the periphery, during an immune response, B-cells can undergo an isotype switch to one of these other isotypes, called class switch recombination (CSR), which is induced by AID (Figure 3).[25] CSR does not change the antigen specificity but influences its effector functions, since different isotypes have different effects on the immune response [8]. This recombination can either occur in a direct process, for example from IgM to IgE, or in an indirect process, for example from IgM to IgG1 and in a subsequent response from IgG1 to IgE.[26] Finally after CSR, B-cells can either differentiate into plasma cells, which produce soluble Ig's, or into

memory B-cells, which upon secondary encounters with the same antigen can initiate a faster and stronger immune response.[10, 27]

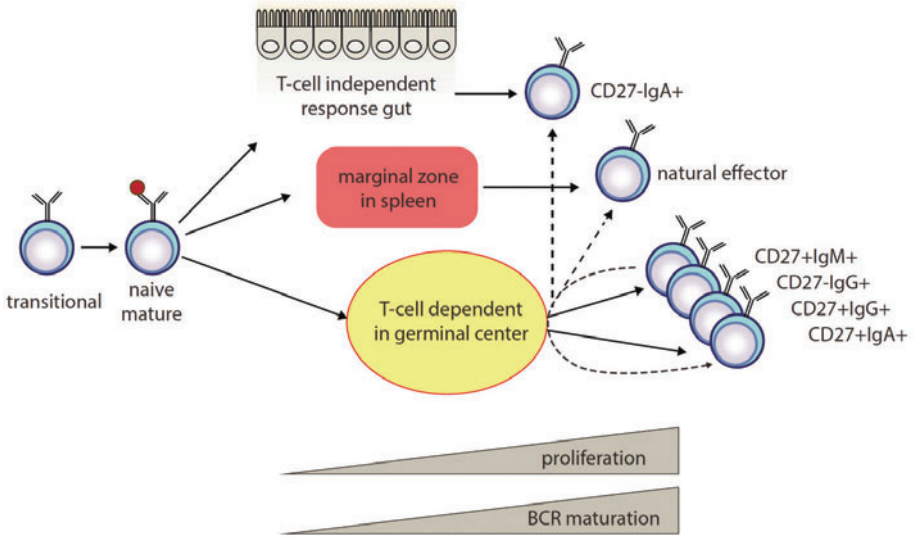


**Figure 4 Memory B-cell formation.** Naïve B-cells can differentiate into memory B-cells through several differentiation pathways resulting in distinct memory B-cell subsets. CD27+IgM+, CD27-IgG+, CD27+IgG+ and CD27+IgA+ memory B-cells develop through a classical germinal center reaction and display signs of high proliferation and BCR maturation status. CD27+IgM+IgD+ (natural effector) B-cells develop in the marginal zone of the spleen, whereas CD27-IgA+ B-cells differentiate from T-cell independent responses in the gut. These subsets display characteristics of less proliferation and BCR maturation.

## Memory B-cell differentiation

Molecular characteristics of purified memory B-cell subsets show evidence that memory B-cells can originate via three differentiation pathways (Figure 4).[28] This can occur via germinal center reactions in a T-cell dependent maturation pathway, yielding memory B-cells with high SHM levels and a high replication. This accounts for IgM only, IgG+CD27-, IgG+CD27+ and IgA+CD27+ memory B-cell subsets, in which IgA+CD27+ and IgG+CD27+ memory B-cells display the highest replication and SHM levels, suggestive of consecutive germinal center reactions. Alternatively, T-cell independent responses in the marginal zone of the spleen lead to the differentiation of natural effector B-cells (IgM+IgD+CD27+), whereas IgA+CD27- memory B-cells originate from T-cell independent responses in the lamina propria of the gut. The latter two subsets display less proliferation history and BCR maturation (Figure 4). Knowledge on IgE+ memory B-cell biology is limited and it is even being discussed if IgE+ memory B-cells in human exist. Moreover, if differentiation of IgE+ memory B-cells is affected in allergic disease is not well known.[29, 30]

## IgE-mediated allergic disease

Based on the immunologic mechanism, allergies can be divided into four categories, namely type I) the IgE mediated immediate allergic response; type II) the cytotoxic allergic reaction; type III) an immune complex mediated allergy; and type IV) delayed type hypersensitivity reaction.[8] IgE-mediated allergies underlie the most common phenotypes of allergic disease: atopic dermatitis, food allergies, hay-fever and allergic asthma (Table 1).[31, 32] These cause a high disease burden and it is estimated that up to 40% of the Western world is affected by one or more allergies, with the highest prevalence in children and adolescents.[1, 33, 34] Atopic dermatitis is often one of the earliest allergies to develop in young children. Of all allergic children, 45% has onset of atopic dermatitis in the first 6 months of life, increasing to 85% before the age of 5 years.[35, 36] The incidence of food allergy seems to be highest in children aged 1-3 years (5-8%), although exact numbers vary greatly due to the difference in self-reported food allergy and food allergy confirmed with a double-blind provocation test.[37, 38] The peak of allergic asthma is at a slightly older age, with a documented prevalence of 13.5% at the age of 13-14 years.[39] IgE-mediated allergic diseases may develop subsequently. This phenomenon is often related to as the atopic march, where infants first develop atopic dermatitis, progressing to allergic asthma in later childhood and allergic rhinitis at adolescence (Figure 5).[40] In general, this complete sequence appears to be quite rare, but it has been shown that about 50% of children with severe atopic dermatitis will develop asthma and about 75% develops allergic rhinitis later in life.[41-44]



**Table I.** Characteristics of IgE mediated allergic diseases

| | Typical age of onset | Route of entry | Common allergens | Symptoms |
|---|---|---|---|---|
| Atopic dermatitis | 1< yrs | Skin/oral | Various (e.g. egg, milk) | Dry skin, itch, redness |
| Food Allergy | 1-3 yrs | Oral | Nuts, shell fish, egg, milk | Vomiting, diarrhea, urticaria |
| Asthma | 6-10 yrs | Inhalation | Dander, pollen | Shortness of breath, airway constriction, mucus production |
| Atopic Rhinitis | 13-14 yrs | Inhalation | House dust mite, pollen | Sneezing, irritation of eyes, itch |
| Anaphylaxis | Variable | Intravenous (sometimes as result of oral absorption) | Insect venom, peanut | Angio edema, vascular permeability (low blood pressure), death |

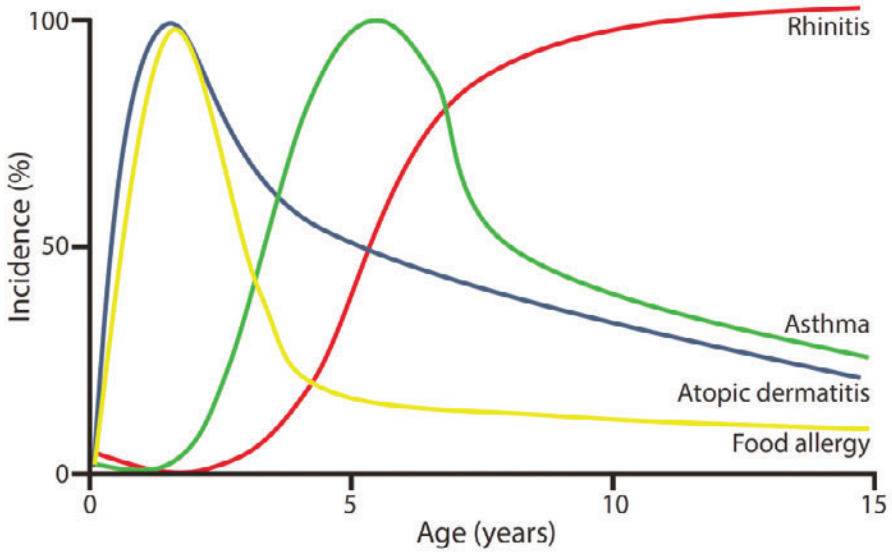


**Figure 5 Atopic march** IgE mediated allergic diseases tend to develop in a subsequent manner. Children aged 1-2 years often first develop atopic dermatitis and food allergy, later at ages 6-10 years develop asthma and at ages 13-14 develop atopic rhinitis.

## Pathogenesis of IgE mediated allergies

The underlying pathogenesis of IgE-mediated allergic disease is multifactorial, with genetic, environmental and immunological factors contributing to the onset of disease.[45-47] Genome wide association studies (GWAS) have contributed to the identification of various susceptibility factors, such as an adequate function of the epithelial and epidermal barrier.[48-50] Mutations in the human filaggrin gene (*FLG*), a structural protein in the stratum corneum of the epidermis, have been identified as the single most significant risk factor for the development of atopic dermatitis and related allergic diseases, such as food allergy and asthma.[51-54] The importance of an intact epidermal barrier is underlined by the described atopic diathesis and elevated IgE serum levels in Netherton syndrome[55], a disease caused by mutations in the 'serine protease inhibitor of kazal type 5' *(SPINK5)* gene, which leads to increased desmosome cleavage and reduced filaggrin proteolytic processing.[56]

Fundamental in the abnormal immune response in allergic disease is the generation of IgE antibodies to allergens such as pollen, peanut or house dust mite (HDM).[57] Hence the central role of total- and specific serum IgE levels in the diagnosis of allergic disease. IgE antibodies bind to high-affinity receptors (FcεRI) on mast cells and basophils, where they can act as a receptor complex which, upon allergen binding, can crosslink and induce degranulation of these cells. The release of mediators and cytokines from granules in the tissue causes allergic symptoms such as itching, sneezing or wheezing.[58]

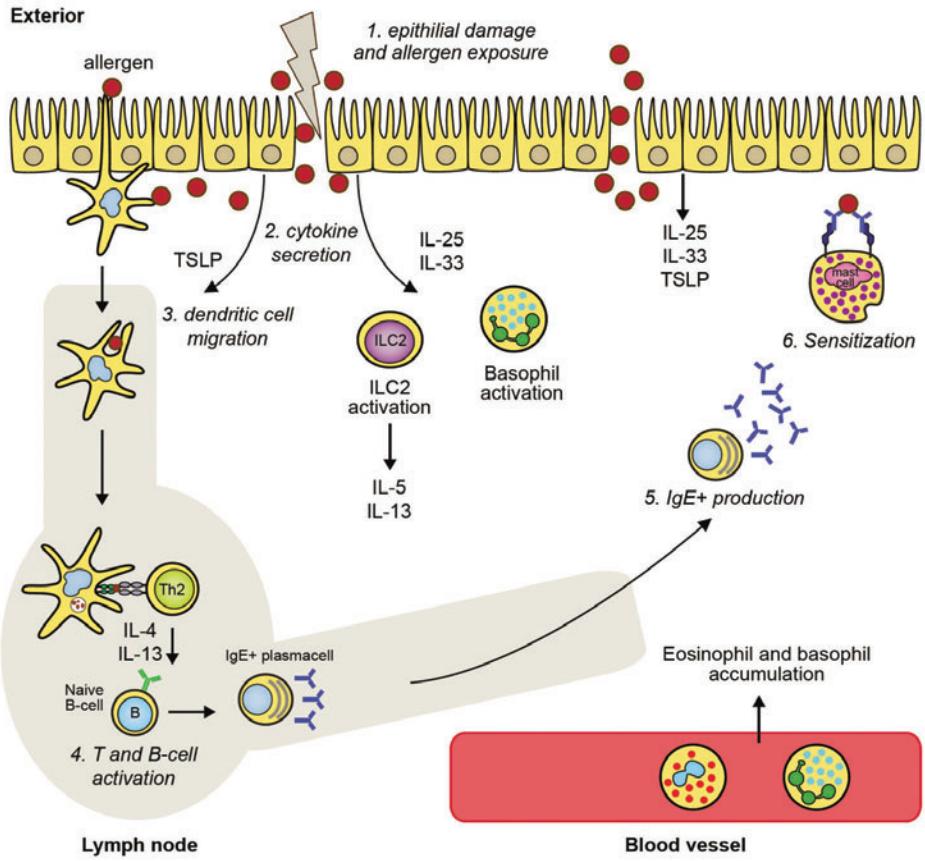


**Figure 6 Mechanism of allergic sensitization** Allergen comes into contact with the immune system through the epithelial cells of the skin or mucosal tissue of the respiratory tract and the gut. Epithelial damage increases allergen penetration and results in cytokine secretion such as IL-25, IL-33 and TSLP which activates cells of the immune system lining the mucosa i.e. dendritic cells, innate lymphoid cells, basophils). Subsequently, dendritic cells can take up allergen and migrate to lymph nodes, where they present allergen epitopes to Th2 cells. Th2 cells produce the cytokines IL-4 and IL-13 and thereby induce class switch recombination of allergen specific naïve B-cells to IgE+ B-cells. Allergen specific soluble IgE produced by plasma cells can bind to mast cells, after which the immune system is sensitized for the encountered allergen.

The generation of allergen-specific IgE and the resulting sensitization is thought to be triggered by enhanced allergen exposure in the affected tissue, mainly due to epithelial damage or inflammation (Figure 6).[59-61] In addition, many allergens have protease activity, which leads to the cleavage of tight junction proteins and thereby can act on epithelial cells by decreasing their barrier function.[62] The resulting increase in allergen exposure promotes the epithelial secretion of cytokines such as interleukin (IL)-33, IL-25 and thymic stromal lymphopoietin (TSLP), which activate dendritic cells, innate lymphoid type 2 cells (ILC2)

and basophils (Figure 6).[63] Some allergens can also directly activate Toll-like receptor (TLR) 4 on epithelial cells and thereby induce the production of specific cytokines.[64, 65] IL-25 and IL-33 are especially potent in stimulating innate lymphocytes to produce the Th2 related cytokines IL4, IL5 and IL13.[63, 66-69] TSLP drives the expansion and differentiation of DCs[70], resulting in their migration and the upregulation of costimulatory molecules.[71] GWAS studies in patients with allergic disease have identified single-nucleotide polymorphisms (SNPs) in genes encoding *TSLP* and *IL1RL1*, ST2 the IL-33 receptor, confirming the importance of these epithelial-derived cytokines in the involvement of allergic diseases.[72, 73] Once DCs lining the epithelial membranes get stimulated, they can migrate to draining lymph nodes where they can present processed allergen peptides via MHC-II to naive T cells. The naive T cells that specifically recognize the MHC-II – peptide complex with their cognate T-cell receptor differentiate into Th2 cells under influence of costimulatory signals and through the cytokine IL4.[74, 75] Simultaneously, naive B-cells in the lymph node can recognize soluble allergens directly with their BCR, without help of APCs. Similar to DCs, B cells can process internalized allergen and display allergen peptides via the MHC-II to the activated Th2 cells. Activated Th2 cells will then produce cytokines (mainly IL-4, IL-13 and IL-21) and can thereby induce the differentiation of naive B-cells into allergen-specific IgE producing plasma cells.[76, 77]

In principal CSR of B-cells is thought to take place in germinal centers in lymph nodes. CSR to IgE is regulated by IL4, IL13 and the tumor necrosis factor receptor (TNFR) superfamily member CD40.[77] CD40 ligation activates NF-κB[78], which together with signal transducer and activator of transcription 6 (STAT6) activated by IL4, induces AID gene expression, essential for CSR.[79, 80] Next to that STAT6 can activate the transcription of the Iε promotor, which is indispensable for Cε germline transcription preceding CSR to IgE. In addition to IL4, also IL13 can induce the activation of STAT6 and thereby initiate CSR to IgE.

Once B-cells are differentiated in IgE producing plasma cells, the secreted allergen-specific IgE will bind to FcεRI on mast cells and basophils, by which the immune system is sensitized for a certain allergen. Upon subsequent exposure to the same allergen, the allergen-specific IgE bound to the surface of mast cells and basophils can cross link and thereby activate these effector cells resulting in the immediate allergic response (Figure 7).[81] In addition, a delayed response is induced involving eosinophils, with chronic inflammation resulting in tissue remodeling e.g. in asthmatic airways (Figure 7).[82] In the latter process, chronic immune activation leads to subepithelial fibrosis, increased smooth muscle mass, epithelial shedding and excessive mucus production, which can prompt asthmatic symptoms irrespective of allergen exposure.[83, 84] Why some individuals generate Th2-mediated IgE responses to harmless allergens and others do not, is not entirely clear. In addition to genetic predisposition, environmental risk factors affecting epithelial cell-DC interaction such as allergen- or cigarette smoke exposure, viral infections and air pollution are important contributors.[59] Central in this process is a disturbed balance between Th1 and Th2-type immunity resulting in the development of IgE-producing B-cells. Investigating IgE+

B-cells and studying IgE+ B-cell differentiation in healthy individuals and in individuals with allergic disease, may enable the identification of processes important in this development

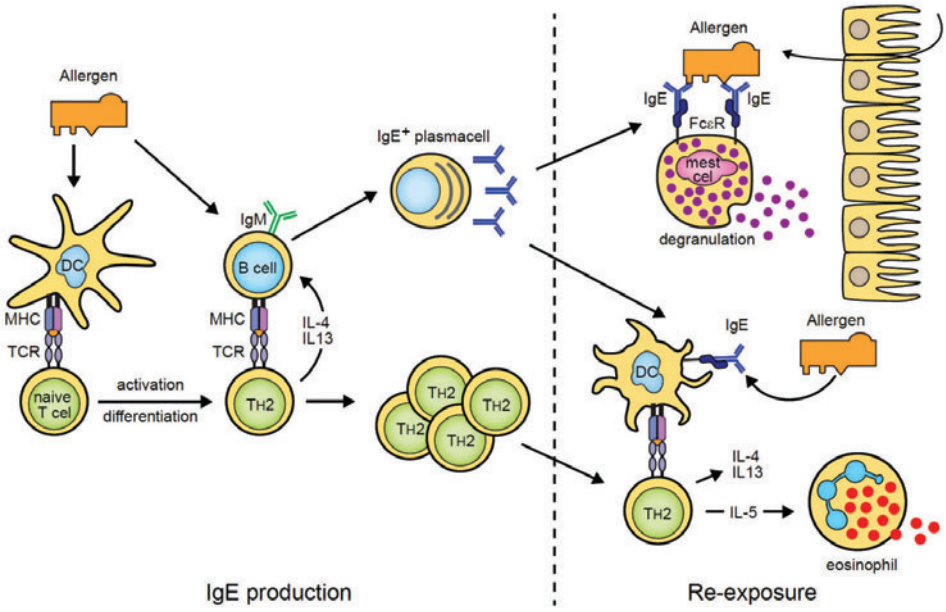


**Figure 7 Mechanism of allergic reaction** Allergen can be internalized by dendritic cells, processed in epitopes and presented to naïve T-cells via MHC class II. Naïve T-cells can recognize the MHC-epitope complex with their T-cell receptor, which, under the right stimuli, leads to their differentiation into Th2 cells. Simultaneously naïve B-cells can recognize allergen with their B-cell receptor, process the allergen and present it to Th2 cells. Activated Th2 cells then produce cytokines such IL-4 and IL-13, which stimulates naïve B-cells to differentiate into IgE producing plasma cells through the regulation of signal transducer and activator of transcription 6 (*STAT6)* . IgE+ plasma cells produce allergen specific IgE, which can bind to high affinity IgE receptors on mast cells and basophils (FcεRI). This results into the sensitization of the immune system for the specific allergen. Upon a re-exposure, allergen will bind FcεRI-bound IgE, which will lead to cross-linking of IgE and degranulation of the effector cells, resulting in allergic complaints. Next to that a late phase reaction can be induced by tissue infiltrating eosinophils.

## IgE+ memory B-cell biology

It has long been under debate if IgE+ memory B-cells are generated in humans or that memory is maintained by long-lived IgE-producing plasma cells in bone marrow and allergen specific IgG+ memory B-cells that switch to IgE production upon secondary encounters.[29, 85] IgE+ memory B-cells are difficult to detect. Three apparent reasons for this are the low numbers of IgE+ B-cells[81]; the low expression of surface IgE on IgE+ B-cells which is due to suboptimal polyadenylation signals downstream of the exons encoding the cytoplasmic tail of IgE leading to instable mRNA and therefore low membrane expression

of IgE[86]; and finally the false positive detection of IgE+ B-cells, as many cells express the low affinity IgE receptor, FcεRII (CD23), making them IgE+ without expressing IgE themselves.[87] To overcome these technical difficulties, several mouse models have been designed to enable reliable detection of IgE+ B-cells. Alternative methods are aimed at intracellular IgE detection or at dissociation of the IgE-FcεRII complexes through a short incubation with cold acid.[88] Based on these approaches, it has been shown that in mice IgE+ B-cells can either develop directly from naive IgM+ B-cells or differentiate from antigen experienced IgG+ B-cells through a secondary immune response.[89-94] How much either differentiation pathway contributes to IgE+ memory B-cell development is unknown. Still, direct CSR to IgE seems predominantly responsible for the production of short lived plasma cells with low affinity, whereas indirect CSR via IgG1 results in the production of IgE with high affinity. In the latter differentiation pathway, IgG1 B-cell differentiation is important for the affinity maturation of the B-cell receptor.[93] In both pathways there is a strong predisposition for the development of IgE-producing plasma cells, with little or no IgE+ memory B-cell development.[94] Possibly this is the result of impaired BCR expression and function with the consequence that IgE+ B-cells are unsuitable to undergo a conventional germinal center reaction.[90] Based on these results it has been postulated that IgE memory function might primarily be the result of IgG1+ B-cells, which are prone to develop into IgE producing plasma cells during a secondary response.[93] In contrast to this, other studies have shown that B-cells can directly develop into IgE+ memory B-cells and IgE+ plasma cells in a germinal center reaction[95], and that IgE+ memory B cells can develop into IgE+ plasma cells independent of a germinal center reaction.[92] Still, all these studies were performed using mouse models, some of which contained modified membrane IgE-expression levels, and that were all exposed to artificially high doses of allergens and sensitization schedules to induce allergic disease. Thus, it still has to be determined how much of these new insights can be translated to human disease. Only few studies have aimed to directly identify IgE+ B-cells in humans, with little data on their immunophenotype and their role in allergic disease.[30, 96] Still, IgE transcripts have been detected in various tissues and in blood of healthy controls and allergic subjects, indicative of the presence of IgE-expressing B cells.[97-100] Furthermore, several studies have shown signs of active Ig-class switch recombination to IgE in bronchial and nasal mucosa.[101-105] Tissue samples showed expression of AID, the enzyme important for IgE class switch recombination and SHM.[102] Furthermore, transcripts from DNA excision circles containing Sε-Sμ and Sε-Sγ switch regions were detected, demonstrating signs of direct IgM to IgE and indirect IgG to IgE class switch recombination.[101] However, data on cellular characteristics and their relation to other subsets of the immune system are lacking, but are critical to explore the possibilities in diagnostic purposes or more important, as target for treatment.

## Treatment of allergic disease

It is not known whether circulating IgE+ memory B-cells sustain the underlying allergy, but in view of the function of memory B-cells in general it is probable that increased circulating IgE+ memory B-cells actively contribute to allergic disease.[106] To achieve desensitization and possibly even cure allergic disease it is therefore important to develop therapies aimed to reduce IgE+ memory B-cells. Omalizumab has shown to decrease the production of IgE in patients with IgE-mediated asthma.[107] However, it mainly targets soluble IgE. Quilizimab is an antibody directed against the M1 prime epitope of membrane IgE, and therefore only targets surface IgE on IgE-expressing memory B cells.[108] Importantly, because Quilizimab does not bind soluble IgE, it could be more efficient in binding to IgE on B cells than Omalizumab. In mouse studies it not only decreased serum IgE, but also depleted IgE producing B-cells.[109] In human trials, Quilizimab also led to a reduction of IgE serum levels, lasting 6 months after treatment cessation. Yet the effect on clinical parameters, such as asthma exacerbations, lung function or quality of life, was variable.[110, 111] The biomarkers that were studied could not identify patient groups with a beneficial treatment effect. However, if IgE+ memory B-cells can be reliably identified, patients that would possibly benefit from anti-IgE treatment could be easily selected.

A different approach to desensitize patients with allergic disease is Specific Immune Therapy (SIT), in which patients are treated with small, but increasing dosages of allergen.[112] This can be either admitted subcutaneous (SCIT) or sublingual (SLIT) and results in the deviation of local and systemic immune responses with an effect on the number and function of effector cells, APCs, T cells and B cells.[113, 114] SIT has been proven to have a therapeutic effect that remains after stopping treatment.[115-117] In addition, SIT can prevent the onset of new sensitizations[118], and has the ability to reduce the development of asthma in patients with allergic rhinitis.[119] Effective immunotherapy has been shown to reverse the Th2 dominance, and to result in anergy of allergen-specific T cells,[120, 121] induction of regulatory T cells[122-124] and production of blocking antibodies of the IgG isotype.[125, 126] Specifically, IL-10 produced by Tregs is pivotal for the successful immune deviation in immunotherapy.[127, 128] The tolerogenic functions of IL-10 are extensive, but IL-10 in combination with IL-4 and IL-13 directs Ig CSR of B cells to IgG4 instead of IgE.[129] Indeed, one of the known effects of SIT is an increase in allergen-specific serum IgG4 and an increase in the serum IgG4/IgE antibody ratio which is associated with successful outcome.[130] Since immunotherapy has been attributed to have long lasting beneficial effects, it is of specific interest to understand if this is the result of changes in immunological memory, i.e. memory B and T cells. Such studies would require reliable detection of IgE and IgG4+ memory B-cells and a deeper understanding of the biology of these cells.

### Is IgG4 always beneficial?

IgG4 is presumed to have an immune dampening effect.[131, 132] The IgG4 molecule displays weak or negligible binding to both C1q and Fcγ receptors.[133, 134] In addition, IgG4 molecules have the exclusive ability to exchange Fab-arms, thus creating monovalent bispecific antibodies that can prevent the formation of immune complexes.[135] Therefore, IgG4 only has a limited ability to stimulate the immune system for the induction of an immune response. However, several diseases have been associated with a possible pathologic role for IgG4 and in 2012 a novel disease entity has been defined, termed IgG4-related disease (IgG4-RD).[136] IgG4-RD patients suffer from tissue fibrosis with atypical infiltration of IgG4+ plasma cells in various organs, but most predominantly in the retroperitoneal space, thyroid, pancreas, salivary glands and orbital tissue.[136] Furthermore, IgG4 serum levels are increased in 50-70% of patients with IgG4-RD.[137, 138] These observations lead to the discussion whether IgG4 is involved in the pathogenesis of the disease. Still it could also be a reflection of chronic inflammation. Since the current limitations in our understanding of IgG4+ B-cells, it is yet unclear how these cells function in alleviation of allergic symptoms following immunotherapy, or in pathogenesis of IgG4-RD.

## AIMS OF THIS THESIS

Allergen-specific IgE plays a central role in the pathophysiology of IgE-mediated allergy. Still, little is known about the cells producing IgE or about memory B-cells which might sustain increased IgE production. Notably, this is the result of the inability to reliably identify IgE+ B-cells, while knowledge on IgE+ B-cell differentiation and their role in allergic disease is crucial to understand the development of IgE mediated allergies. Moreover, treatment of allergic disease is typically aimed at symptom relief and none of the current therapies is able to cure allergic disease. Therefore, more insights into IgE-expressing B cells and their relation to other immune cells are needed to understand disease pathogenesis. This knowledge might be readily translated into diagnostic purposes or uncover targets for treatment. In **Chapter II** we aimed to reliably identify and immunophenotype IgE-producing plasma cells and IgE+ memory B-cells. Based on our knowledge on mature B-cell development, we identified distinct IgE+ B-cell subsets, studied their differentiation pathways based on molecular characteristics, and investigated their abnormalities in atopic dermatitis. In **Chapter III** we aimed to increase our knowledge of IgE+ B-cell involvement in three major types of IgE-mediated allergic diseases. We studied the immune compartment of children with atopic dermatitis, food allergy and asthma, focusing on IgE+ B-cells and their relation to T-cell and innate cell subsets involved in the allergic response. Since a disturbed epithelial barrier results in increased allergen exposure, we sought to study the effect of barrier dysfunction on IgE+ B-cell development in **Chapter IV**. Here, we studied the peripheral

immune compartment of patients with Netherton disease, a disease caused by mutations in the *SPINK5* gene which results in a disrupted epidermal barrier.

Subsequently, we investigated the effect of different treatment strategies on the immune compartment of children with allergic disease. **Chapter V** describes the observed effects of multidisciplinary outpatient treatment at sea level and inpatient treatment at high altitude in children with moderate to severe atopic dermatitis. To further investigate the effect of desensitization on IgE+ and IgG4+ memory B-cells, we performed a longitudinal study in patients with hay-fever receiving SLIT. In **Chapter VI** we discuss the results of this therapy on various B- and T-cell subsets.

In **Chapter VII** and **Chapter VIII** we studied pathological aspects of IgG4+ B-cells in chronic inflammatory diseases. We performed detailed cellular and molecular studies in patients with IgG4-RD. The implications of these studies are discussed in the **General Discussion**, which also speculates on future directions.

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# PART II

## THE IDENTIFICATION OF IGE+ B-CELLS AND THEIR CELLULAR CHARACTERISTICS

# CHAPTER 2

# Human IgE+ B-cells are derived from T cell-dependent and T cell–independent pathways

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## ABSTRACT

*Background* The prevalence of IgE-mediated diseases increases worldwide. Still, the IgE-expressing B cells are poorly characterized, mainly due to their scarcity and low membrane IgE levels.

*Objective* To study the immunobiology of human IgE-expressing B cells in health and allergic disease.

*Methods* Stepwise approach for flow cytometric detection and purification of human IgE-expressing B cells in controls, CD40L-deficient patients and patients with atopic dermatitis. Molecular analysis of replication histories, somatic hypermutations (SHM) and Ig class switching.

*Results* Using multi-color flow cytometry, we reliably detected IgE-expressing plasma cells and two IgE-expressing memory B-cell subsets. These IgE-expressing cells showed molecular and phenotypic signs of antigen responses. The replication history and SHM levels of $IgE^+$ plasma cells and $CD27^+IgE^+$ memory B cells fitted with a germinal center (GC-)dependent pathway, often via an IgG intermediate, as evidenced from Sγ remnants in Sμ-Sε switch regions. $CD27^-IgE^+$ cells showed limited proliferation and SHM, and were present in CD40L-deficient patients, indicating a GC-independent origin. Patients with atopic dermatitis had normal numbers of blood $IgE^+$ plasma cells and $CD27^+IgE^+$ memory B cell, but increased $CD27^-IgE^+$ memory B cells with high SHM loads as compared to healthy controls and patients with psoriasis.

*Conclusions* We delineated GC-dependent and GC-independent $IgE^+$ B-cell responses in health, and indicated involvement of the GC-independent pathway in a human IgE-mediated disease. These findings provide new insights into the pathogenesis of IgE-mediated diseases, and may contribute to accurate monitoring of $IgE^+$ cells in patients with severe disease undergoing anti-IgE treatment.

## INTRODUCTION

Allergic disease incidence has increased over the past decades in most Western countries.[1] It has been estimated that currently ~8% of adults in the United States suffer from asthma,[2] and 30-40% of the world population is affected by one or more allergies.[3] Allergic responses are mediated by an expansion of T helper type 2 ($T_H2$) cells and isotype switching of B cells to generate allergen-specific immunoglobulin (Ig)E.

Allergic patients have IgE with specificities towards harmless food- or airborne particles (allergens) that underlie the disease.[4] Despite the crucial role of IgE, little is known about the cellular aspects of $IgE^+$ B-cell responses.[5] The three main reasons for this are: 1) the presumed scarcity of the IgE-expressing B cells,[6] 2) the poor expression of membrane IgE due to suboptimal polyadenylation signals downstream of the exons encoding the cytoplasmic tail of IgE,[7] and 3) the presence of the low-affinity receptor FcεRII (CD23) on B cells that binds IgE and results in detection of IgE on non-IgE-producing B cells.[8]

Class-switching towards IgE is primarily thought to take place in lymphoid germinal centers (GC), where it is mediated by IL-4, IL-13, IL-21 and CD40L on activated $T_H2$ cells.[9-12] In addition, co-stimulatory signals can be provided by the interaction between CD23 and CD21 on B cells, as well as signaling via CD86.[13,14] Still, respiratory mucosa and the gastrointestinal tract also show molecular signs of IgE class-switching.[15] This is reminiscent of mucosal IgA responses that can take place independently of cognate T-cell help, possibly against non-peptide antigens.[16,17]

In recent studies with mouse models, IgE-producing plasma cells in lymph nodes were found to be derived from GC reactions. These cells were mostly short-lived and their participation in GCs seemed transient. Long-term memory was generated in the form of post-GC $IgG1^+$ cells that switched to IgE upon subsequent antigen encounter.[18,19] Direct IgMàIgE class-switching was found as well,[20,21] but the generation of high-affinity IgE antibodies depended on indirect IgMàIgG1àIgE switching.[22] $IgE^+$ memory B cells have only been observed by Talay *et al.*,[23] which is potentially due to the manipulated IgE allele.[24]

Due to continuous antigenic pressure, human lymph nodes have persistent GCs, and large numbers of memory B cells can be found in blood. These features limit direct extrapolation from (manipulated) mouse models to human disease. We have recently delineated the human memory B-cell compartment by direct *ex vivo* analysis of Ig genes in sorted B-cell subsets.[25] The Ig genes are targets of unique processes that leave permanent marks in the genomic DNA: V(D)J recombination for Ig repertoire development, somatic hypermutation (SHM) for affinity maturation, and Ig class-switch recombination (CSR) for effector function. $CD27^-IgA^+$ B cells and $CD27^+IgM^+IgD^+$ 'natural effector' B cells (at least in part) were derived from local and systemic GC-independent responses, respectively; $CD27^-IgG^+$ and $CD27^+IgM^+IgD^-$ 'IgM-only' B cells were derived from primary GC responses and $CD27^+IgG^+$ and $CD27^+IgA^+$ B cells (at least in part) from consecutive GC responses.

We now developed an 8-color flowcytometry-based strategy to reliably detect $IgE^+$ plasma cells and $CD27^-IgE^+$ and $CD27^+IgE^+$ memory B-cell subsets following stepwise exclusion of $IgM^+$, $IgD^+$, $IgG^+$ and $IgA^+$ B cells. These $IgE^+$ B cells lacked CD23 expression and carried IgE transcripts. We employed molecular analysis of their replication history, SHM, and CSR profiles and studies in CD40L-deficient and in atopic dermatitis patients to reveal new insights into their maturation pathways and involvement in allergic disease.

# METHODS

## Patients

Peripheral blood samples from patients and healthy donors, and childhood tonsils were obtained with informed consent and according to the guidelines of the Medical Ethics Committee of Erasmus MC.

All CD40L-deficient patients lacked CD40L protein expression on activated T cells as measured after 5-hour stimulation with phorbol 12-myristate 13-acetate (Sigma-Aldrich) and calcium ionophore (Sigma-Aldrich), and carried hemizygous mutations in their *CD40L* gene.

Patients with atopic dermatitis and psoriasis vulgaris were selected from the outpatient clinic of Dermatology and Venereology at Erasmus MC in Rotterdam. The diagnosis of psoriasis vulgaris was made by certified dermatologists by clinical examination only. Patients with atopic dermatitis fulfilled the criteria by Hanifin and Rajka.[26] Disease severity was scored for both groups by the attending physician. Total serum IgE and IgE against common inhalant allergens (Phadiatop) were measured with ImmunoCAP 250 (Phadia, AD Sweden) according to the manufacturer's manual.

## Flow cytometric immunophenotyping and cell sorting

Absolute counts of blood $CD3^+$ T cells, $CD16^+/56^+$ NK cells and $CD19^+$ B cells were obtained with a diagnostic lyse-no-wash protocol. Eight-color flow cytometry of memory B cells from peripheral blood and plasma cells from tonsils was performed using monoclonal antibodies. Intracellular staining was performed upon fixation and permeabilization with the FIX&PERM® Kit (An der Grub).

$CD19^+CD38^{dim}CD27^+IgD^-IgM^-IgE^+$ and $CD19^+CD38^{dim}CD27^-IgD^-IgM^-IgE^+$ were purified (>95% purity) on a FACSAria cell sorter (BD Biosciences) following AutoMACS (Miltenyi Biotec) enrichment of CD19+ B cells from post-ficoll mononuclear cells of healthy donors.[25, 27]

## Molecular analysis of replication history and Ig gene rearrangements

DNA was isolated from each sorted B-cell subset with a mammalian DNA Miniprep Kit (Sigma-Aldrich) to assess the replication history with the Kappa-deleting Recombination Excision Circles (KREC) assay, and the frequency of mutated *IGK* alleles was determined with the Igκ

restriction enzyme hot-spot mutation assay (IgκREHMA).[27] RNA from $CD19^{+}CD38^{dim}CD27^{+}IgD^{-}IgM^{-}$ and $CD19^{+}CD38^{dim}CD27^{-}IgD^{-}IgM^{-}$ B cells and total plasma cells was isolated using a mammalian RNA Miniprep kit (Sigma-Aldrich) and reversely transcribed with random hexamers. Complete *IGH* gene rearrangements were amplified in a semi-nested approach with previously described L-VH forward primers,[28] and two different *IGHE* primers in the first (5'-CATCACCGGCTCCGGGAAGTAG-3') and second (5'-GTTTTTGCAGCAGCGGGTCAAG-3') reactions, or in a one-step PCR with either *IGHM*, *IGHA* or *IGHG* consensus reverse primers.[25] PCR products were cloned into the pGEM T-easy vector (Promega) and sequenced on an ABI Prism 3130XL (Applied Biosystems). Obtained sequences were analyzed with the IMGT database (www.imgt.org),[29] and the JoinSolver program.[30]

### Ig switch region analysis

Hybrid Sμ-Sε regions were amplified from genomic DNA in a nested long range PCR (LR-PCR) using two Sμ-Sε primer sets for the first and second reactions.[31, 32] PCR products were sequenced on the ABI Prism 3130XL and the results were aligned with the *IGH* reference sequence (NG_001019)



### In vitro plasma cell differentiation

Naive mature, $IgG^{+}$ and $IgE^{+}$ B-cell subsets were purified, and 10,000-25,000 cells of each subset were cultured in 100μl IMDM supplemented with 10% fetal calf serum, 10 μg/ml anti-CD40 (Bioceros B.V.) and 50 ng/ml IL-21 (PeproTech).[33] Plasma cell maturation was assessed with flow cytometry after 6 days.

### Statistical analyses

Statistical analyses were performed with the Mann-Whitney U test, paired t test or $\chi 2$ test as indicated in detail in Table and Figure legends. p values $<0.05$ were considered statistically significant.

## RESULTS

### IgE+ plasma cells in childhood tonsils

The identification of $IgE^{+}$ B cells by flow cytometry has been controversial because of the scarcity of these cells, the low IgE expression levels and the presence of cells that have captured IgE with their Fcε receptors.[6,7] We acknowledged these difficulties by the design of a novel 8-color flow cytometric approach to detect $IgE^{+}$ plasma cells in childhood tonsil and $IgE^{+}$ memory B-cell subsets in blood (Fig 1 and Fig 2). We identified plasma cells based on the $CD19^{+}CD38^{high}$ phenotype. Subsequently, we selected $IgM^{-}IgD^{-}$ cells within the plasma-cell gate, and analyzed IgE, IgA and IgG expression on these cells (Fig 1A and Fig E1). In childhood

tonsil, CD19$^{+}$CD38$^{high}$ plasma cells constituted ~4% of total CD19$^{+}$ cells. These plasma cells were mostly CD138$^{-}$, of which 66% co-expressed IgM and IgD. About 30% of the total plasma cells was surface Ig negative, but expressed cytoplasmic IgG (not shown). The remaining plasma cells either expressed IgG (2.6%), IgA (1.2%) or IgE (1.6%) on their membrane.

Sequencing of *IGHE* transcripts from plasma cells yielded diverse clones that nearly all contained SHM in their *IGHV* regions (Fig 1B and C). In both childhood tonsil and adult blood, the SHM levels were similar to IgM$^{+}$ plasma cells, and significantly lower than in IgA$^{+}$ and IgG$^{+}$ plasma cells. Still, targeting of SHM to hypermutable motifs and selection against replacement mutations in CDR regions were similar in all plasma-cell subsets (not shown).

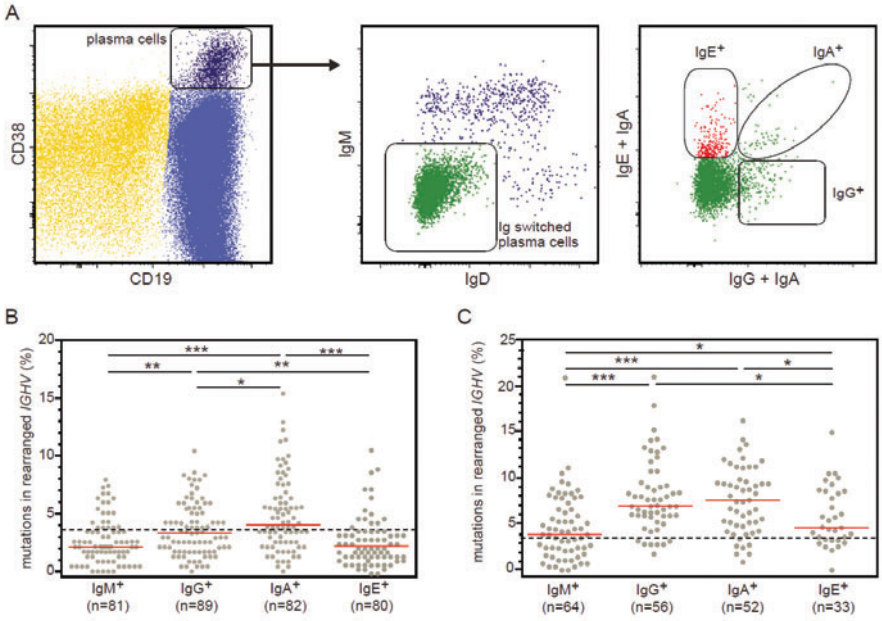


**Figure 1.** Detection of IgE$^{+}$ plasma cells in tonsil and peripheral blood. (**A**) Representative images from flow cytometric analysis of plasma-cell subsets in childhood tonsil. IgM$^{-}$IgD$^{-}$ cells were gated within the total CD19$^{+}$CD38$^{high}$ plasma cell fraction and analyzed for surface IgG, IgA and IgE expression. (**B and C**) SHM frequencies in rearranged *IGHV* genes in *IGHM*, *IGHG*, *IGHA* and *IGHE* transcripts from childhood tonsil (**B**) and adult blood (**C**). Each dot represents an individual sequence, red lines represent median values and the dashed line indicates the median SHM frequency in centrocytes. Data were statistically analyzed with the Mann-Whitney test. *, p<.05, **, p<.01, ***, p<.001.

## Two IgE+ memory B-cell subsets in blood

Subsequently, we analyzed blood of healthy adult donors, who carry large numbers of circulating memory B cells.[25] Within the CD19$^{+}$CD38$^{dim}$IgM$^{-}$IgD$^{-}$ class-switched memory B cells we detected two subsets of IgE$^{+}$ B cells with differential expression of CD27: CD27$^{+}$IgE$^{+}$ and CD27$^{-}$IgE$^{+}$ B cells (Fig 2A).

In young children (<6 years), $CD27^+IgE^+$ cells were scarce with a median frequency of 0.03% of total $CD19^+$ B cells (Fig 2B). This frequency increased with age and reached 0.1% of total B cells in adults of 31-40 years. In contrast, $CD27^-IgE^+$ cells were more abundant in children than in adults, with the highest frequency of 0.12% of total B cells in children of 6-15 year. Thus, two subsets of $IgE^+$ B cells can be reproducibly detected in blood of healthy individuals and show distinct kinetics with age.

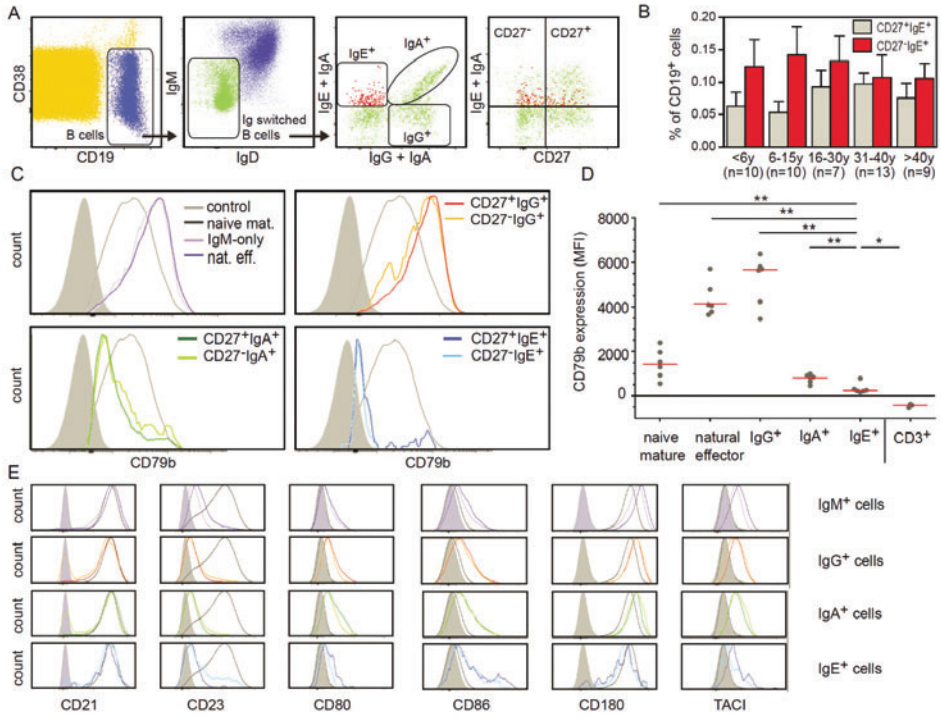


**Figure 2.** Two $IgE^+$ memory B-cell subsets in blood. (**A**) Stepwise gating strategy for idenfification of $CD27^+IgE^+$ and $CD27^-IgE^+$ subsets. (**B**) $CD27^+IgE^+$ and $CD27^-IgE^+$ B-cell frequencies in healthy children and adults. Bars represent mean values with SEM. (**C**) Expression levels of CD79b on $IgM^+$, $IgG^+$, $IgA^+$ and two $IgE^+$ memory B-cell subsets. Each histogram is composed of merged data from 6 healthy donors, and contains a light grey filled histogram representing $CD3^+$ T cells, and a dark grey histogram representing naive mature B cells. (**D**) Median fluorescence intensity of CD79b on B-cell subsets and T cells from 6 individual donors. Data were statistically analyzed with the Mann-Whitney test. *, p<.05; **, p<.01. (**E**) Expression levels of selected markers on $IgM^+$, $IgG^+$, $IgA^+$ and $IgE^+$ memory B-cell subsets. Light grey filled histograms represent isotype controls; dark grey histograms represent naive mature B cells.

IgE expression seemed dim on memory B cells, which could be due to suboptimal polyadenylation signals in transcripts encoding membrane IgE.[7] To study whether B-cell receptor levels were indeed low, we determined the expression levels of the B-cell antigen

receptor complex member, CD79b, on all memory B-cell subsets. CD79b expression was high on $IgG^+$ and natural effector B cells, intermediate on naive mature B cells, reduced on $IgA^+$ cells and clearly the lowest on $IgE^+$ memory B cells (Fig 2C and D). The differences in CD79b expression were not due to steric hindrance of the anti-IgA and anti-IgE isotypes (Supplemental Fig 1D). The low IgE membrane levels might have prevented prior detection of $IgE^+$ memory B cells.

Subsequently, we studied the immunophenotype of the $IgE^+$ memory B cells, and compared this with naive mature B cells and the 6 previously defined memory subsets.[25] All analyzed B-cell subsets had high expression of CD21 (Fig 2E). A minor fraction of $CD27^-IgA^+$ and $CD27^-IgG^+$ cells lacked CD21 expression, confirming previous observations that part of the $CD21^{low}$ B-cell subset is negative for IgM and IgD.[34] CD23 was highly expressed on naive B cells, but not on memory B cells.[35,36] The detection of IgE on $CD27^+$ and $CD27^-$ memory B-cells subsets is therefore not the result of FcεR-bound molecules. Furthermore, $CD27^+IgE^+$ and $CD27^-IgE^+$ B cells showed an immunophenotype that was characteristic for activated cells; with increased expression of the B7 family members CD80 and CD86, and TNF receptor superfamily member TACI as compared to naive mature B cells. In contrast to other memory B cells, $IgE^+$ B cells did not show upregulation of TLR-related CD180. Still, $CD27^+IgE^+$ and $CD27^-IgE^+$ B cells carry a typical memory B-cell immunophenotype with absence of CD23, presence of CD21 and upregulation of activation markers.[25,37]

## Replication history, SHM and class switching patterns in IgE+ memory B cells

$CD27^+IgE^+$ and $CD27^-IgE^+$ memory B cells were purified from healthy individuals for molecular analysis of their *in vivo* replication history and Ig gene maturation. $CD27^+IgE^+$ memory B cells showed a replication history of ~9 cell divisions (Fig 3A). This was similar to $CD27^+$IgM-only and $CD27^-IgG^+$ B cells and higher than centrocytes. In contrast, $CD27^-IgE^+$ B cells had a limited replication history of only ~4 cell divisions, which was similar to $CD27^-IgA^+$ memory B cells. In agreement with the replication history, the frequency of mutated *IGKV3-20* alleles and SHM levels in rearranged *IGHV* genes of $CD27^+IgE^+$ were similar to memory B cells from primary immune responses (Fig 3B and C). In contrast, $CD27^-IgE^+$ B cells showed few mutated *IGKV3-20* alleles and low SHM levels in *IGHV*, reminiscent of GC-independent responses. Thus, both the replication history and SHM levels of $CD27^+IgE^+$ memory B cells suggest that these are post-GC cells, whereas $CD27^-IgE^+$ and $CD27^-IgA^+$ B cells share an origin from local T-cell independent (non-GC) responses.

Further analysis of SHM revealed high ratios of replacement versus silent mutations (R/S) in IGHV-CDRs of $CD27^+IgE^+$ (2.5) and $CD27^-IgE^+$ (4.7) memory B cells, and were indicative of antigenic selection.[25] Interestingly, detailed analysis of the SHM targeting showed less abundant targeting of RGYW motifs in $IgE^+$ memory B cells than in most of the other memory B-cell subsets, suggesting lower activity of AID in the formation of these cells.

IgM+, IgG+ and IgA+ memory B-cell subsets in man have undergone selection against long IgH-CDR3 regions.[25] The median IgH-CDR3 sizes of both CD27+IgE+ and CD27−IgE+ memory B cells were 16 amino acids, which was similar to centrocytes, but larger than the 14-15 amino acids found in other memory B-cell subsets (Fig 3D). Thus, selection mechanisms for IgE+ memory B cells seem different than for other memory B-cell subsets.

Class switching to IgE can occur indirectly via an upstream *IGHG* or *IGHA* constant region (Fig 3E). Theoretically, this would leave a Sα or Sg remnant in about half of the Sμ-Sμ switch regions. Therefore, we studied these molecular signs of indirect switching in hybrid Sμ-Sμ switch regions in genomic DNA of purified IgE+ memory B cells. CD27−IgE+ memory B cells hardly contained molecular signs of indirect switching (5%; Fig 3E). However, a substantial fraction of Sμ-Sε switch regions in CD27+IgE+ memory B cells (32%) contained remnants of an Sγ (mainly Sγ1) region. Thus, CD27−IgE+ cells seem to be almost exclusively generated directly from IgM+ cells, whereas IgE class-switching via an IgG intermediate is a frequent event in CD27+IgE+ memory B cells.



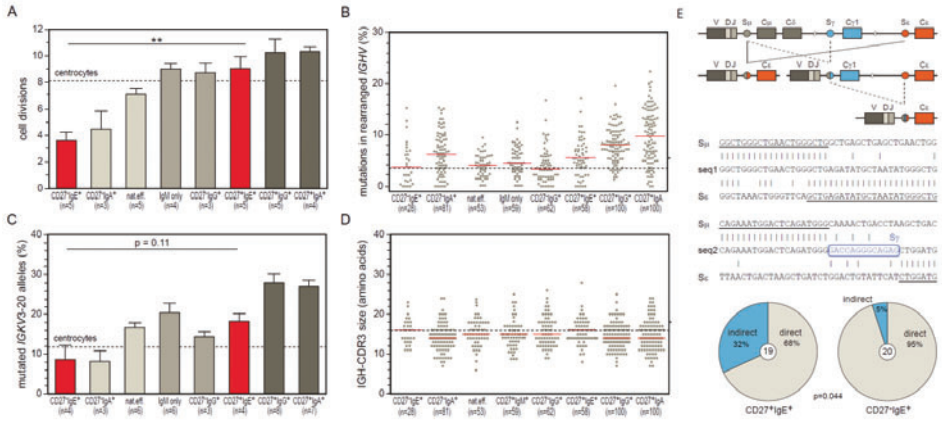


**Figure 3.** Distinct levels of molecular maturation in CD27+IgE+ and CD27−IgE+ memory B cells. (**A**) Replication histories of memory B-cell subsets as measured with the KREC assay.[25,27] Bars represent mean values with SEM. Dashed lines represent values for centrocytes (n=55).[27] (**B**) Frequencies of mutated nucleotides in rearranged *IGHV* genes. Each grey dot represents a unique rearrangement, and red lines indicate median values. (**C**) Frequencies of mutated *IGKV3-20* genes as measured with the IgκREHMA assay.[27] (**D**) IgH-CDR3 size distributions in memory B-cell subsets. Differences between CD27+IgE+ and CD27−IgE+ cells were statistically analyzed with the Mann-Whitney test. **, p<.01 (**E**) Schematic representation of direct (solid lines) and indirect (dashed lines; via IgG1) class-switching to *IGHE*. Examples of hybrid Sμ-Sε switch regions generated from direct (upper sequence) and sequential (lower sequence) class switching to *IGHE*. Frequencies of direct and indirect class-switching in CD27+IgE+ and CD27−IgE+ memory B cells. The numbers of analyzed sequences are indicated in the inner circles. Data were statistically analyzed with the χ2 test.

## T-cell independent generation of CD27-IgE+ memory B cells

To study whether $CD27^{-}IgE^{+}$ memory B cells can indeed be formed independently of cognate T-cell help, we studied these in blood of 5 CD40L-deficient patients (Fig 4A). These patients lack $IgG^{+}$ memory B cells and carry strongly reduced $IgM^{+}$ and $IgA^{+}$ memory B-cell numbers.[25] While the $CD27^{+}IgE^{+}$ events were on the borderline of detection, $CD27^{-}IgE^{+}$ B cells were reliably detected with ~30% reduced frequencies as compared to healthy age-matched controls (Fig 4B). From this, we concluded that $IgE^{+}$ B cells can be formed in the absence of cognate T-cell help and these cells are nearly all CD27 negative.

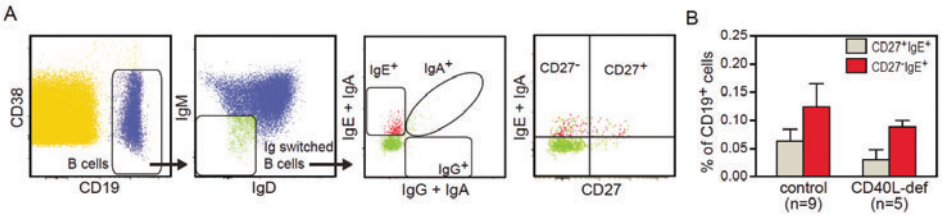


**Figure 4.** Germinal center-dependent and -independent origin of $IgE^{+}$ memory B-cell subsets in blood. (**A**) Representative images of flowcytometric analysis of circulating memory B-cell subsets in CD40L-deficient patients. (**B**) $CD27^{+}IgE^{+}$ and $CD27^{-}IgE^{+}$ B cell frequencies in CD40L-deficient patients and healthy age-matched donors (0-3y). Bars represent mean values with SEM.

## Efficient plasma cell differentiation of IgE+ memory B cells

Plasma cell differentiation and Ig class switching can be mediated by the cytokines IL4, IL13 and IL21.[10,13,38] The receptor for IL13 was not expressed on naive and memory B-cell subsets, IL4R was weakly expressed and IL21R was clearly detectable (Fig 5A). To study whether the IgE memory B cells could differentiate into plasma cells, we cultured FACS-purified cells for 6 days in the presence of anti-CD40 and IL21. Both IgG and IgE memory B cells generated $CD38^{hi}CD27^{+}$ plasma cells (Fig 5B), although the $CD27^{+}$ subsets seemed more efficient than the CD27- cells.

## Increased circulating CD27–IgE+ memory B cells in patients with atopic dermatitis

We exploited the reliable detection of $IgE^{+}$ memory B-cell subsets in blood to study 23 patients with atopic dermatitis, an allergic response often accompanied by increased IgE serum levels.[39] $IgE^{+}$ B-cell frequencies were compared with 16 age-matched healthy controls, and with 14 patients with $T_H1/T_H17$-mediated psoriasis. Frequencies of total $IgE^{+}$ memory B cells and $CD27^{+}IgE^{+}$ B cells were not significantly different between the groups (Fig 6A). However, the median frequency of $CD27^{-}IgE^{+}$ cells in atopic patients (0.12%) was significantly higher than in healthy donors (0.8%; $p<0.05$) and psoriasis patients (0.5%; $p<0.05$).

Patients with a 'high' disease severity according to the criteria by Hanifin and Rajka had more $CD27^{+}IgE^{+}$ and $CD27^{-}IgE^{+}$ cells than patients with the 'mild' or 'moderate' manifestation

of the disease (data not shown).[26] Due to low numbers of patients per group, these differences did not reach statistical significance. The frequencies of IgE$^+$ memory B cells did not correlate with total or allergen-specific IgE serum levels (Fig 6B).

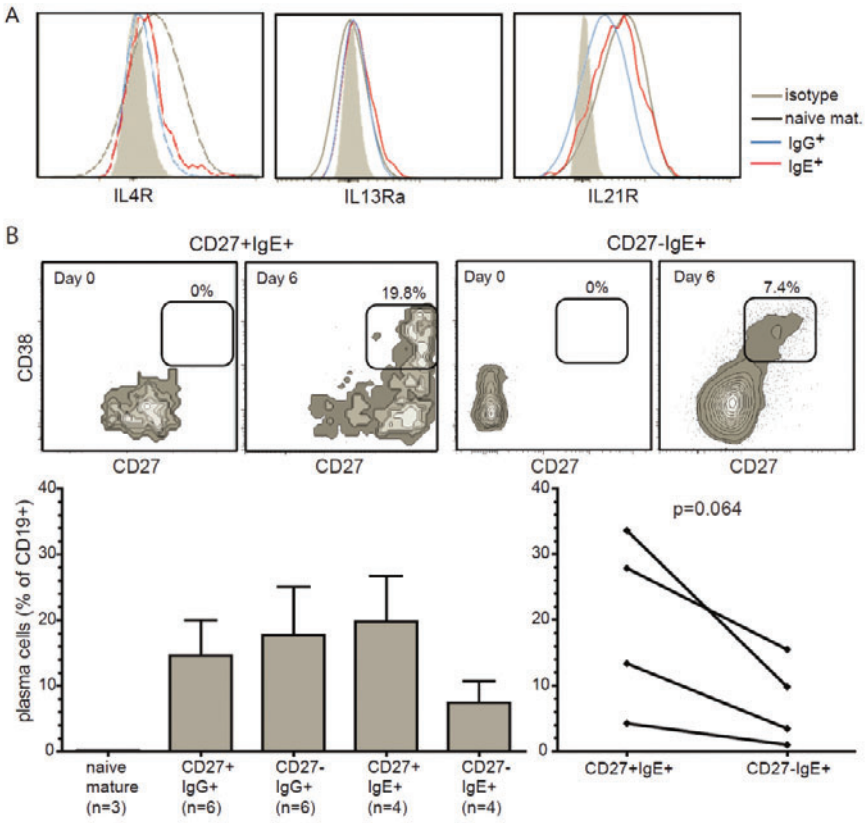


**Figure 5.** (**A**) Expression levels of IL4R, IL13Ra, and IL21R on naive mature, and IgG$^+$ and IgE$^+$ memory B cells. (**B**) The generation of plasma cells defined as CD38$^{hi}$CD27$^+$ after 6-day cultures. The average values for independent experiments with SEM are shown, as well as paired analysis of CD27$^+$IgE$^+$ and CD27$^-$IgE$^+$ memory B-cell subset (paired t test).

To study the effects of chronic disease on the IgE responses, we analyzed SHM in variable regions of *IGHE* transcripts amplified from 5 atopic dermatitis patients with a selective increase in CD27$^-$IgE$^+$ memory B cells (at least 6-fold more CD27$^-$IgE$^+$ than CD27$^+$IgE$^+$ B cells). The frequency of SHM in *IGHV* genes of CD27$^-$IgE$^+$ in atopic dermatitis patients was significantly higher than in control CD27$^-$IgE$^+$ memory B cells, and similar to control CD27$^+$IgE$^+$ B cells (Fig 6C). Patients' transcripts showed molecular signs of affinity maturation with a high IGHV-CDR R/S ratio (3.0). Thus, patients with atopic dermatitis show increased levels of circulating CD27$^-$IgE$^+$ memory B cells that show molecular signs of extensive antibody maturation.

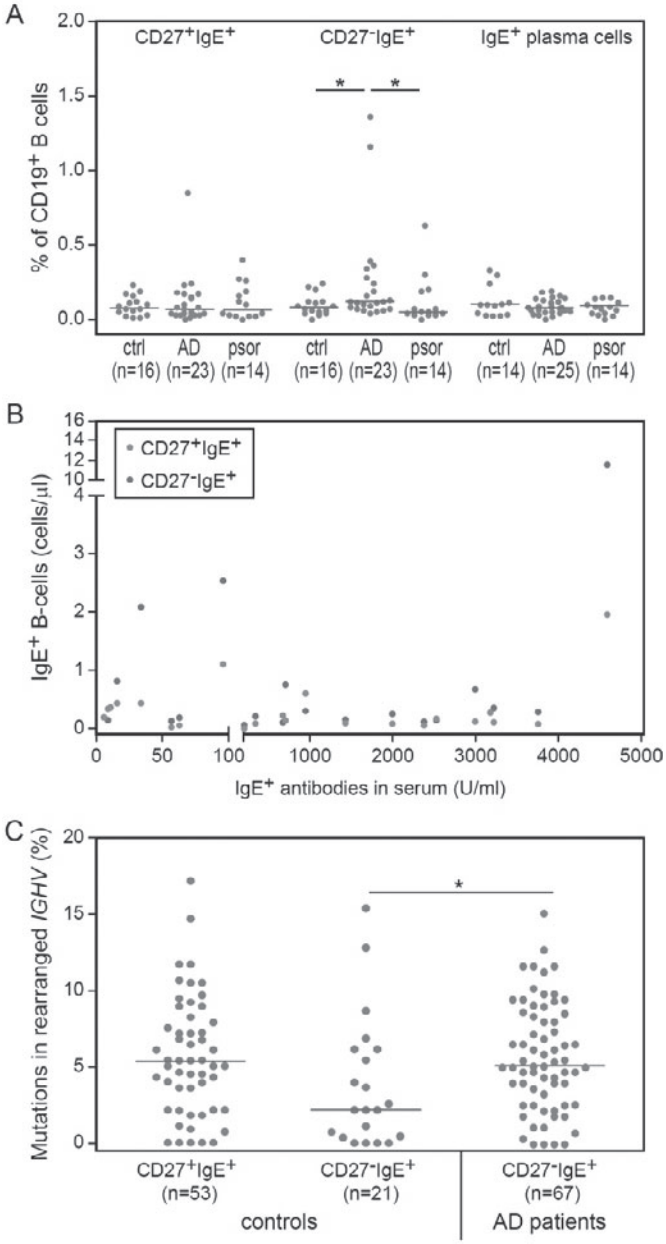


**Figure 6.** Increased frequencies of $CD27^{-}IgE^{+}$ memory B cells in atopic dermatitis patients. (**A**) Frequencies of $CD27^{+}IgE^{+}$ and $CD27^{-}IgE^{+}$ memory B cells and $IgE^{+}$ plasma cells in atopic dermatitis (AD) patients, psoriasis (psor) patients and healthy age-matched donors (ctrl). Individual data points are shown as grey dots with red lines indicating median values. (**B**) Correlation between $CD27^{+}IgE^{+}$ and $CD27^{-}IgE^{+}$ memory B-cell numbers, and IgE serum levels in 23 patients with atopic dermatitis. (**C**) Frequencies of mutated nucleotides in rearranged *IGHV* genes. Data were statistically analyzed with the Mann-Whitney test; *, $p<.05$.

### Model of IgE+ plasma cell and memory B-cell formation in human

To recapitulate our findings, we propose a model of $IgE^+$ plasma cell and $IgE^+$ memory B-cell formation in human (Fig 7). Based on the distinct molecular features and similarity to previously characterized memory B-cell subsets, we distinguish $CD27^+IgE^+$ memory B cells derived from a primary GC-dependent pathway and $CD27^-IgE^+$ memory B cells from local (mucosal) tissue. $IgE^+$ plasma cells are likely to be derived from both the GC and non-GC maturation pathways.

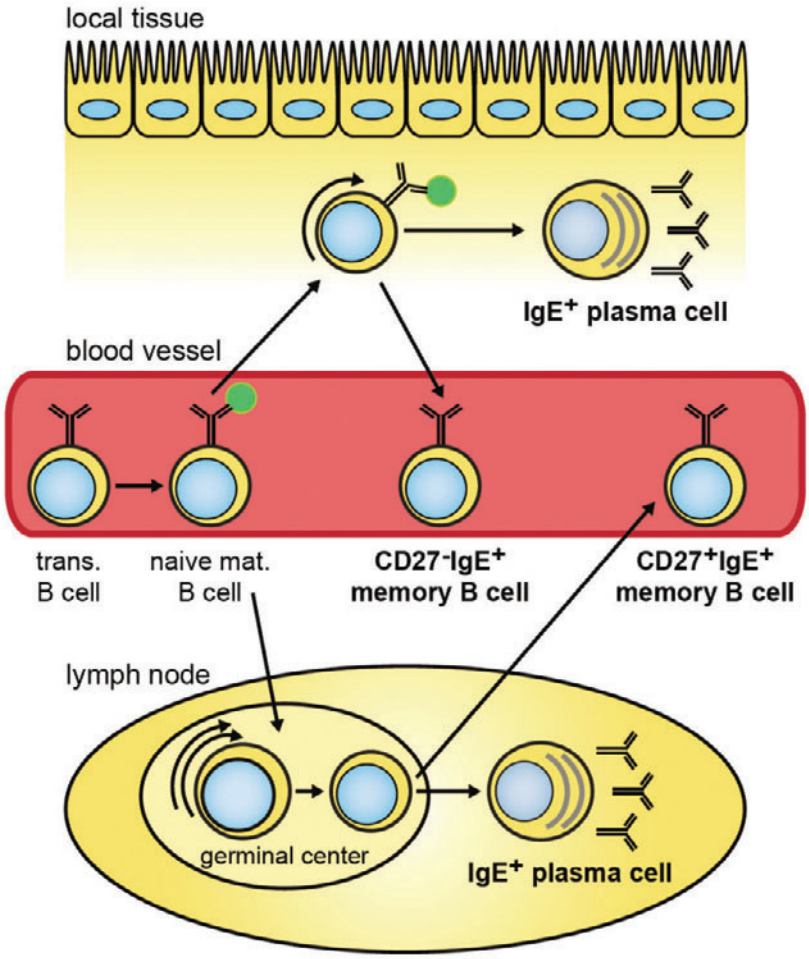


**Figure 7.** Model for GC-dependent and GC-independent maturation of $IgE^+$ B cells. Naive mature B cells scavenge the peripheral lymphoid system for antigens. Responses to peptide allergens with T-cell help result in extensive proliferation and affinity maturation in GCs, generating $IgE^+$ plasma cells and $CD27^+IgE^+$ memory B cells, potentially via an IgG intermediate. Antigen responses independently from T-cell help in local tissue (e.g. mucosa, skin) induce limited proliferation, SHM and direct IgE switching, generating plasma cells or $CD27^-IgE^+$ memory B cells.

## DISCUSSION

Using a novel 8-color flow cytometry strategy, we reliably detected $IgE^+$ plasma cells and two $IgE^+$ memory B-cell subsets. Purification and molecular analysis of these subsets allowed us to dissect their origins from GC responses ($IgE^+$ plasma cells and $CD27^+IgE^+$ memory B cells) and GC-independent responses ($CD27^-IgE^+$). The GC-dependent responses generated more SHM and frequently involved Ig CSR via IgG1, contrasting non-GC responses generating $CD27^-IgE^+$ memory B cells. These $CD27^-IgE^+$ cells were increased and showed extensive SHM in patients with atopic dermatitis.

Prior to flow cytometric selection of $IgE^+$ B cells, we performed a stepwise exclusion of $IgM^+$, $IgD^+$, $IgG^+$ and $IgA^+$ cells to increase the specificity of detection of rare $IgE^+$ B cells. $IgE^+$ B cells carried low surface Ig levels as compared with other B cells, possibly due to the suboptimal polyadenylation signals in membrane IgE transcripts.[7] Moreover, with this strategy, we excluded $CD23^+$ naive B cells that stained positively for IgE due to CD23-mediated capture from serum.[35] Thus far, the detection of $IgE^+$ B cells has been problematic, and often depended on cold acid wash to disrupt CD23-IgE binding,[40] which can be very damaging to the cells. Our direct flow cytometric approach on otherwise untreated cells allowed detailed immunophenotyping and molecular studies.

The majority of plasma cells in childhood tonsils had an immature phenotype with surface Ig and low/absent CD138 expression, which is commonly seen in plasma cells in lymph nodes, but not in blood and bone marrow,[41,42] and likely reflects recent generation. The IgE transcripts showed low mutation frequencies similar to IgM. Furthermore, blood $IgE^+$ plasma cells had intermediate SHM levels as compared to the two $IgE^+$ memory B-cell subsets. Therefore, $IgE^+$ plasma cells are likely a mixture of cells from primary GC-dependent and –independent responses.[25,27]

In blood, we identified circulating $IgE^+$ B cells that could be separated into $CD27^+$ and $CD27^-$ subsets. Both subsets showed immunophenotypic and molecular signs of B-cell memory: (1) high expression of activation and costimulatory molecules; (2) antigen-driven proliferation; and (3) SHM levels with high R/S ratios in IGHV-CDRs. The existence of $IgE^+$ B-cell memory has been disputed, although some indirect evidence exists from the kinetics of allergic responses.[43,44] Moreover, several studies report the detection of low numbers of circulating $IgE^+$ B cells displaying a plasma cell-like phenotype.[45,46] The $IgE^+$ B cells we identified showed a memory B-cell phenotype with low CD38 expression and upregulation of CD80, CD86 and TACI. In addition, FACS-purified cells expressed IgE-switched transcripts with SHM. Thus, $IgE^+$ memory B cells can be formed in healthy individuals and circulate in peripheral blood.

The two circulating $IgE^+$ memory B-cell subsets did not merely differ in their CD27 expression: the replication history, SHM levels, and frequency of indirect class-switching were significantly higher in $CD27^+IgE^+$ than in $CD27^-IgE^+$ memory B cells. In $CD27^+IgE^+$

memory B cells, these levels were slightly higher than in centrocytes, but lower than in $CD27^+IgG^+$ and $CD27^+IgA^+$ memory B cells, indicating that the majority of these cells in healthy adults are potentially derived from primary GC responses. This GC-dependent IgE response is most likely the source of high-affinity IgE in man. In mouse, switching to IgE takes place in post-GC $IgG^+$ B cells.[22] Because $CD27^+IgE^+$ memory B cells show high frequencies of indirect switching via an IgG intermediate, it is possible that IgE switching does not take place in the GC, but is a post-GC process.

$CD27^-IgE^+$ memory B cells had a limited replication history (3-4 cell divisions) and low SHM levels, and were present in CD40L-deficient patients, supporting their origin from a T-cell independent pathway. Until recently, CD27 was considered a pan-memory B-cell marker,[47] but there is growing evidence that also $CD27^-$ Ig class-switched cells display memory B-cell characteristics.[25,48] Importantly, the replication history and SHM levels of $CD27^-IgE^+$ memory B cells were similar to $CD27^-IgA^+$ memory B cells. Furthermore, both subsets showed predominantly direct Ig class-switching, and the frequently used *IGHA2* region in $CD27^-IgA^+$ memory B cells is genomically close to *IGHE*.[25] These comparable features support a similar origin of $CD27^-IgA^+$ and $CD27^-IgE^+$ memory B cells from local mucosal responses.[25] This contrasts with $CD27^-IgG^+$ cells that are fully dependent on T-cell help and share clonal relationships with $CD27^+IgG^+$ B cells.[25,49] Still, it cannot be excluded that such clonal relationships are also present between $CD27^+IgE^+$ and $CD27^-IgE^+$ subsets. Multiple studies demonstrated local IgE production in the respiratory tract of patients with atopic rhinitis and asthma,[50,51] and in the gastrointestinal tract of patients with food allergy, and showed increased local IL-4 and IL-13 production in these pathological conditions.[52,53] Of these, IL-4 is crucial for CD40-independent class-switching and BAFF- or APRIL-induced switching to IgE.[54,55] $CD27^-IgE^+$ memory B cells were the predominant $IgE^+$ subset in children. Since GC-dependent responses remain underdeveloped in young children, involvement of $CD27^-IgE^+$ cells in local responses could possibly explain early onset of some allergic diseases.

Atopic dermatitis patients showed increased frequencies of $CD27^-IgE^+$ memory B cells with high levels of SHM. While this increase was not found in psoriasis, the nature of these cells in atopic dermatitis remains unclear. The $CD27^-IgE^+$ phenotype could reflect the mainly local manifestation of this allergic disease. These cells would then be abnormally expanded and gained increased SHM levels. Alternatively, the $CD27^-IgE^+$ memory B cells in these patients are derived from a GC response with normal SHM levels, but display an abnormal phenotype. Still, these findings imply that analysis of $CD27^-IgE^+$ memory B-cell numbers can contribute to diagnosis and therapy monitoring of these patients, especially in cases with low IgE serum levels. Furthermore, $IgE^+$ memory B-cell measurements could prove valuable in monitoring of patients undergoing immunotherapy or anti-IgE antibody therapy (e.g. with omalizumab). A recent study indicated that the beneficial effect of omalizumab is the

result of reduced IgE production.[56] Therefore, the kinetics of reduction of $IgE^+$ memory B-cell levels could be an early marker to predict therapy outcome for patients with severe allergies.

Thus, by implementing a new strategy to reliably detect IgE-expressing B cells, we provided new insights into the biology of human $IgE^+$ plasma cells and dissected the molecular differences of the two memory B-cell subsets potentially derived from T-cell dependent and T-cell independent pathways. These insights open new possibilities to study the pathophysiological mechanisms underlying IgE-mediated diseases, which can be directly translated into patient care.

## ACKNOWLEDGEMENTS

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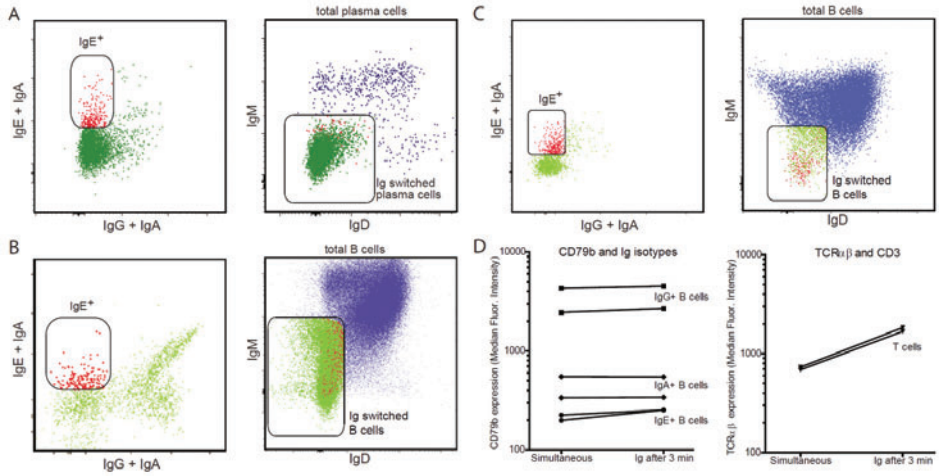
## SUPPLEMENTAL DATA




**Supplemental Figure 1. Immunophenotyping details of IgE+ memory B cells and plasma cells.** Backgating of IgE+ plasma cells from tonsil (**A**), memory B cells from blood of a healthy control (**B**) and a CD40L-deficient patient (**C**) within the IgM-IgD- gate. (**D**) Evaluation of steric hindrance of CD79b (left) and TCRαβ expression. Cells from 2 healthy donors were incubated simultaneously with anti-CD79b and anti-Ig isotypes or 3 min with CD79b prior to addition of anti-Ig isotypes. Pre-incubation with anti-CD79b prior to addition of the anti-Ig isotype antibodies did not lead to higher expression levels. In contrast, pre-incubation of T cells with anti-TCRαβ clone IP26 prior to addition of anti-CD3 clone OKT3 did lead to increased TCRαβ expression levels.

# CHAPTER 3

# IgE-expressing memory B cells and plasmablasts are increased in blood of children with asthma, food allergy and atopic dermatitis

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## ABSTRACT

Despite the critical role of soluble IgE in the pathology of IgE-mediated allergic disease, little is known about abnormalities in the memory B-cells and plasma cells that produce IgE in allergic patients. We here applied a flowcytometric approach to cross-sectionally study blood IgE+ memory B-cells and plasmablasts in 149 children with atopic dermatitis, food allergy and/or asthma, and correlated these to helper-T(h)2 cells and eosinophils. Children with allergic disease had increased numbers of IgE+CD27- and IgE+CD27+ memory B-cells and IgE+ plasmablasts, as well as increased numbers of eosinophils and Th2 cells. IgE+ plasmablast numbers correlated positively with Th2 cell numbers. These findings open new possibilities for diagnosis and monitoring of treatment in patients with allergic diseases.

## INTRODUCTION

IgE is commonly used in the diagnostic work-up of patients with allergic disease, but little is known about the cells producing it.[1, 2] All antibodies, including IgE, are produced by plasma cells.[3] IgM, IgG and IgA expressing memory B cells and plasma cells can be readily detected in tissue or blood of healthy children and adults. In contrast, IgE-expressing B cells have been enigmatic.[4] Still, based on the presence of IgE transcripts isolated from various tissues and blood of healthy controls and allergic subjects[5-7], the presence of IgE-expressing B cells has been anticipated.[8] We developed a flowcytometric strategy that enabled the detection of IgE-expressing $CD27^{+}CD38^{hi}$ plasmablasts, CD27+ and CD27- memory B cells.[9] Although T-cell help is required to generate the former subsets, CD27-IgE+ B cells can be formed independent of cognate T-cell help.[9, 10]

More insights into IgE-expressing B cells and their interactions with other immune cells are needed to better understand the pathogenesis of allergic disease, which could be useful for diagnostic purposes or to uncover targets for treatment. Similar to immunotherapy, anti-IgE antibody treatment can potentially target IgE+ memory B-cells and thereby might resolve the underlying allergy.[11]

We performed a cross-sectional study into IgE-expressing B cells in allergic children and healthy controls. Patients were separated into three groups based on distinct allergic phenotypes of asthma, food allergy and/or atopic dermatitis. We studied IgE+ memory B-cell and IgE+ plasmablast numbers with flow cytometry, their maturation status with molecular studies, and related these to clinical phenotype and other clinical and immunological parameters.

## METHODS

Peripheral blood samples were obtained from children aged 6-18 years who were recruited from the allergy outpatient clinics (KinderHaven and allergy department) of the Erasmus University Medical Center Rotterdam. All children were recruited from a tertiary referral center for children with allergic disease and were doctor's diagnosed as moderate to severe allergic disease. All children had allergic disease based on clinical examination and confirmed either by atopy skin test (HEP >0.21), specific IgE serum levels (>0.35 IU/L), immune solid-phase allergy chip (ISAC) or, in the case of food-allergy, double blind food provocation test. Based on symptoms, clinical findings and IgE-specificity, children with allergic disease were divided into three groups: 1) children with asthma in absence of food allergy (FA) and atopic dermatitis (AD): 'Asthma', n=68; 2) children with asthma in combination with FA and/or AD: 'Asthma + FA/AD', n=48; and 3) children with FA and/or AD in absence of asthma; 'FA/AD', n=33. Age-matched non-allergic controls were recruited among children undergoing

surgery in the Sophia Children's Hospital in Rotterdam. Exclusion criteria for the study were: age <6 years, systemic immunosuppressive therapy and immunological diseases, such as immunodeficiencies and auto-immune diseases.

All peripheral blood samples were collected after written informed consent was obtained and with approval of the Medical Ethical Committee of the Erasmus Medical Center, which complies with the Helsinki declaration.

Detailed methods on flow cytometry, molecular analysis of IgE transcripts and statistics can be found in the supplemental data.

## RESULTS

### Patient characteristics

A total of 149 allergic and 15 non-allergic children were included. All allergic children were sensitized for either aero- or food-allergens, as detailed per group in Table S1.

### Increased numbers of eosinophils, T-cells and B-cells in children with allergic disease

Total leukocyte counts were higher in patients with allergic disease than in non-allergic controls (Figure 1A). Similarly, total T- and B-cell numbers were significantly higher in each of the 3 groups with allergic disease than in non-allergic controls (Figure 1B). Total numbers and frequencies of eosinophils were significantly increased in allergic children as compared to non-allergic children (Figure 1C and Figure S2). Leukocyte subsets and total T- and B-cell numbers did not differ between the three allergic groups.

Absolute CD4+ T-cell numbers and subsets within CD4+ T-cells were significantly higher in children with allergic disease than in non-allergic children (Figure 1D). Still, frequencies of CD4 T-cells within CD3+ T-cell were not different from healthy children (Figure 1E). In addition, frequencies of Th1 and Th2 subsets within CD4+ T cells were not different between healthy children and children with allergic disease (Figure 1F). As a result, we did not observe a difference in Th2/Th1 cell ratios (Figure 1G) between patients and controls.

### Increased numbers of circulating IgE+ memory B-cells and IgE producing plasmablasts

IgE+ memory B-cells were defined within the $CD19^{+}CD38^{dim}$ fraction and IgE+ plasmablasts within the $CD19+CD38+CD27^{hi}$ fraction following exclusion of CD19+ B-cells expressing IgM, IgD, IgA or IgG (Figure 2A).[9]

Median cell numbers of both CD27+IgE+ and CD27-IgE+ memory B-cells were significantly higher in all three groups of allergic children than in non-allergic controls (Figure 2B). In addition, IgE+ plasmablast numbers were significantly increased in allergic

children (Figure 2C). IgE+ memory B-cell and IgE+ plasmablast numbers did not significantly differ between patient groups. The increases in IgE+ B-cell numbers in allergic children were not only the result of higher total B-cell counts, because their relative numbers within CD19+ B cells were also significantly higher in all patient groups (Figure 2D,E).

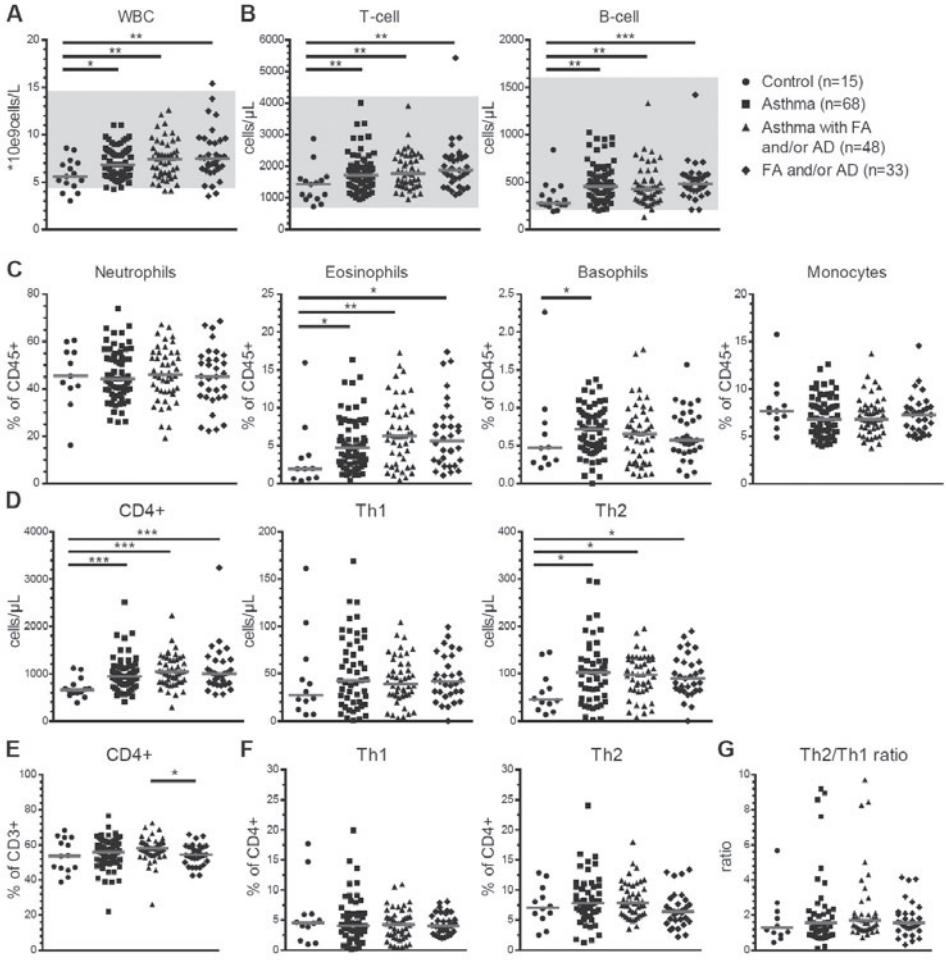


**Figure 1.** Absolute numbers and frequencies of leukocyte subsets and CD4+ helper-T(h) subsets. **A.** White blood cell count. **B.** Absolute numbers of T cells and B cells. **C.** Percentages of granulocyte subsets and monocytes within total CD45+ cells. **D.** Absolute cell numbers of CD4+ T-cells, Th1 and Th2 cells **E.** Percentage of CD4+ T-cells within total CD3+ T-cells. **F.** Percentage of Th1 and Th2 cells within total CD3+ T-cells. **G.** ratio of Th2/Th1 cells. Each dot represents one individual and red lines indicate median values. Grey surface represent reference values. Statistical analysis was performed with Mann Whitney *U* test. *P<0.05, **P<0.01, ***P<0.001

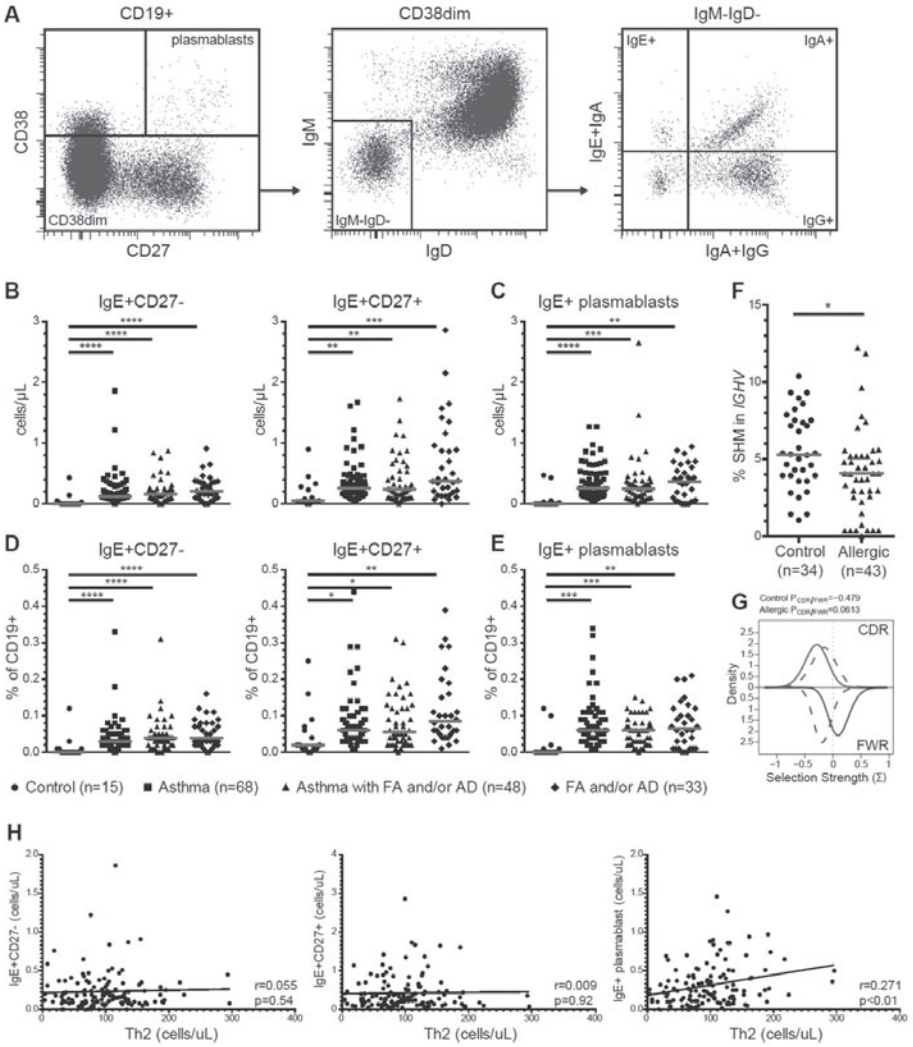


**Figure 2.** IgE+ B-cell subsets. **A.** Representative flowcytometry image and gating strategy of IgE+ memory B-cells. **B.** Absolute numbers and percentages of IgE+ memory B-cells. **C.** Absolute numbers and percentages of IgE+ plasmablasts. **D.** Somatic hypermutation (SHM) levels in the *IGHV* gene of IgE+ plasmablasts from healthy children and children with allergic disease (n=5 children each). **E.** Selection for replacement mutations in the CDR (red) and FR (blue) regions. **F.** Correlation of Th2 cell numbers with IgE+CD27-, IgE+CD27+ and IgE+ plasmablasts in children with allergic disease. In panels B, C and F each dot represents one individual; red lines indicate median values; and black lines in panel F indicate linear correlations. Statistical analysis was performed with Mann Whitney *U* test. *P<0.05, **P<0.01, ***P<0.001, ****P<0.0001. Correlation was calculated with Spearman R.

To investigate whether the increased numbers constituted normal plasmablasts, we studied the levels of somatic hypermutation (SHM) in IgE transcripts of sorted cells from allergic children (n=43 unique transcripts) and from non-allergic controls (n=34 unique transcripts). Transcripts from patients carried fewer mutations in their *IGHV* genes than controls (Figure 2F). As a measure for antigen-driven selection of the mutations in the *IGHV* genes, we analyzed the selection for replacement mutations in the complementarity determining regions (CDR) with the BASELINe program. Transcripts from healthy controls ($P_{CDR/FWR}$ -0.48) nor from children with allergic disease ($P_{CDR/FWR}$ 0.06) showed more replacement mutations than expected by random chance in the CDR regions, i.e. absence of positive selection (Figure 2G). Thus, the higher numbers of circulating IgE-expressing B-cells in children with allergic disease did not show molecular signs of enhanced antigen receptor maturation.

Since type 2 cytokines produced by helper-T(h) cells are important for IgE class switch recombination (CSR) and children with allergic disease had increased numbers of IgE+ B-cells, we investigated the correlation between Th cells and IgE+ B-cells. We observed a correlation between IgE+ plasmablast numbers and Th2 cells (r 0.271; $P$ <0.01), whereas there was no correlation between IgE+ memory B-cell subsets and Th2 cells (Figure 2H).



## DISCUSSION

In the present study we extended the immunological phenotype of allergic children. In addition to Th2 cells, eosinophils, basophils and serum IgE, we found increased numbers of circulating IgE+ memory cells and IgE+ plasmablasts, and a positive correlation between IgE+ plasmablasts and Th2 cell numbers.

In our patient group, type 2 sensitization was confirmed by high IgE serum levels, sensitization to various aero- and food allergens, high numbers of eosinophils in blood and increased Th2 cell numbers.

Our findings of increased numbers of circulating IgE+ memory B cells extend previous observations.[9, 12, 13] Importantly, our flowcytometric gating strategy enabled exclusion of IgE-binding cells from the true IgE-expressing B cells, which we confirmed by the presence of mature IgE transcripts. We showed that this occurs in children, and is independent of the allergic phenotype. A cause for the increased numbers of circulating IgE+ memory B cells could be the chronic inflammation. This is specifically thought to occur in patients with atopic dermatitis, and to some extent also in asthma. Here we found increased numbers of both CD27+ and CD27- IgE-expressing memory B cells in allergic patients. It has been shown that CD27- IgE+ memory B-cells display a limited proliferation history and lower SHM levels in their immunoglobulin genes[9], which suggest that they originate from mucosal tissue, analogous to CD27-IgA+ memory B-cells.[14] This is supported by previous detections

of IgE transcripts and Sμ-Sε switch circles in nasal tissue of patients with allergic rhinitis and in bronchial tissue of patients with allergic asthma.[8, 15] Several studies have shown that sensitized individuals who are exposed to allergens via nasal tissue or the respiratory tract, display a substantial increase in systemic serum IgE levels, thus indicating the direct activation of an established local pool of IgE memory cells with defined specificities as an underlying mechanism.[16]

We did not observe a correlation between IgE+ memory B-cell and Th2 cell numbers, but we did find a positive correlation between Th2 cells and IgE+ plasmablasts. Previous studies show that type 2 innate lymphoid cells (ILC2) might play an important role in the local CSR towards IgE.[17, 18] Furthermore expansions of ILC2 populations have been observed in allergic disease and are known to produce type 2 cytokines and ILC2 cells facilitate sensitization to local, but not systemic Th2 inducing allergen exposures.[19] Next to that, T follicular helper (Tfh) cells have been linked to IgE class switching through the production of IL-4.[20, 21] Tfh cells express the B-cell follicle homing receptor CXCR5 and mainly reside in germinal centers. Thus possibly ILC2 cells might be important in local IgE CSR, Tfh cells in germinal center IgE CSR, whereas Th2 cells are important in plasma cell differentiation. In future studies it would be interesting to study ILC2 cells in mucosal tissue and correlate these to IgE+ memory B-cell numbers.

It is uncertain if circulating IgE+ memory B-cells sustain the underlying allergy, but in vitro stimulated IgE+ memory B-cells can differentiate into IgE+ plasma cells.[9] Therefore it is possible that increased circulating IgE+ memory B-cells actively contribute to allergic disease.[22] To achieve desensitization and possibly even cure allergic disease, it might therefore be important to develop therapies to reduce IgE+ memory B-cells. Omalizumab treatment in humans has shown to decrease serum IgE in patients with IgE-mediated asthma[23], however it mainly targets soluble IgE. This is not the case for Quilizimab, which only recognizes surface-expressed IgE on B cells[11], and has shown to deplete IgE producing B-cells in mouse studies.[24] Although human clinical trials with Quilizimab reduced IgE serum levels for 6 months after treatment cessation, the effects on clinical parameters were variable.[25, 26] Our present findings can possibly contribute to patient selection and treatment monitoring of anti-IgE treatment.

## AUTHOR CONTRIBUTIONS

JJH, JCdJ, JJMvD and MCvZ designed research. JJH and LR performed research. JJH, LR and MCvZ analyzed data. NJA, SGP, JCdJ and GJD evaluated and included patients in the study. JJH and MCvZ wrote the paper, and all authors commented on the paper and approved the final version.

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# SUPPLEMENTAL DATA

## Supplemental Materials and Methods

*Flow cytometry of blood samples*

White blood cell (WBC) counts were determined with a Coulter cell counter (Beckman Coulter) within 24 hours of blood sampling. Absolute counts of CD45+ leukocytes, CD3+ T-cells and CD19+ B-cells were obtained with a diagnostic lyse-no-wash protocol using TruCount tubes (BD Biosciences, San Jose, Calif). For detailed 11-color flow cytometry, red blood cells were lysed with NH4Cl prior to incubation of 2 million nucleated cells for 15 minutes at room temperature in a total volume of 100µL. After preparation, cells were measured on a 4-laser LSRFortessa flow cytometer (BD Biosciences) using standardized settings. Data were analyzed with FACSDiVa software V8.0 (BD Biosciences). Our previously published sequential flowcytometric gating strategy was applied to identify IgE+ B-cells.

*Molecular analysis of IGH gene rearrangements*

RNA was isolated from post-Ficoll mononuclear cells with a GenElute mammalian RNA kit (Sigma-Aldrich, St Louis, Mo) and reverse transcribed to cDNA with random primers (Invitrogen Life technologies). Alternatively, IgE+ plasma cells (CD19+CD38hiCD27+IgE+) were single cell sorted on a FACSAria III (BD Biosciences) into 96-well PCR plates (VWR European, Radnor, PA) containing ice-cold 0.5x PBS, 10mM DTT (Invitrogen, Carlsbad, Calif) and 8 U RNAsin® (Promega, Madison, Wis). Total RNA from these single cells was reversed transcribed into cDNA using 5µM random hexamer primers (Applied Biosystems, Foster City, Calif), 0.5% Triton X (Sigma-Aldrich, St Louis, Mo), 1x first strand buffer (Invitrogen), 1.25mM dNTPs, 10mM DTT (Invitrogen), 16 U RNAsin® (Promega) and 70 U SuperScript® III reverse transcriptase (Invitrogen).

IGHV gene rearrangements were amplified in a semi-nested multiplex PCR approach using 4 different IGHV-family specific leader forward primers in combination with IGHE-specific reverse primers (first PCR: 5'-CATCACCGGCTCCGGGAAGTAG-3'; second PCR: 5'-ACGGAGGTGGCATTGGAGGGAAT-3'). PCR products were cloned into a pGEMT easy vector (Promega, Madison WI), amplified by colony PCR, and sequenced on an ABI Prism 3130XL (Applied Biosystems, Foster City, CA). The sequences were analyzed with the IMGT database (www.imgt.org) and BASELINe program (selection.med.yale.edu/baseline) to quantify somatic hypermutations (SHM) and selection for replacement mutations.

*Statistical analysis*

Frequencies and absolute cell numbers had a non-Gaussian distribution. Therefore, all results were expressed as median (interquartile range) if applicable and were analyzed using

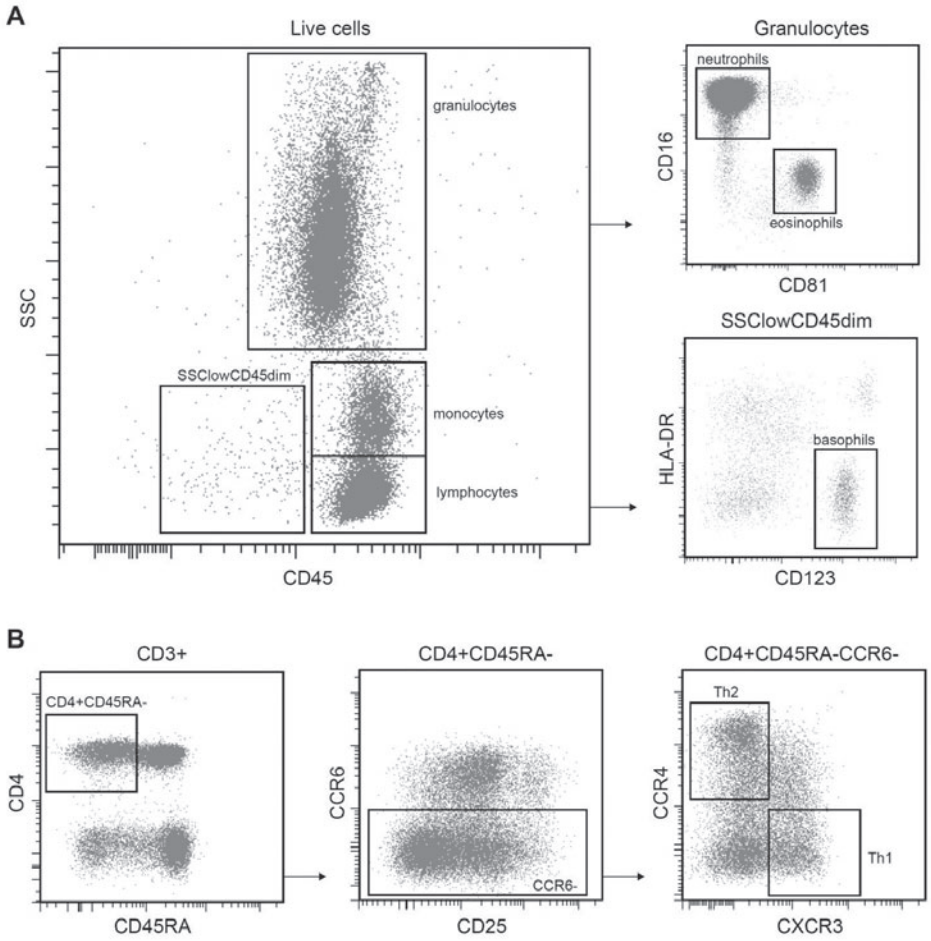
the Mann-Whitney U test. Linear regression was used to study the strength of association between cell-subsets. Spearman rho was used to define if the correlation was significant. All indicated P-values were two-tailed and considered statistically significant if values were lower than 0.05. Statistical analysis was performed using GraphPad Prism software, version 6 (GraphPad Software, La Jolla, Calif).

**Supplemental Table 1.** Patient characteristics

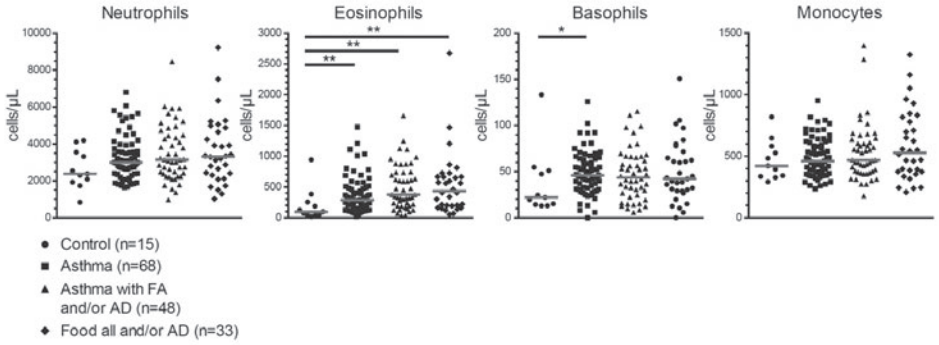
| | Control | | Asthma | | Asthma with food allergy and/or atopic dermatitis | | Food allergy and/or atopic dermatitis | |
|---|---|---|---|---|---|---|---|---|
| | (n=15) | | (n=68) | | (n=48) | | (n=33) | |
| | | | | | | | | |
| **Gender – *n (%)*** | | | | | | | | |
| Male | 7 | (47%) | 46 | (68%) | 31 | (65%) | 20 | (61%) |
| Female | 8 | (53%) | 22 | (32%) | 17 | (35%) | 13 | (39%) |
| | | | | | | | | |
| **Age – *mean yr (range)*** | | | | | | | | |
| | 11.1 | (6-16) | 11.4 | (6-17) | 10.9 | (6-16) | 9.0 * | (6-15) |
| | | | | | | | | |
| **Sensitization to aero-allergens – *n (%)*** | | | | | | | | |
| HDM | n.d. | | 51 | (75.0%) | 28 | (58.3%) | 0 | (0.0%) |
| Pollen | n.d. | | 46 | (67.6%) | 31 | (64.6%) | 18 | (54.5%) |
| Dander | n.d. | | 43 | (63.2%) | 28 | (58.3%) | 13 | (39.4%) |
| | | | | | | | | |
| **Sensitization to food allergens – *n (%)*** | | | | | | | | |
| Milk | n.d. | | 3 | (4.4%) | 11 | (22.9%) | 6 | (18.2%) |
| Eggs | n.d. | | 3 | (4.4%) | 13 | (27.1%) | 8 | (24.2%) |
| (shell)fish | n.d. | | 0 | (0.0%) | 9 | (18.8%) | 1 | (3.0%) |
| Fruits | n.d. | | 1 | (1.5%) | 4 | (8.3%) | 0 | (0.0%) |
| Peanut | n.d. | | 7 | (10.3%) | 23 | (47.9%) | 10 | (30.3%) |
| Nuts | n.d. | | 3 | (4.4%) | 25 | (52.1%) | 18 | (54.4%) |
| Wheat | n.d. | | 0 | (0.0%) | 6 | (12.5%) | 2 | (6.1%) |
| Soy | n.d. | | 1 | (1.5%) | 5 | (10.4%) | 2 | (6.1%) |
| | | | | | | | | |
| **Serum immunoglobulins – *mean IU/ml (range)*** | | | | | | | | |
| Total IgE | n.d. | | 782 (6-9,082) | | 2561 (13-13,452) | | 1,346 (252-9,140) | |

HDM, house dust mite; n.d. not determined

* significantly lower than Asthma and Asthma with FA and/or AD ($P<0.01$)




**Supplemental Figure 1** Flowcytometry gating strategy for leukocyte subsets and T helper subsets. **A.** Analysis of leukocyte subsets using antibodies listed in Supplemental Table 2 (Leukocyte subsets). **B.** Analysis of T-helper subsets using antibodies listed in Supplemental Table 2 (T-cell subsets)




**Supplemental Figure 2.** Absolute counts of neutrophils, eosinophils, basophils and monocytes per microliter of blood. Each dot represents one individual and red lines indicate median values. Statistical analysis was performed with Mann Whitney *U* test. *P<0.05, **P<0.01

# CHAPTER 4

# Netherton syndrome: a local skin barrier problem rather than a systemic immunodeficiency - *A case series of 14 patients with Netherton syndrome in the Netherlands*

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*Manuscript in preperation*

## ABSTRACT

*Background* Comel-Netherton syndrome (NS) is a rare autosomal disease, characterized by severe skin conditions, hair shaft defects, atopic diathesis and increased susceptibility for infections.

*Objective* Detailed clinical and immunological analysis of the cohort of NS patients in the Netherlands with the aim to identify if there is an immune defect underlying recurrent infections.

*Methods* Phenotypes were scored for skin severity, specific hair shaft defect, atopy and infections. Patients' blood samples were collected for quantification of serum Ig levels, specific antibodies against *Streptococcus pneumoniae* and allergen-specific IgE, as well as detailed immunophenotyping of blood leukocyte and lymphocyte subsets with flowcytometry.

*Results* 14 patients were included with age range 3-46 years and varying degrees of skin involvement. All patients presented with atopic symptoms (food allergy, n=13; hay fever, n=10; asthma, n=7). Recurrent skin infections were common, particularly in childhood (n=12). Absolute numbers of lymphocytes, lymphocyte subsets and serum immunoglobulin levels were al l within nomal ranges. Low levels of specific antibodies against *S. pneumoniae* were found in 10 of the evaluated 11 patients.

*Conclusion* Multidisciplinary evaluation of our national cohort showed no evidence for a severe systemic immunodeficiency We hypothesize that in Dutch NS patients the increased risk for infections results from the skin barrier disruption and that increased allergen penetration predisposes to allergic sensitization.

## INTRODUCTION

Comel-Netherton syndrome (NS) (OMIM #256500) is a severe genodermatosis typically characterized by chronic skin inflammation (ichtyosis and scaly erythroderma), specific hair shaft defects (trichorrhexis invaginata) and atopic diathesis with elevated serum IgE levels.[1, 2] The disease is caused by mutations in the *SPINK5* gene (serine protease inhibitor of kazal type 5) encoding LEKTI (lympho-epithelial kazal type related inhibitor), which is expressed in the stratified epithelium of the skin, the mucosa and in the Hassal corpuscles of the thymus.[3-6]

The consequence of LEKTI deficiency is a loss of inhibition of serine proteinases such as plasmin, trypsin, subtilisin A, cathepsin G and elastase.[7-11] This particularly leads to unopposed activity of kallikrein-related peptidase 5 (KLK5), which activates KLK7, KLK14 and elastase 2 (ELA2).[12-14] This leads to increased degradation of corneodesmosomal cadherins through increased degradation of desmoglein 1, increased desmosome cleavage and reduced filaggrin proteolytic processing.[15-17] The result is an abnormal skin homeostasis and detachment of the stratum corneum, which contributes to a defective skin barrier and thereby enabling microbe and allergen penetration.[18] KLK5 also activates protease-activated receptor 2 (PAR-2) which is expressed on the surface of keratinocytes.[19] Observations from SPINK5 knock-out mouse embryos indicate a KLK5-Par2 cascade, leading to enhanced production of thymic stromal lymphopoietin (TSLP), a T-helper 2 (Th2) related cytokine, enhancing the allergic predisposition.[20] Furthermore, Par-2 leads to increased expression of TNF-α, IL-8 and ICAM-1, thereby augmenting an inflammatory process.[21]

The effects of defective LEKTI expression in the thymus and its effect on T-cell maturation are less well described. However, apart from dermal and allergic inflammation, the disease has also been associated with immunodeficiency and increased susceptibility to skin-, respiratory tract and systemic infections.[22-25] Although recurrent skin infections with *S. aureus* can be related to altered skin homeostasis, some have attributed these observations to intrinsic immune defects, with improvement after intravenous immunoglobulin treatment.[22] Described immune defects include decreased numbers of natural killer (NK) cells, an immature phenotype of NK cells with reduced lytic function, reduced numbers of memory B cells and reduced responses to pneumococcal vaccinations.[22-26] Although a premature senescent state of T-cells has been proposed, there have been no reports on dysfunctional T-cell properties. This is likely due to the fact that NS is a rare disease, the described patient cohorts are small (mostly 2-9 patients) and mainly consist of children.

To examine the potential immunodeficiency in NS, we actively recruited all known patients in the Netherlands for detailed clinical and immunological examinations including skin phenotype, allergic manifestations and blood leukocyte immunophenotyping.

# METHODS

## Study design and patient characteristics

The study was designed as a cross-sectional study. All known sixteen patients with NS in the Netherlands were invited to participate in the study. Patients were actively recruited through the Erasmus University Medical Centre Rotterdam, an acknowledged national expert center for patients with NS, through the patients association and by social media. Patients were diagnosed based on the presense of germline mutations in *SPINK5*, LEKTI-deficiency in skin biopsy and/or trichorrhexis invaginata (ISRCTN12831121).[27] Patients were included after obtaining written informed consent and with approval of the Medical Ethical Committee of the Erasmus MC (MEC-2013-026), which complies with the Helsinki declaration. Control blood samples were obtained from healthy volunteers amongst department staff (MEC-2016-022).

All patients received questionnaires about their medical and psychological wellbeing, including medical history, (daily) medication use, daily skin appearance, growth rate, frequency of infections, hospital admissions and (food)allergies. Subsequently patients were invited for blood sampling and a multidisciplinary evaluation by a the (pediatric-) dermatologist and (pediatric-) immunologist of our Netherton team.

## Skin assessment and recurrent infections

To objectively score skin lesions, the body surface area (BSA in %), total lesional sign score for NS (TLSS-NS) and the investigator's global assessment for NS (IGA-NS) *(*Supplemental Table 1) were used.[28-30]

Recurrent infections were defined as follows: recurrent skin infections: > 1 per year; recurrent respiratory infections > 3 per year for adults, > 6 per year for children; recurrent ear nose and throat (ENT) infections > 2 per year; recurrent gastro-intestinal infections > 1 per year.

## Immunoglobulin serology

Specific IgE for inhalation and food allergens were determined using ImmunoCAP and/or the Immuno Solid-phase Allergen Chip (ImmunoCAP ISAC, Phadia, Uppsala, Sweden), according to manufacturer's instructions.

Total IgM, IgG and IgA serum levels were measured by immunonephelometry with a Siemens BN II nepholometer according to manufacturer guidelines. Specific antibody titers against S. pneumoniae were analyzed using a Luminex assay according to a protocol adapted from Borgers et al.[31]

## Vaccination response

Patients were immunized using a polysaccharide vaccine against S. pneumoniae (Pneumovax). Eleven out of 14 patients received Pneumovax vaccine and 4 to 6 weeks

after immunization specific antibody titers against S. pneumonia were measured and compared to pre-immunization titres. A 4-fold increase in titres reaching at least 1.00 µg/mL in a minimum of 7 of the 13 measured serotypes (type 1,3,4,5,6A,6B,7F,9V,14,18C,19A,19F,23F) 4 to 6 weeks after immunization was determined adequate.[32, 33]

### Flowcytometric immunophenotyping of blood leukocytes

Absolute numbers of granulocytes, monocytes, lymphocytes, as well as NK-cells (CD16+/CD56+), T-cells (CD3+) and B-cells (CD19+) were obtained with a diagnostic lyse-no-wash protocol using commercial Trucount tubes (BD Biosciences, San Jose, Calif). For detailed 11-color flow cytometry,[34] red blood cells were lysed with $NH_4Cl$ prior to incubation of 1 million nucleated cells with antibody cocktails for 15 minutes at room temperature in a total volume of 100µL. After preparation, cells were measured on a 4-laser LSRFortessa flow cytometer (BD Biosciences) using standardized settings.[35] Data were analyzed with FACSDiva software V8.0 (BD Biosciences).

### Statistical analyses

Frequencies and absolute cell numbers were assumed a non-Gaussian distribution. All results are expressed as median values with interquartile range if applicable. Results were analyzed using the non-parametric Mann-Whitney U test. All P-values are two-tailed and were considered statistically significant if values were lower than 0.05. Statistical analysis was performed using GraphPad Prism software, version 6 (GraphPad Software, La Jolla, CA).



## RESULTS

In the present study we evaluated 14 patients with NS in the Netherlands (6 males, 8 females). All known patients with NS in the Netherlands (n=16) were invited, however due to private circumstances two patients refused to participate. Ages ranged from 3 to 46 years (median, 24 years), including four children, which were all female (Table 1).

### Phenotypes

Ten patients were born with generalized erythroderma and two patients developed erythroderma within several hours after birth (Table 1). The skin severity varied between patients: six patients had an affected Body Surface Area (BSA) of 100% and 5 patients of >50% with a median IGA-NS score of 3 (IQR 3-4). All patients had trichorrhexis invaginata. Seven out of 14 patients noticed improvement of their skin over time with a median of 6.5 years. All patients reported daily invalidation on a scale 1 to 10 (median 5; IQR 3-6), a daily pain numerical rating scale (NRS) median of 7.0 (IQR 2.8-8.0) and pruritus NRS median of 4.0 (IQR 3.0-7.5) (Supplemental Table 1).

**Table 1.** Patient characteristics and clinical manifestations

| patient | sex | age (yr) | diag-nosis | skin at birth | skin | hair | Atopic manifestation | IgE serum Level (IU/ml) | BSA | TLSS | IGA | age of improve-ment |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| 1 | M | 43 | DNA | ED | ED | TI, short, easily broken | rhinitis, food allergy | 22 | total | E3I2L2 | 4 | none |
| 2 | F | 46 | DNA | other | ILC+ED | TI | asthma, rhinitis, atop. dermatitis, food allergy | 159 | >50% | E1I1L0 | 2 | none |
| 3 | M | 21 | DNA | ED | ILC+ED | TI, easily broken | atop. dermatitis, food allergy | >5000 | total | E3I3L2 | 4 | none |
| 4 | F | 9 | DNA | normal | ILC+ED | TI, short | rhinitis, food allergy | 4524 | total | E1I1L2 | 4 | none |
| 5 | F | 8 | DNA | other | ED | TI, short, easily broken | asthma, rhinitis, food allergy | >5000 | total | E2I1L2 | 4 | 1.5 yrs |
| 6 | F | 24 | DNA | ED | ILC+ED | TI, short, easily broken | rhinitis, food allergy | 4083 | >75% | E3I3L2 | 3 | 5 yrs |
| 7 | F | 22 | DNA | ED | ILC+ED | TI | food allergy | >5000 | total | E2I2L2 | 3 | none |
| 8 | M | 24 | DNA | ED+ILC | ILC | TI, easily broken | asthma, rhinitis, atop. dermatitis, food allergy | >5000 | >75% | E2I2L2 | 3 | 12 yrs |
| 9 | M | 36 | DNA | normal | ILC | TI | none | >5000 | total | E2I1L2 | 2 | none |
| 10 | F | 6 | DNA | ED | ILC+ED | TI, short, easily broken | asthma, rhinitis, atop. dermatitis, food allergy | missing | >50% | E2I3L3 | 3 | 1 yr |
| 11 | F | 3 | DNA | ED | ILC+ED | TI | atop. dermatitis, food allergy | >5000 | <50% | E2I1L1 | 3 | 1 yr |
| 12 | M | 39 | DNA | ED | ILC+ED | TI, short easily broken | asthma, rhinitis, atop. dermatitis, food allergy | 3522 | >50% | E1I2L3 | 3 | 12 yrs |
| 13 | F | 43 | DNA | ED | ILC | TI, longer length | asthma, rhinitis, atop. dermatitis, food allergy | missing | missing | missing | missing | 14 yrs |
| 14 | M | 36 | DNA | ED | ILC | TI, easily broken | asthma, rhinitis, atop. dermatitis, food allergy | >5000 | <25% | E2I2L0 | 2 | 25 yrs |

ED, erythroderma; ILC, ichthyotic linearis circdumflexa; TI, trichorrhexis invaginata; BSA, body surface area; TLSS, total lesional sign score; IGA, investigator's global assessment

When compared to the normal growth chart for children and the average height for adults (data of the Netherlands Organization for Applied Scientific Research), patients seemed to be smaller than the average population (Supplemental Table 2). Three patients had a length within 1 SD of the general population. All 4 children had normal weight-length ratios. Eight out of 10 adults had a normal BMI between 18.5 and 25; two patients were overweight with a BMI above 25 (Supplemental Table 2).

### Atopic syndrome

Ten out 14 patients reported hay fever and 7 patients reported asthma. All but one patient reported reactions to food (especially nuts, cow milk, eggs and fish) with symptoms occurring within two hours after intake (nausea, stomach pain or edema of the nasopharynx) (Table 1 and Supplemental Table 3). Most patients had refused double blind food provocation tests due to clear acute reactions. Therefore, additional laboratory tests were performed to confirm IgE sensitization. Eleven out of the 12 tested patients had elevated total immunoglobulin E (IgE) above 100 IE/ml and one patient had a normal IgE level of 22 IE/ml (Table 1). All 11 patients tested, had IgE sensitization to food allergens (Supplemental Table 3).



### Treatment

All 14 patients used emollients for daily skin treatment, and 12 out of 14 patients needed topical corticosteroids, ranging from hydrocortisone acetate to clobetasol proprionate. One patient used tacrolimus ointment and one patient used coal tar. Three patients received treatment with oral antihistamines and all received treatment with inhalation medication. At the time of study inclusion, none of the patients received systemic immunosuppressive treatment.

### Immunological evaluation

A manifest amount of infections were reported in all but one patient, particularly recurrent skin infections are a common problem, with a median of 4.1 infections requiring treatment with antibiotics every year (IQR 2.8-5.0; Table 2). Seven patients reported recurrent ENT infections since early childhood for which repetitive antibiotic treatments were required. In one patient, recurrent ENT infections persisted throughout adulthood. Several patients reported the need of regular cleaning of the external auditory meatus every 4 weeks to prevent external ear infections. Before the age of 6 years patients did not report frequent respiratory infections. Of the 12 patients above the age of 6 years, 7 patients suffered from recurrent skin infections in the previous 12 months. One patient reported recurrent gastro-intestinal infections as a child, none reported these during adulthood. Similarly, one patient reported severe systemic infections during childhood, but none were reported during adulthood. No patients described a period of persistent fever after vaccinations (Table 2).

**Table 2.** Infectious manifestation and immunological characteristics

| patient | recurrent infections | | | | | | | | | | treatment with IVIG | serum Ig levels (g/L) | | | Specific antibodies against S. *pneumoniae* |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | skin | | resp tract | | ENT | | gastro intestinal | | severe invasive | | | IgM | IgG | IgA | |
| | ≤ 6 yrs | > 6yrs | ≤ 6 yrs | > 6yrs | ≤ 6 rs | > 6yrs | ≤ 6 yrs | > 6yrs | ≤ 6 yrs | > 6yrs | | | | | |
| 1 | yes | yes | - | - | - | - | - | - | - | - | - | 0.52 | 22.0 | 5.70 | **low** |
| 2 | | yes | - | yes | yes | yes | - | - | - | - | - | 1.98 | 11.0 | 2.82 | **low** |
| 3 | yes | yes | - | - | yes | - | - | - | - | - | - | 1.19 | 7.0 | 2.54 | n.d. |
| 4 | yes | yes | - | - | yes | - | yes | - | - | - | - | 0.97 | 12.2 | 1.80 | normal |
| 5 | yes | - | yes | - | yes | yes | - | - | yes | - | - | 0.97 | 13.9 | 1.84 | n.d. |
| 6 | yes | yes | - | - | - | - | - | - | - | - | - | 0.92 | 9.6 | 1.24 | **low** |
| 7 | yes | yes | - | - | yes | - | - | - | - | - | yes | 1w.36 | 16.8 | 2.11 | **low** |
| 8 | - | - | - | - | - | - | - | - | - | - | - | **0.34** | 12.4 | 2.35 | **low** |
| 9 | yes | yes | - | - | - | - | - | - | - | - | - | 0.47 | 13.7 | 3.87 | **low** |
| 10 | yes | yes | - | - | - | - | - | - | - | - | yes | 0.67 | 10.7 | 1.81 | **low** |
| 11 | yes | yes | - | - | yes | yes | - | - | - | - | - | 1.13 | 12.2 | 1.53 | **low** |
| 12 | yes | - | - | - | yes | - | - | - | - | - | - | **0.26** | 14.2 | 1.71 | **low** |
| 13 | yes | - | - | - | - | - | - | - | - | - | - | n.d. | n.d. | n.d. | n.d. |
| 14 | - | yes | yes | - | - | - | - | - | - | - | - | 0.51 | 9.0 | 1.89 | **low** |

Values below the normal range are depicted in bold font; above normal range in italics

None of the 13 tested patients showed an overt antibody deficiency. All patients had normal to high levels of IgG and IgA, and only 2 patients had reduced IgM serum levels (Table 2). Still, in 10 of 11 tested patients, levels of specific antibodies against *S. pneumoniae* were low after Pneumovax vaccination (i.e. less than a 4-fold increase in titres reaching at least 1.00 µg/mL in a minimum of 7 of the 13 measured serotypes) (Table 2).

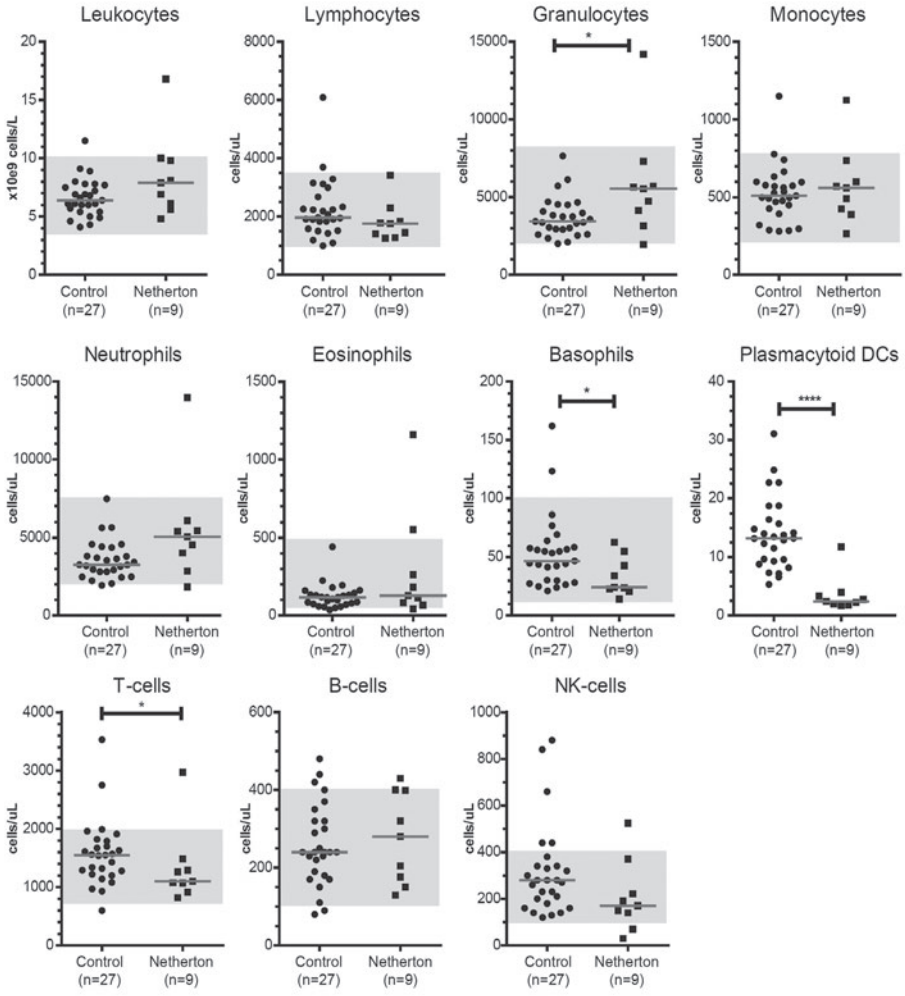


**Figure 1.** Absolute numbers of leukocytes and leukocyte subsets in healthy individuals and in adult patients with NS. Each symbol represents an individual with red lines indicating median values. Normal ranges are depicted in grey shades. Statistics, Mann-Whitney U test; *, $p<0.05$; ****, $p<0.0001$.

### Patients with NS have higher numbers of granulocytes

Blood leukocytes and their subsets were studied in 9 of the 10 adult patients (Figure 1). One patient had a leukocyte count above the normal range, which was caused by an elevated granulocyte count. Compared to the healthy individuals, patients with NS had a significantly higher granulocyte count (*P* 0.04). Absolute numbers of total lymphocytes and monocytes were in the normal range. Within the granulocyte subset, mainly the numbers of neutrophils seemed higher, although this was not significantly different from controls. Two patients had an increased eosinophil count. All patients had a normal basophil count, yet the median count was significantly lower than that of the control group (*P*=0.02). Numbers of plasmacytoid dendritic cells (pDCs) were dramatically decreased in the patient group (*P*<0.001). Within the lymphocyte subset one patient had an increased B-cell count and one patient had an increased T-cell count, yet as a group the median T-cell count was significantly lower than that of healthy controls (*P*=0.04). In our study cohort, NK-cell numbers were quite diverse, but statistically different from the control group. Still one patient had increased NK-cell numbers, whereas two patients had reduced NK-cell numbers.

### Patients with NS have lower IgM-only memory B-cell numbers and increased IgE+CD27- memory B-cell numbers

Within the B-cell compartment we studied naive, memory and effector B-cell subsets (Figure 2). Absolute numbers of naive B-cell subsets (transitional B-cells and naive mature B cells) were comparable to that of healthy controls. In the memory compartment, median cell numbers of IgM-only memory B-cells (IgM+IgD-CD27+) were significantly lower than that of healthy controls (*P*=0.03), whereas cell numbers of other memory B-cell subsets (IgM+IgD+CD27+ Natural Effector B-cells, IgG+ and IgA+ memory B-cells) were comparable to those of healthy controls (Figure 2A). In contrast, the median cell number of IgE+CD27- memory B-cells was significantly higher in the patient group (Figure 2B; P=0.01). Plasma blast numbers seemed much lower in patients with NS. One patient had a high plasma blasts count, therefore as a group there was no significant difference between patients and controls.

### Patients with NS have lower Th1 cell numbers

Since LEKTI is highly expressed in the thymus, the primary organ for T-cell development, we performed detailed analysis of the T-cell compartment (Figure 3). Although total T-cell numbers were significantly lower in the total group of patients as compared to controls, all patients but one, had T-cell numbers still within the normal range. In line with this, total CD4+ and CD8+ T-cells, as well as numbers of naive, central memory and effector memory subsets within CD4+ and CD8+ T-cells were not different between patients and controls (Figure 3A and B).

Within the T-helper subset, numbers of Th1 cells were significantly lower in patients with NS, whereas Th2 cell numbers were not affected (Figure 3C). In addition, Th17 cell numbers were not different between patients and controls. Next to that also regulatory T-cell numbers were comparable between patients and controls (Figure 3C).

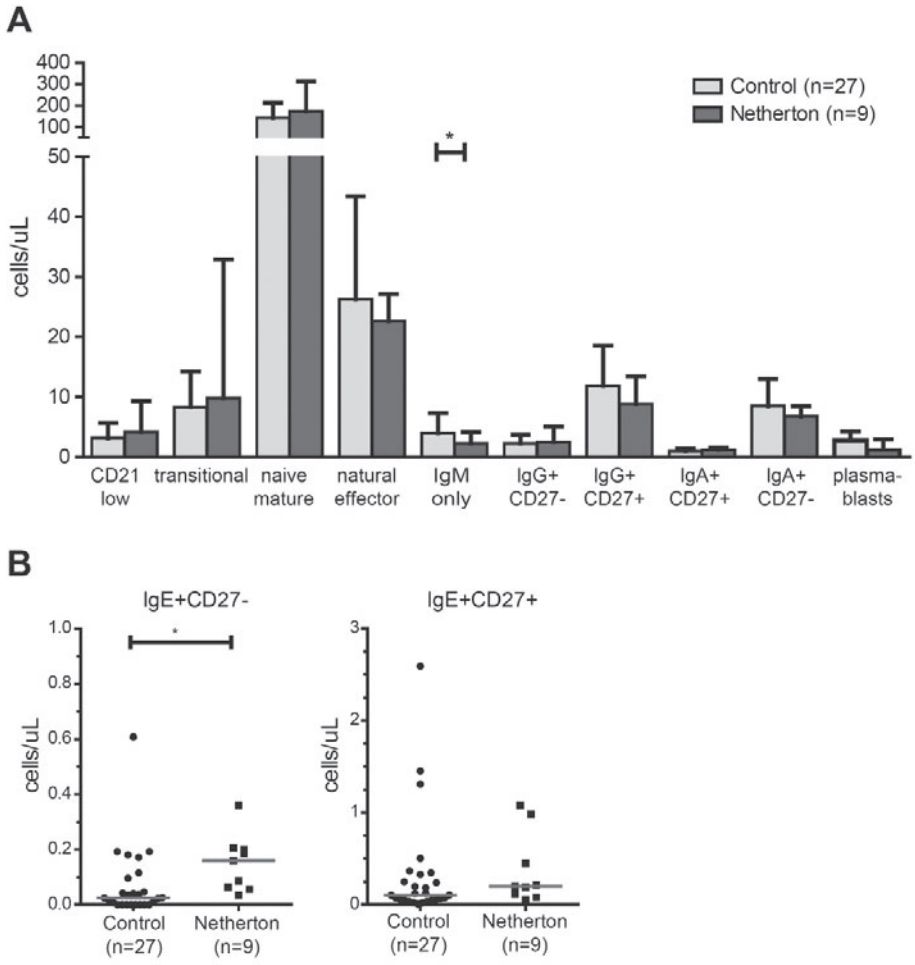


**Figure 2.** Absolute numbers of B-cell subsets in healthy individuals and in adult patients with NS. **A**. Absolute numbers of naive and memory B-cell subsets and plasma cells. Columns indicate median values with interquartile range. **B.** Absolute numbers of IgE+ memory B-cells. Each symbol represents an individual with red lines indicating median values. Statistics, Mann-Whitney U test; *, $p<0.05$

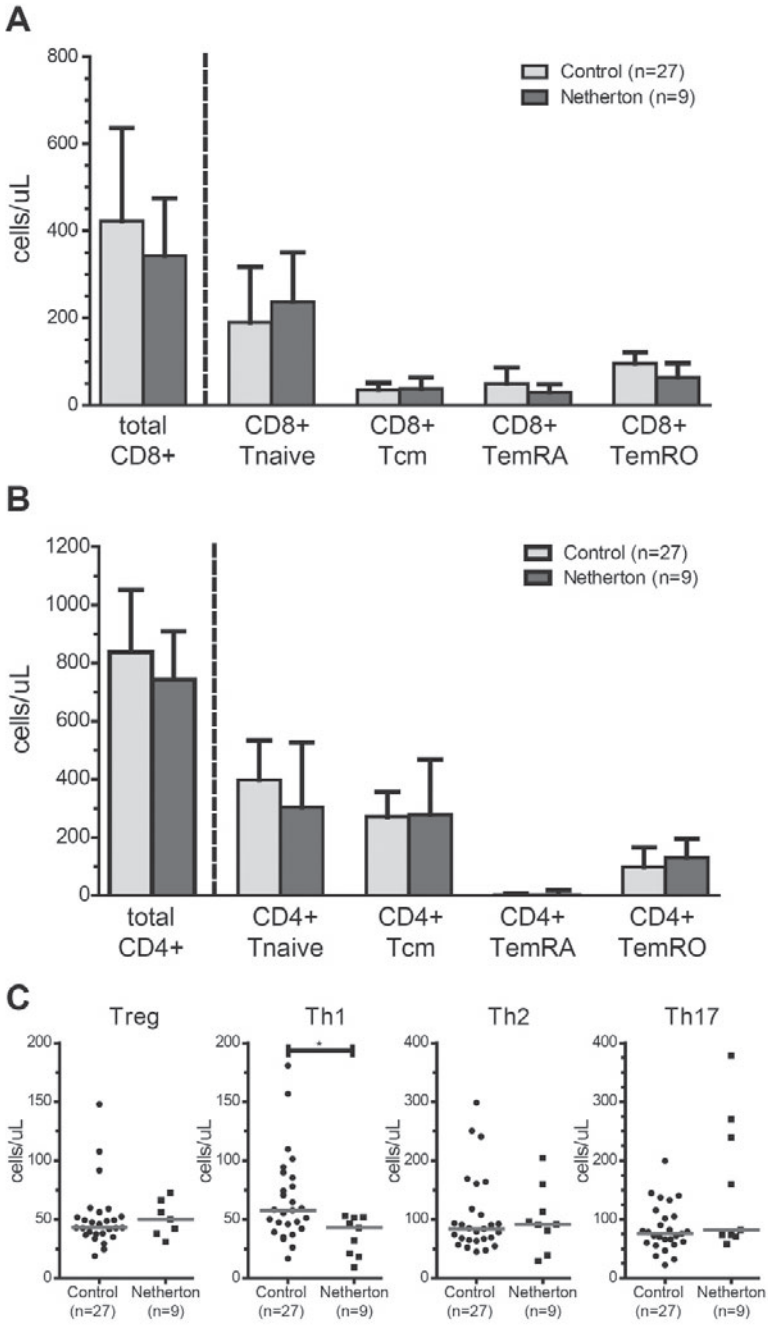


**Figure 3.** Absolute numbers of T-cell subsets in healthy individuals and in adult patients with NS. **A.** of total CD8+ T-cells and CD8+ Tnaive, Tcm, TemRO and TemRA. **B.** total CD4+ T-cells and CD4+ Tnaive, Tcm, TemRO and TemRA. Columns represent median values with interquartile range. **C.** counts of regulatory T-cells, Th1, Th2 and Th17 T-cells. Each symbol represents an individual with red lines indicating median values. Statistics, Mann-Whitney U test; *, $p<0.05$

## DISCUSSION

In this case series, 14 of the 16 known NS patients in the Netherlands were evaluated for their clinical phenotypes, atopic diathesis and immunological characteristics. To our knowledge this is the first described national NS patient cohort. Their history and clinical presentation are an illustration of the differences in the spectrum in patients with NS concerning clinical features, disease severity and follow-up.[26]

Immunological evaluation of our patients showed no evidence for a severe systemic immunodeficiency, even in the more severely affected patients. This in contrast to other studies [22, 26] Although low titers of specific antibodies against *S. pneumoniae* were measured, the current evaluation could not confirm the presence of a clinically relevant humoral immunodeficiency, based on the fact that patients did not suffer from an increased number of severe infections caused by encapsulated bacteria, which we see in antibody deficient patients.[22] Functional assays evaluating potential NK-cell disturbances were not performed, however NK-cell numbers were not affected, thus this does not explain the difference between our observations with previous studies. An explanation might be that due to the national recruitment we experienced less selection bias resulting in more diversity in our cohort hence giving a better reflection of the whole spectrum of NS, at least in the Netherlands. The dramatically decreased numbers of pDCs can be explained by the fact that these cells are highly sensitive to corticosteroid therapy.[36]

All patients in the Dutch cohort were below the age of 50 years. We hypothesized that NS patients may have an accelerated aging of the immune system due to the severe skin barrier defect and/or by the defective LEKTI expression in the thymus. This was not confirmed by our data as the numbers of naive T-cells as well as effector T-cells were within normal range. This study does not explain the young age of the adult patients.

The increased manifestation of skin and ENT infections, especially in childhood, observed in our cohort may still imply an immune dysregulation according to the guidelines of European Society for Immunodeficiencies (ESID).[37] Although in our cohort it is not known by which pathogens the infections were caused. As patients were born erythroderm, the observed change in skin phenotypes in some patients from erythroderma into Ichthyosis linearis circumflexa (ILC) are in line with previous described NS patients. Our cohort includes less severely affected NS patients and half of our patients reported improvement of their skin with age (median age 6.5 years). This might explain why less skin infections were reported in adults. We also hypothesized an impact of natural maturation of the immune system. In general, an increased susceptibility to infections during childhood has previously been described in healthy children, especially for respiratory infections and nasal immune responses in the first 2 years of life.[38] The recurrent ear infections in children and adults

with NS might also be caused by obstruction of the external auditory canal as a result of excessive skin scaling.

Evaluation of atopic manifestations showed a remarkable high percentage of sensitization to food allergens, which is in line with previous observations.[39] The increased risk of an atopic constitution corresponded with an elevated total IgE in 11 of the 12 tested patients as well as increased IgE+ memory B-cell numbers. Similar observations have been described in patients with atopic dermatitis.[40] As described, the impaired epidermal skin barrier may open aberrant routes of entrance for allergens.[41, 42] Combined with a different local immune response with enhanced risk of Th2 imprinting could lead to further sensitization to (food-) allergens.[20, 41, 43] In our cohort food allergies for nuts, cow milk, eggs and fish were most common. The types of allergens do not differ from the general population in which 90% of the allergic responses to food are caused by cow's milk, soy, eggs, wheat, peanuts, tree nuts, fish and shellfish.[44-47] This suggests that the role of the skin barrier and sub-epithelial environment with a more Th2 profile might be greater than assumed for the atopic constitution in NS.

### Strengths and limitations

To our knowledge this national cohort of 14 NS patients is the largest described until now.[22, 23, 26, 39, 48-53] Because of active recruitment we assume less selection bias and greater diversity in severity in our cohort. For example, some patients (n=2) were not in care before active recruitment and received their topical treatment from their general practitioner. Another strength is the multidisciplinary approach of this study. Evaluation of anamnestic information was partially based on retrospective data and could be considered as a limitation.

**Clinical implications:** Evaluation of patients with Netherton syndrome in the Dutch cohort showed no evidence for a severe systemic immunodeficiency. Rather it seems more likely a severe skin disease due to a local impaired skin barrier with an increased risk for infections and sensitization to food allergens, combined with reduced maturation of the immune system at a young age especially at a young age.

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## SUPPLEMENTAL DATA

**Supplemental Table 1**. Numerical scores for pain and pruritus

| patient | NRS pain | NRS pruritus |
|---|---|---|
| 1 | 1 | 4 |
| 2 | 7 | 4 |
| 3 | 7 | 4 |
| 4 | 9 | 10 |
| 5 | 2 | missing |
| 6 | 7 | 8 |
| 7 | 8 | 3 |
| 8 | 2 | 3 |
| 9 | 5 | 8 |
| 10 | 9 | 4 |
| 11 | 3 | 2 |
| 12 | 4 | 6 |
| 13 | 8 | 9 |
| 14 | 7 | 2 |

NRS: numerical ratings scale (score 1-10)

**Supplemental Table 2**. Patients' height and weight

| patient | child/adult | age | sex | growth (in SD) | length (cm) | BMI |
|---|---|---|---|---|---|---|
| 1 | adult | 43 | M | < -2 SD | 170 | 23.88 |
| 2 | adult | 46 | F | -1 SD and 0 SD | 163 | 30.49 |
| 3 | adult | 21 | M | < -2 SD | 173 | 20.05 |
| 4 | child | 9 | F | < -2 SD | 121 | 17.76 |
| 5 | child | 8 | F | < -2 SD | 92 | 34.26 |
| 6 | adult | 24 | F | -2 SD and 1 SD | 160 | 19.92 |
| 7 | adult | 22 | F | -1 SD and 0 SD | 165 | 22.04 |
| 8 | adult | 24 | M | unknown | 173 | 21.72 |
| 9 | adult | 36 | M | -1 SD and 1 SD | 172 | 21.97 |
| 10 | child | 6 | F | -1 SD and 0 SD | 102 | 15.19 |
| 11 | child | 3 | F | < -2 SD | 99 | 16.32 |
| 12 | adult | 39 | M | unknown | 178 | 22.72 |
| 13 | adult | 43 | F | -2 SD and 1 SD | 172 | 20.28 |
| 14 | adult | 36 | M | -1 SD and 1 SD | 189 | 26.32 |

SD: standard deviation; BMI: body mass index

**Supplemental Table 3**. Atopic sensitization measured with Immuno-solid-phase Allergy Chip (ISAC)

| Patient | 1 | 2 | 3 | 4 | 5 | 6 | 7 | 8 | 9 | 10 | 11 | 12 | 13 | 14 |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| Grass pollen | low | moder-ate | very high | n.d. | n.d. | very high | very high | very high | very high | n.d. | und. | very high | n.d. | very high |
| Birch pollen | und. | und. | very high | n.d. | n.d. | very high | very high | very high | und. | n.d. | low | very high | n.d. | very high |
| Dog | und. | low | high | n.d. | n.d. | moderate | und. | very high | very high | n.d. | und. | moderate | n.d. | very high |
| Cat | und. | und. | moderate | n.d. | n.d. | moderate | high | very high | high | n.d. | und. | und. | n.d. | very high |
| Egg white | und. | und. | moderate | n.d. | n.d. | very high | und. | very high | und. | n.d. | moderate | und. | n.d. | und. |
| Egg yolk | und. | und. | moderate | n.d. | n.d. | und. | und. | very high | und. | n.d. | moderate | und. | n.d. | und. |
| Cod | und. | und. | und. | n.d. | n.d. | low | und. | very high | und. | n.d. | low | und. | n.d. | und. |
| Shrimp | und. | und. | und. | n.d. | n.d. | und. | und. | very high | moderate | n.d. | und. | moderate | n.d. | und. |
| Cashew nut | und. | und. | low | n.d. | n.d. | und. | und. | und. | und. | n.d. | moderate | und. | n.d. | low |
| Brazil nut | und. | und. | und. | n.d. | n.d. | und. | low | moderate | und. | n.d. | moderate | und. | n.d. | moderate |
| Hazelnut | und. | und. | moderate | n.d. | n.d. | very high | low | moderate | und. | n.d. | moderate | und. | n.d. | und. |
| Walnut | und. | und. | moderate | n.d. | n.d. | und. | very high | high | und. | n.d. | very high | moderate | n.d. | und. |
| Sesame | und. | und. | und. | n.d. | n.d. | und. | und. | very high | und. | n.d. | very high | low | n.d. | und. |
| Soybean | und. | und. | very high | n.d. | n.d. | und. | und. | moderate | und. | n.d. | moderate | und. | n.d. | und. |
| Wheat | und. | und. | und. | n.d. | n.d. | und. | moderate | moderate | und. | n.d. | und. | und. | n.d. | und. |
| Apple | und. | und. | very high | n.d. | n.d. | very high | very high | very high | und. | n.d. | und. | moderate | n.d. | und. |
| Peach | und. | und. | very high | n.d. | n.d. | very high | very high | very high | und. | n.d. | und. | moderate | n.d. | very high |
| Kiwi | und. | und. | und. | n.d. | n.d. | moderate | und. | very high | und. | n.d. | moderate | moderate | n.d. | und. |
| House dust Mite | und. | high | very high | n.d. | n.d. | very high | very high | very high | very high | n.d. | very high | moderate | n.d. | high |

und: undetectable; n.d.: not determined

*ISAC Standardized Units (ISU-E): <0.3 undetectable; 0.3-0.9 low; 1-9 moderate; 10-14.9 high; ≥ 15 very high

# PART III

## THE EFFECT OF TREATMENT ON THE PERIPHERAL IMMUNE COMPARTMENT IN IGE MEDIATED DISEASE

# CHAPTER 5

# Treatment for moderate to severe atopic dermatitis at alpine and moderate maritime climates differentially affect helper T cells and memory B cells in children

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## ABSTRACT

Background: Treatment of atopic dermatitis (AD) is focused on topical anti-inflammatory therapy, epidermal barrier repair and trigger avoidance. Multidisciplinary treatment in both moderate maritime and alpine climates can successfully reduce disease activity in children with AD. However, it remains unclear whether abnormalities in B- and T-cell memory normalize and if this differs between treatment strategies.

Objective: To determine whether successful treatment in maritime and alpine climates normalises B- and T-lymphocytes in children with moderate to severe AD.

Methods: The study was performed in the context of a trial (DAVOS trial, registered at Current Controlled Trials ISCRTN88136485) in which eighty-eight children with moderate to severe AD were randomized to 6 weeks of treatment in moderate maritime climate (outpatient setting) or in the alpine climate (inpatient setting). Before and directly after treatment, disease activity was determined with SA-EASI and serum TARC, and T- and B-cell subsets were quantified in blood.

Results: Both treatment protocols achieved a significant decrease in disease activity, which was accompanied by a reduction in circulating memory Treg, transitional B-cell and plasmablast numbers. Alpine climate treatment had a significantly greater effect on disease activity and was accompanied by a reduction of blood eosinophils, and increases in memory B-cells, CD8+ TemRO, CD4+ Tcm and CCR7+ Th2 subsets.

Conclusions and clinical relevance: Clinically successful treatment of AD induces changes in blood B- and T-cell subsets reflecting reduced chronic inflammation. In addition, multidisciplinary inpatient treatment in the alpine climate specifically affects memory B-cells, CD8+ T cells and Th2 cells. These cell types could represent good markers for treatment efficacy.

## INTRODUCTION

Atopic dermatitis (AD) is a common inflammatory skin disease, which affects up to 10-20% of children with a lifetime prevalence estimated at 15-30%.[1]{Bieber, 2010 #1337;Odhiambo, 2009 #1348;Leung, 2003 #1757}(1)(1, 2) The underlying pathogenesis of AD is multifactorial and includes environmental factors, genetic predisposition, skin barrier dysfunction, and altered local and systemic immune responses.[2-6] It remains unclear if reported abnormalities in the immune response are the cause of the disease, a result of reduced skin barrier function, or a combination of both. Still, it is generally accepted that AD is characterized by a skewing towards T-helper 2 (Th2) responses with increased immunoglobulin (Ig)E serum levels and eosinophilia.[6, 7]

The main principles in AD treatment are based on epidermal barrier repair with anti-inflammatory topical treatment, emollients, antimicrobial therapy and trigger avoidance.[5,8] Only in more severe cases, or when insufficient control of AD is achieved, systemic immunosuppressive treatment may be required.[5, 8-11] Treatment in the alpine climate has been used in children with AD and asthma for decades,[12] with long term improvement in disease control and health related quality of life.[13] On the short term, disease activity has been shown to decrease significantly, which is accompanied by a significant reduction in eosinophils and fractional exhaled nitric oxide (FeNO).[13] Despite the rationale of these treatments to dampen abnormal immune responses, data on underlying immunological effects are scarce.[14, 15]



In the acute phase of AD, dermal and epidermal lesions are predominated by Th2 cells and Th2 cytokines, such as interleukin (IL)-4, IL-5 and IL-13.[16, 17] This is also reflected in peripheral blood with increased numbers of CD4+ T-cells producing IL-4 and IL-13.[18-22] In children, this increase specifically concerns T cells expressing the cutaneous lymphocyte antigen (CLA).[23] In addition, expansions of CLA+ Th22 cells have been observed in adults with severe AD.[23] During chronic stages of the disease, T-helper cell skewing changes, with an increase of Th1 cells, which is referred to as the biphasic T-cell polarization.[24] The production of IL-22 induces epidermal hyperplasia through their effect on keratinocytes, whereas Th2 cytokines induce barrier disruption.[17, 25] Furthermore, regulatory T-cells (Tregs) are consistently found to be increased in blood of patients with AD.[26-28] Possibly, this is caused by chronic inflammatory signals, because numbers decline with treatment and correlate well with disease severity.[29, 30]

Under influence of the Th2 cytokines IL-4 and IL-13, activated B cells can undergo Ig class switching to IgE.[31] Indeed IgE-producing plasmablasts are increased in AD,[32] and elevated total and allergen-specific IgE serum levels are a hallmark of the disease. These B-cell responses also result in higher numbers of IgE+ memory B-cells in blood of patients with AD.[32, 33] Furthermore, numbers of transitional B-cells, which are attributed to have regulatory capacities through the production of IL-10, have been reported to be increased in AD.[32] Still

it has been shown that dupilumab, an antibody blocking IL-4/IL-13 receptors, has equal potency in treatment of either extrinsic AD (with increased IgE levels) as intrinsic AD (without increased IgE levels). This can be explained by data showing that Th2 cytokines diminish the expression of barrier proteins such as filaggrin, loricrin, involucrin and antimicrobial peptides[34]. Hence, this illustrates the clinical and immunological diversity of the disease.

Despite these insights into T-cell and B-cell abnormalities in disease, data about the effects of treatment on the immune response in patients with AD are limited. Most studies evaluated lesional skin biopsies and focused on T-cell subsets.[35-40] Evaluation of alpine climate treatment has been focused on a reduction in eosinophil numbers, but data on T- and B-cell subsets are limited.[14, 15]

Here we investigate blood T- and B-cell subsets in children with moderate to severe AD before and after an intensive 6-week treatment protocol. Specifically, we compared the effects of two personalized integrative multidisciplinary treatment protocols: inpatient treatment in the alpine climate and outpatient treatment in moderate maritime climate (DAVOS trial).[41] Here we report on the identification of common and treatment-specific effects on blood B- and T-cell subsets.

# METHODS

## Study design

This study is part of the DAVOS trial, ISRCTN88136485, which is a pragmatic randomized controlled trial for children with moderate to severe, difficult to treat AD. A detailed description of the study design has been published previously.[41] In short, patients were enrolled between September 2010 and October 2014 and randomized to either a 6 week personalized integrative multidisciplinary inpatient treatment at a high altitude clinic in Switzerland (alpine climate) at 1,560 meters (intervention, NAD group), or to a 6 week personalized integrative multidisciplinary outpatient treatment program in the Netherlands at sea level (moderate maritime climate) (control, WKZ group). Patients were assessed before the start of treatment (T0) and within 72 hours after the end of the 6 week treatment (T1). The study assessments, including blood sampling, were all performed in the Netherlands. The trial design was in line with current health care practice in the Netherlands, which is outpatient whenever possible, whereas alpine climate treatment is always provided in an inpatient setting.

During the 6 week inpatient treatment period, children in the NAD group had weekly individual treatment sessions with a pediatrician, a psychologist (alternating with the psychomotor therapist) and a physiotherapist (if needed). A nurse monitored correct application of topical treatment in individual sessions with each child twice daily. In these individual sessions health education was provided with a varying weekly theme. During

their stay at the high altitude clinic, children attended an integrated school. Prior to outdoor activities, sun cream was applied on exposed skin to protect for high UV exposure when necessary. Children in the WKZ group visited the outpatient clinic on a weekly basis for 6 weeks. They had three consultations with the dermatologist, five consultations with the dermatological nurse, three consultations with the pediatric allergist and three consultations with the psychologist. In the third week, separate group consultations were scheduled for the children and their parents about coping with AD and compliance.

### Participants

Dutch children and adolescents with difficult to treat AD were eligible for participation in the trial. We defined difficult to treat as use of at least a class 3 topical corticosteroid and not being able to step down, or current use of systemic immunosuppressive treatment, or repeated treatment with potent topical corticosteroids or systemic immunosuppressive treatment, or a history of use of systemic treatment, or a significant impact of AD on the child's or the families quality of life, or seemingly unresponsive to conventional therapy according to current guidelines. All patients and their parents provided written informed consent for participation in the trial, which was reviewed and approved by the Medical Ethics Committee of the University Medical Center Utrecht, the Netherlands (reference 09-192/K).



### Clinical outcome measures

Data regarding gender, age, asthma, allergic rhinitis, food allergy and use of topical or systemic medication during the treatment period were extracted from electronic patient files in the Wilhelmina Children's Hospital. Disease severity was measured with the objective Self-Administered Eczema Area and Severity Index (SA-EASI) by the research nurse prior to (T0) and after 6 weeks of treatment (T1).[42]

### Blood measurements

Total lymphocyte and eosinophil counts were determined with a Coulter cell counter (Beckman Coulter) within 24 hours of blood sampling. Serum Thymus- and activation-regulated chemokine (TARC) levels were measured with an Enzyme-Linked Immuno Sorbent Assay. Total serum IgE levels were measured with an ImmunoCAP 250 (Phadia) on T0. Peripheral blood mononuclear cells (PBMCs) of all patient samples were isolated by Ficoll-plaque density centrifugation, stored in liquid nitrogen, and used for 11-color flow cytometry. For this, one million PBMCs were incubated with antibody cocktails against B-cell or T-cell markers for 15 minutes on room temperature in 100µL total volume. Flow cytometric analyses were performed on a 4-laser LSRFortessa (BD Biosciences) using standardized measurement settings,[43] and data were analyzed using FacsDiva V8.0 (BD Biosciences). The absolute lymphocyte counts obtained from fresh samples were used to calculate absolute numbers of the CD3+ T-cell and CD19+ B-cell subsets.

### Statistical analysis

Statistical analysis was performed in SPSS version 21.0 (IBM Corp, Armonk, NY). The course of AD disease activity after treatment was determined by assessing the SA-EASI score on T1 versus T0 with the Paired Samples T-test. Changes in the absolute counts of T- and B-cell subsets after treatment in the total cohort or within the NAD and WKZ groups were analyzed with the Wilcoxon Signed Rank Test. Differences in kinetics of the T- and B-cell subsets before and after treatment between the NAD and WKZ groups were analyzed with Analysis of covariance. This analysis was corrected for possible pre-existing differences between the groups on T0 and the use of systemic medication during the treatment period. Systemic medication was defined as a dichotomous variable, with patients classified as using systemic medication when they had used prednisone and/or cyclosporine at any point during the six-week treatment period. P-values of <0.05 were considered statistically significant. Data analysis was supervised by a senior statistician.

## RESULTS

### Study subjects

A total of 88 patients were enrolled in the Davos trial (Supplementary Figure 1).[13] Of these, 79 started treatment; 38 in the alpine climate (NAD) and 41 in moderate maritime climate (WKZ). Two patients (both WKZ) did not complete treatment, and data from one or more blood samples were lacking from 13 patients. This resulted in a total of n=64 patients in this study: NAD group, n=31; WKZ group, n=33 (Supplementary Figure 1). Patient characteristics of children with missing blood samples did not differ from the study group. Patient gender and age were equally distributed in both groups; 51.6% males in the NAD group and 51.5% males in the WKZ group with a mean age of 12.7 years in both groups (Table 1). Most patients (85.9%) had one or more additional allergic comorbidities (Table 1). The use of topical corticosteroids before the start of treatment was similar between the groups, with most patients using a class III corticosteroid. In addition, 4 patients (12.1%) in the WKZ group used cyclosporine at T0, whereas none did in the NAD group (Table 1).

### Disease activity decreases after treatment

In the total study cohort (n=64), we observed a reduction of the SA-EASI score. The median score of 34.4 out of 96 before the start of treatment (T0) decreased to a median score of 6.0 after treatment (T1) (P <.001; Figure 1A). SA-EASI scores before treatment were similar between both treatment groups (median score NAD 31.4; WKZ 39.15, P 0.82). Both treatments resulted in significantly reduced SA-EASI scores at T1, yet the decrease was significantly greater in the NAD group than in de WKZ group (P <.01). This resulted in a significantly

**Table 1.** Baseline Patient Characteristics

| Characteristics | | Total group (n = 64) | NAD group (n = 31) | WKZ group (n = 33) |
|---|---|---|---|---|
| Gender | | | | |
| Male | *n (%)* | 33 (51.6) | 16 (51.6) | 17 (51.5) |
| Female | *n (%)* | 31 (48.4) | 15 (48.4) | 16 (48.5) |
| | | | | |
| Age (years) | *mean ± SD (range)* | 12.7 ± 2.4 (8 – 18) | 12.7 ± 2.4 (8 – 16) | 12.7 ± 2.5 (8 – 18) |
| | | | | |
| SA-EASI (<17 mild; 18-46 moderate; >47 severe) | *mean ± SD (range)* | 37.2 ± 19.9 (3.8 – 79.8) | 36.7 ± 20.8 (5.4 – 79.8) | 37.6 ± 19.3 (3.8 – 77.0) |
| | | | | |
| Other atopic diseases | | | | |
| Asthma | *n (%)* | 55 (85.9) | 27 (87.1) | 28 (84.8) |
| Allergic rhinitis | *n (%)* | 55 (85.9) | 27 (87.1) | 28 (84.8) |
| Food allergy | *n (%)* | 43 (67.2) | 21 (67.7) | 22 (66.7) |
| | | | | |
| Sensitization profile† | | | | |
| Birch | *n (%)* | 51 out of 60 (85.0) | 24 out of 31 (77.4) | 27 out of 29 (93.1) |
| Timothy | *n (%)* | 46 out of 60 (76.7) | 23 out of 31(74.2) | 23 out of 29 (79.3) |
| Mugwort | *n (%)* | 32 out of 60 (53.3) | 17 out of 31 (54.8) | 15 out of 29 (51.7) |
| House dust mite | *n (%)* | 54 out of 60 (90.0) | 28 out of 31 (90.3) | 26 out of 29 (89.7) |
| Cat | *n (%)* | 46 out of 59 (78.0) | 22 out of 31 (71.0) | 24 out of 28 (85.7) |
| Dog | *n (%)* | 55 out of 60 (91.7) | 26 out of 31 (83.9) | 29 out of 29 (100) |
| Common food allergens | *n (%)* | 57 out of 62 (91.9) | 22 out of 31 (71.0) | 25 out of 31 (80.6) |
| | | | | |
| Topical corticosteroid use on T0 | | | | |
| No topical medication | *n (%)* | 1 (1.6) | 0 (0.0) | 1 (3.0) |
| Hydrocortisone | *n (%)* | 0 (0.0) | 0 (0.0) | 0 (0.0) |
| Triamcinolone/Emovate/ Tacrolimus | *n (%)* | 3 (4.7) | 1 (3.2) | 2 (6.1) |
| Cutivate/Elocon | *n (%)* | 23 (35.9) | 16 (51.6) | 7 (21.2) |
| Betnelan/Betamethasone | *n (%)* | 33 (51.6) | 13 (41.9) | 20 (60.6) |
| Dermovate | *n (%)* | 4 (6.3) | 1 (3.2) | 3 (9.1) |
| Systemic medication use on T0 | | | | |

† for these parameters some patients had missing values

**Table 1.** Continued.

| Characteristics | | Total group (n = 64) | NAD group (n = 31) | WKZ group (n = 33) |
|---|---|---|---|---|
| No systemic medication | *n (%)* | 59 (92.2) | 29 (93.5) | 29 (87.9) |
| Prednisone | *n (%)* | 1 (1.6) | 1 (3.2) | 0 (0.0) |
| Cyclosporine | *n (%)* | 4 (7.8) | 0 (0.0) | 4 (12.1) |
| | | | | |
| serum TARC (<510 pg/ml) | *median (range)* | 1080.5 (170.0 – 14100.0) | 1149.0 (170.0 – 7217.0) | 861.0 (339.0 – 14100.0) |
| | | | | |
| Eosinophils (0.03-0.35x $10^9$/L) | *median (range)* | 0.62 (0.10 – 1.80) | 0.61 (0.18 – 1.37) | 0.51 (0.10 – 1.80) |
| | | | | |
| Total IgE (<700 U/ml ) | *median (range)* | 2849.0 (150.0 - 17260.0) | 2872.0 (150.0 - 16718.0) | 2614.0 (150.0 - 16718.0) |

† for these parameters some patients had missing values

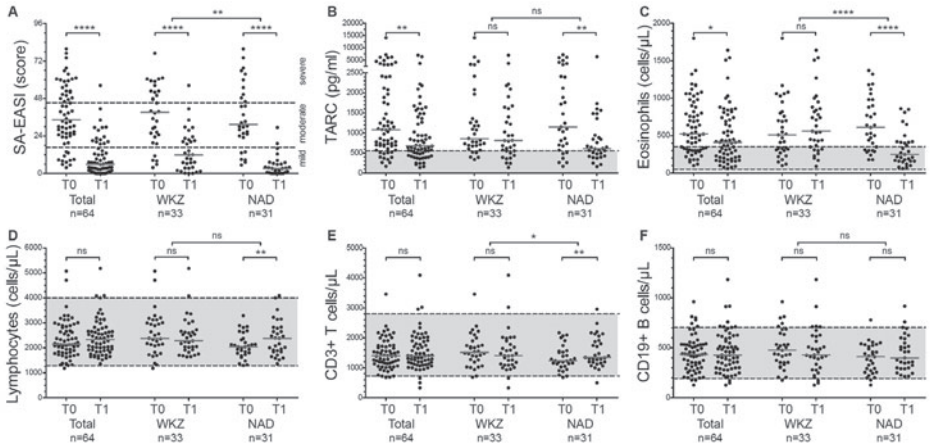


**Figure 1.** Six weeks of multidisciplinary treatment both in an alpine climate (NAD) and at moderate maritime climate (WKZ) decrease disease severity with only the former resulting in lower blood eosinophil numbers. A. Self-Administered Eczema Area and Severity Index (SA-EASI). B. Thymus and activation regulated chemokine (TARC). C. Eosinophils. D. Total lymphocytes E. Total T-cells F. Total B-cells. T0, baseline; T1, 6 weeks. Each dot represents one individual, and red lines median values. Dashed lines with grey surface indicate reference values. Statistical analysis between the groups was performed with the Wilcoxon signed rank test, analysis between the groups was performed with analysis of covariance. *P<0.05, **P<0.01, ***P<0.001, ****P<0.0001

lower median SA-EASI score in the NAD group at T1 (median of 3.2) than in the WKZ group (median 11.9) (Figure 1A).

Serum TARC levels varied considerably but correlated significantly with the SA-EASI score on T0 and T1 (Supplemental Figure 2). TARC levels also decreased after treatment, but were differently affected by treatment protocols (Figure 1B). A significant decrease was observed in the NAD group with a median of 1149.0 pg/ml at T0 and a median of 610 pg/ml at T1 (P <.01), whereas the serum TARC levels were not significantly lower after treatment in the WKZ group.

### Reduction of eosinophils and increase in T-cells after alpine climate treatment

Numbers of blood eosinophils decreased after alpine climate treatment; from a median of 610 cells/μL at T0 to 250 cells/μL at T1 (P <.001) (Figure 1C). The reduction in eosinophil numbers in the NAD group was accompanied by a significant increase in lymphocyte numbers, from a median of 2040 cells/μL at T0 to 2380 cells/μL at T1 (P <.01) (Figure 1D). This effect was significantly different from the WKZ group, in which both eosinophil and lymphocyte numbers did not change after treatment (Figure 1C and D). Within total lymphocytes, specifically T-cells were affected in the NAD group with an increase from a median of 1236.9 cells/μL before treatment to 1345.7 cells/μL after treatment (P <.01) (Figure 1E). Total B-cell numbers did not change following either treatment (Figure 1F). Although patients were randomized to a treatment protocol, the total lymphocyte counts were significantly different between the WKZ and NAD groups at T0 (P <.05). This difference could not be explained by patient characteristics or technical work-up as these were similar between both groups. Therefore, we corrected for pre-existing differences between the groups on T0 in our statistical analysis.

### Blood T- and B-cell subsets are affected by treatment

To study the immune system in more detail we focused on total and treatment-group specific effects on subsets within the T-cell and B-cell lineages. Within the CD3+ T-cell compartment, naive (Tnaive), central memory (Tcm), CD45RA- effector memory (TemRO) and CD45RA+ effector memory (TemRA) populations were defined in both the CD4+ and the CD8+ lineages (Supplemental Figure 3).[44] Naive T cell numbers were not affected by therapy. After treatment, absolute numbers of CD8+ TemRO T-cells were increased in the NAD group (median at T0 of 108.2 cells/μL and median at T1 of 123.5 cells/μL; P <.05) (Supplemental Figure 3A). Other CD8+ T-cell subsets were not affected by treatment.

### Treatment affects Th2 and memory Treg cells

CD4+ Tcm were differentially affected by treatment (P <.05); the absolute numbers decreased slightly in the WKZ group (from a median of 189 cells/μL to 167.2 cells/μL; P =0.5), and significantly increased in the NAD group (median T0 136.9 cells/μL and median

T1 171.9 cells/μL; P <.01) (Supplemental Figure 3B). Other memory CD4+ T-cell subsets did not significantly change with treatment in either of the treatment groups (Supplemental Figure 3B).

Next, we studied if Th subsets were affected by treatment. Th1, Th2 and Th17 cells were defined within the CD45RA- memory compartment based on differential expression of the chemokine receptors CCR4, CCR6 and CXCR3 (Figure 2A and B).[45] Whereas no effects of treatment were found on Th1 and Th17 cells, numbers of Th2 cells were significantly different after treatment. Importantly, treatment of the WKZ group in moderate maritime climate reduced Th2 cell numbers (from a median of 84.9 cells/μL to 75.8 cells/μL; P <.05), whereas alpine climate treatment of the NAD group resulted in a (non-significant) increase of Th2 cells (from a median of 78.5 cells/μL to 89.0 cells/μL; P 0.09) (Figure 2A). As a result, the treatments also had significantly different effects on the Th1/Th2 cell ratios between the WKZ and the Davos group (P <.05) (Figure 2A). To further investigate the increase in Th2 cells after alpine climate treatment we analyzed the expression of the lymphoid homing receptor CCR7. We found that CCR7+ Th2 cells attributed most to the increase of peripheral Th2 cells in the NAD group (Figure 2C), in line with the total increase in CD45-CCR7+ Tcm cells (Supplementary Figure 3). The reduction of Th2 cells in the WKZ group was the result of a decrease of both the CCR7+ and CCR7- subsets (Figure 2C).

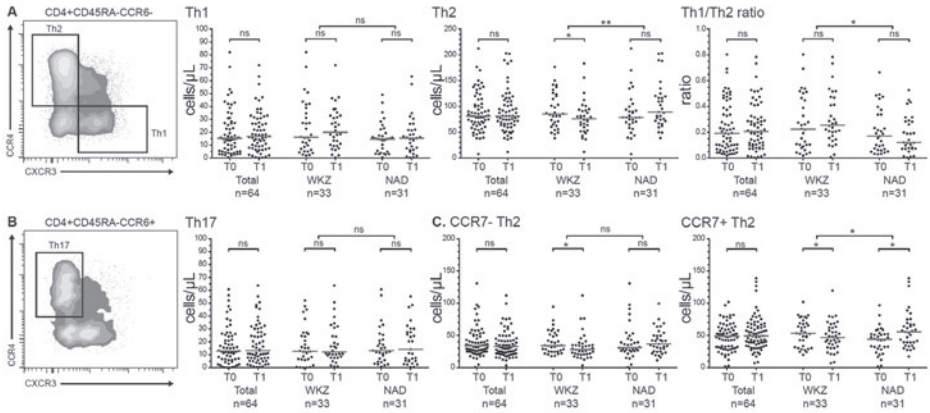


**Figure 2.** Decreased Th2 cell numbers following treatment at moderate maritime climate. A. Flowcytometric analysis of Th1 and Th2 cells. B. Flowcytometric analysis of Th17 cells. C. Absolute counts of the CCR7- and CCR7+ Th2 cell subsets. Each dot represents one individual, and red lines median values. Statistical analysis between the groups was performed with the Wilcoxon signed rank test, analysis between the groups was performed with analysis of covariance. *P<0.05, **P<0.01

Regulatory T cells were studied based on the phenotype CD4+CD25+CD127- (Figure 3A).[45] Numbers of total Tregs decreased slightly but not significantly after treatment (Figure 3B). However, numbers of CD45RA- memory Tregs significantly decreased following

treatment, irrespective of the type of treatment (P <.01; Figure 3D), whereas numbers of naive Tregs did not change after treatment (Figure 3C).

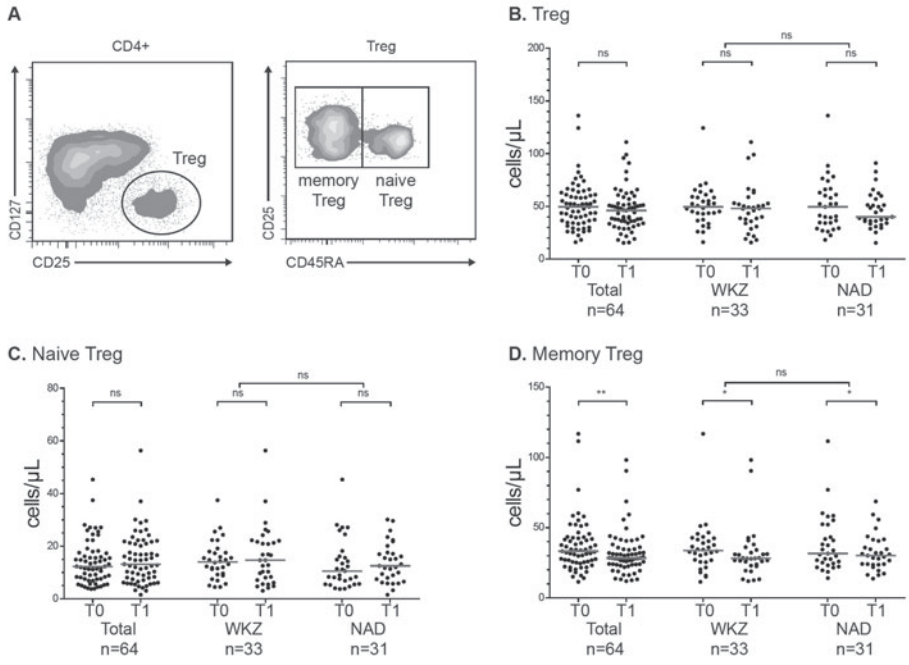


**Figure 3.** Reduction in memory regulatory T-cells (Tregs) following treatments both at moderate maritime and in an alpine climate. A. Gating strategy for detection of Tregs B. Absolute counts of total Tregs. C. Absolute counts of naive Tregs (Treg CD45RA+) D. Absolute counts of memory Tregs (Treg CD45RA-). Each dot represents one individual and red lines median values. Statistical analysis between the groups was performed with the Wilcoxon signed rank test, analysis between the groups was performed with analysis of covariance. *P<0.05, **P<0.01

## Decreased transitional B-cell and plasmablast numbers after treatment

Within CD19+ B-cells, multiple naive and memory B-cell subsets and plasmablasts were defined (Figure 4A and 5A). Numbers of $CD21^{low}$ and naive mature B-cells did not change after treatment (Figure 4B). However, numbers of transitional B-cells (P <.01) and plasmablasts (P <.05) were significantly reduced in the total cohort (Figure 4B). From the plasmablast subsets, only IgE+ plasmablasts significantly decreased after alpine climate treatment (Supplemental Figure 4). Transitional B-cell numbers were also solely affected by high alpine treatment (NAD; P <.01), whereas the slight decreases in plasmablast numbers were not significant when treatments were analyzed separately. When comparing alpine climate treatment and moderate maritime climate, an opposing effect on natural effector B-cell numbers was found (P <.05), with treatment at maritime climate leading to a decrease and alpine climate leading to an increase (Figure 5B).

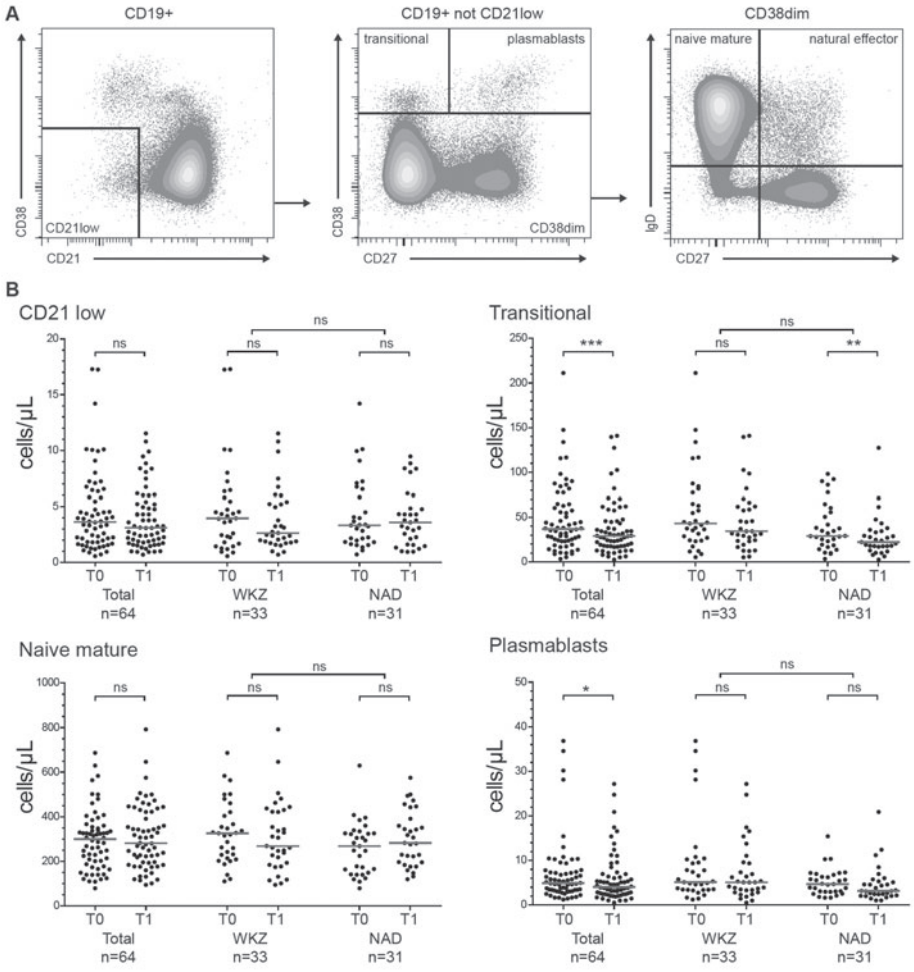


**Figure 4.** Reduced numbers of blood transitional B-cells and plasmablasts following treatment. A. Flow cytometric gating strategy of major B-cell subsets. B. Absolute counts of major B-cell subsets. Each dot represents one individual, and red lines median values. Statistical analysis within the groups was performed with the Wilcoxon signed rank test, analysis between the groups was performed with analysis of covariance. *P<0.05, **P<0.01, ***P<0.001

## Increased IgM, IgG and IgA memory B cell numbers following alpine climate treatment

Within the memory B-cell compartment, we defined 8 distinct subsets based on differential expression of the 5 Ig isotypes and CD27 (Figure 5A).[33, 46, 47] Of these 8 subsets, only numbers of IgG+CD27+ memory B cells were significantly increased after treatment (P <.05). This effect

was significantly greater following alpine climate treatment than treatment at maritime climate (P <.05). In addition, alpine climate treatment led to significantly increased numbers of Natural Effector (P <.05), IgA+CD27- (P <.05) and IgA+CD27+ (P <.05) memory B cell subsets, as compared to maritime climate treatment (Figure 5B-E). No effects were found on either CD27+ or CD27- IgE-expressing memory B cells. Thus, treatment resulted in reduced transitional B cells and plasma cells, whereas alpine climate treatment specifically led to slightly increased numbers of IgA and IgG memory B cells. A summary of the significant effects of treatment on the various outcomes are displayed in Table 2.

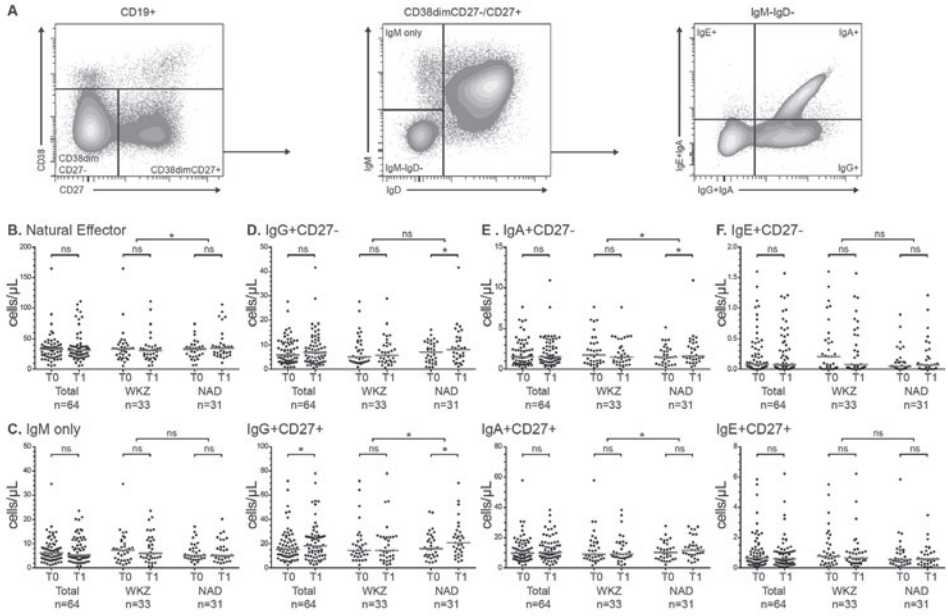


**Figure 5.** Increased numbers of CD27-IgG+, CD27+IgG+ cells and CD27-IgA+ memory B-cell numbers following treatment in alpine climate. A. Flow cytometric gating strategy of memory B-cell subsets. B. Absolute counts of CD27+IgM+IgD+ Natural Effector B-cells. C. Absolute counts of IgM-only B-cells D. Absolute counts of CD27- and CD27+ IgG memory B-cells. E. Absolute counts of CD27- and CD27+ IgA memory B-cells. F. Absolute counts of CD27- and CD27+ IgE memory B-cells. Each dot represents one individual, and red lines median values. Statistical analysis between the groups was performed with the Wilcoxon signed rank test, analysis between the groups was performed with analysis of covariance. *P<0.05

**Table 2.** Summary of significant effects after treatment

| Parameter | WKZ | NAD | Different effects between groups |
|---|---|---|---|
| | | | |
| SA-EASI | ↓ | ↓ | * |
| TARC | - | ↓ | |
| Eosinophils | - | ↓ | * |
| | | | |
| T-cell subsets | | | |
| Total CD3+ T-cells | - | ↑ | * |
| CD8+ | - | ↑ | |
| CD8+ TemRO | - | ↑ | |
| CD4+ Tcm | - | ↑ | * |
| Memory Tregs | ↓ | ↓ | |
| Th2 | ↓ | - | * |
| Th2 CCR7- | ↓ | - | |
| Th2 CCR7+ | ↓ | ↑ | * |
| | | | |
| B-cell subsets | | | |
| Transitional | - | ↓ | |
| IgA+CD27- memory | - | ↑ | * |
| IgG+CD27- memory | - | ↑ | |
| IgG+CD27+ memory | - | ↑ | * |
| IgE+ plasmablasts | - | ↓ | |

↑ indicates significant increase; ↓ indicates significant decrease; - indicates no change;
* indicates significant different effect between groups after treatment

WKZ, outpatient treatment at moderate maritime climate; NAD inpatient treatment at alpine climate

## DISCUSSION

We here report that clinically successful treatment protocols of children with atopic dermatitis, either at maritime climate (outpatient treatment) or at alpine climate (inpatient treatment), differentially affect the immune system. Reduced disease activity after 6 weeks of treatment quantified by SA-EASI in both groups, and by serum TARC levels after alpine climate treatment, was accompanied by a decrease in circulating memory Tregs, transitional B-cells and plasmablasts. Moreover, 6 weeks of alpine climate treatment resulted in significantly lower disease activity scores and blood eosinophil counts than treatment in

moderate maritime climate, and was associated with higher numbers of Th2 and memory B cells (for summary of significant effects see Table 2). Thus, inpatient alpine climate treatment appears to have unique effects on the patient's immune system.

A notable reduction was found in memory Treg cell numbers following therapy, irrespective of the treatment protocol. Patients with AD have increased numbers of Tregs in blood as compared to healthy controls.[26-28, 48] Furthermore, Treg numbers positively correlate with AD disease activity.[30] It is thought that the increase in Treg numbers is a response to the chronic inflammation, and the numbers go down upon suppression.[29, 49] Although it cannot be excluded that therapy induces a Treg influx into the skin and thereby locally reduces skin inflammation. Still, considering the decrease in disease activity following treatment in our patients, the reduction in Treg numbers seems a reflection of successful treatment. Although it is not entirely sure if Tregs migrate to skin. Hence, Treg cell numbers could be a good general marker for treatment evaluation.

In addition to Tregs, transitional B-cells and plasmablasts also decreased after treatment. Transitional B-cells are recent bone marrow emigrants that can further develop into naive mature B-cells.[46] These functionally immature cells respond poorly to IgM stimulation and can exert regulatory functions via the production of IL-10, thereby dampening immune responses and inflammation.[50, 51] Transitional B-cells are reported to be expanded in patients with AD, although reports on this are not conclusive.[32, 47] Similar to memory Tregs, the decline in transitional B-cells can be the result of a decrease in disease activity. It remains to be studied if treatment directly affects survival of transitional B cells or the B-cell output from the bone marrow.

Plasmablasts are antibody producing cells that are mostly short-lived and can be precursors to long-lived plasma cells. Although our measurements were performed after freezing and thawing of cells, which could affect plasmablast numbers, there are no indications that study samples are differently affected by this. In the case of AD, where high levels of allergen specific IgE is one of the hallmarks of the disease, plasma cells are potentially important contributors to the disease.[32] Blood plasma cell numbers increase during active immune responses, as illustrated by acute infections and following vaccinations.[52, 53] Our finding that plasmablast numbers decrease upon treatment are in line with the previously established correlation with active inflammation. In addition, we stained plasmablasts for IgE, IgA and IgG and found that the largest reduction was found in IgE+ plasmablasts. However, since surface immunoglobulin staining of plasmablasts is controversial the latter result must be interpreted with some caution, although other studies also report high numbers of IgE+ plasmablasts in patients with AD.[32]

Besides the common effects of treatment, we also observed several differences in B- and T- cell subsets between the two treatments. The significantly larger decreases in SA-EASI and serum TARC levels after alpine climate treatment were accompanied by an increase in total CD3+ T-cells. This was mainly due to increased numbers of CD8+ TemRO T-cells

and T-helper 2 cells. As these are memory T cell subsets, their increased numbers could reflect increased maturation following an immune response. However, this is unlikely as the patients showed decreased disease activity and inflammation. An alternative cause for their increased numbers in blood is their re-localization from tissue. The skin harbors large numbers of immune cells, and especially Th2 cell numbers are increased in AD lesions.[16] In addition, CD8+ T-cells have been found to infiltrate lesional skin of patients with AD.[54, 55] This can even result in decreased numbers of blood CD8+ T-cells and a higher CD4/CD8 ratio than healthy controls.[56] The increase of CD8+ TemRO and CD4+ Th2 cells following alpine climate treatment could reflect the normalization of AD skin lesions and redistribution of the memory T cells to the peripheral blood.[57-59]

Another hypothesis could be that these patients with chronic AD were skewed towards Th1 dominance prior to treatment. Following treatment in the alpine climate with reduced disease provoking allergens, this chronic Th1 response may have subsided and the immune system could have reverted to the original state that was prone to Th2 predominance.[60] Due to the reduced allergenic pressure in the alpine climate the Th2 response is minimal and markers of the Th2 response, such as TARC and eosinophils, are reduced. This hypothesis would also explain the lack of long term differences between alpine climate treatment and the outpatient treatment program at moderate maritime climate.[13] Treatment in the NAD group may have dampened inflammation, but not have reset the immune system. Rather, our results show that the immune response remained skewed towards Th2, which could underlie the renewed inflammation upon return to maritime climate with higher allergen exposure.

In addition to memory T cells, alpine climate treatment also resulted in increased memory B-cell numbers. IgA+CD27-, IgG+CD27- and IgG+CD27+ memory B-cells were significantly increased, and the effects on natural effector and IgA+CD27+ memory B cells were significantly different from treatment at moderate maritime climate. Since almost 80% of our patient population was additionally diagnosed with asthma, it could be speculated that the IgA+ memory B-cells may have migrated from the lungs into the peripheral circulation after clinical improvement of asthma. This is supported by additional analyses demonstrating that improvement of asthma, measured as lower exhaled nitric oxide levels, correlated with an increase of IgA+CD27- and IgA+CD27+ memory B-cells in peripheral blood. Yet, we here did not directly investigate any skin homing markers on B-cell subsets. Importantly, despite the observed effects on memory B cells, IgE+ B-cells were not affected by either treatment. As the CD27-IgE+ subset has been shown to be significantly increased in patients with AD,[33] the current data suggests that these numbers are not normalized following intensive treatment for 6 weeks. Since the favorable outcome of alpine climate treatment on disease severity did not persist until 6 months after treatment,[13] our observed effects on the immune system are most likely a result of reduced disease activity rather than a cause for reduction in disease severity.

However explanatory studies are needed to identify the main responsible factor for the observed improvement since there was a difference in treatment setting in both study arms. Beneficial factors ascribed to treatment in the NAD group are the inpatient setting and the characteristics of the alpine climate, which include reduced air concentrations of allergens and pollutants and a higher exposure to UV radiation.[61] In asthma, and to a lesser extent AD, reduced allergen exposure has been linked to a decrease in peripheral eosinophilia.[62-67] Indeed also in our study, after treatment, blood eosinophils were significantly lower in the NAD group. Furthermore, UV radiation exposure can lead to immunosuppressive effects, and specifically can activate local apoptosis in T and B-cells and result in decreased cytokine productions involved in lymphocyte activation and trafficking.[68-70] However, direct sunlight exposure to the skin was limited and patients were protected with sun cream.

Both treatment protocols involved intensive treatment by multidisciplinary teams of healthcare professionals, and resulted in decreased disease activity scores after 6 weeks of treatment with sustained beneficial effects lasting for >6 months.[13] Based on the observed immunological changes and the greater clinical improvement in the alpine climate treatment group, in this study alpine climate treatment seems a more effective treatment on the short-term. In the present study we did not investigate the long-term effects on the immune compartment, but it would be interesting to study the effects 6 weeks and 6 months after treatment completion and observe if alpine climate treatment led to an altered peripheral immune system.



### Conclusion

Intensive treatment of children with moderate to severe AD affected blood T- and B- cell subsets. In addition to changes in Treg, transitional B cells and plasmablasts upon treatment in both a moderate maritime and an alpine climate, the latter also resulted in additional changes in circulating CD8+ TemRO, Th2 and memory B-cells.

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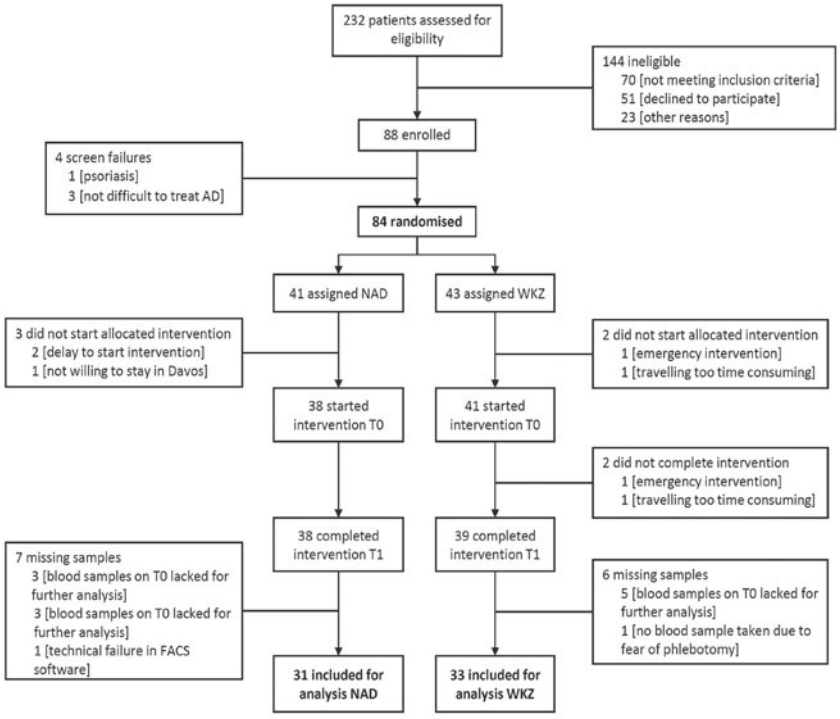
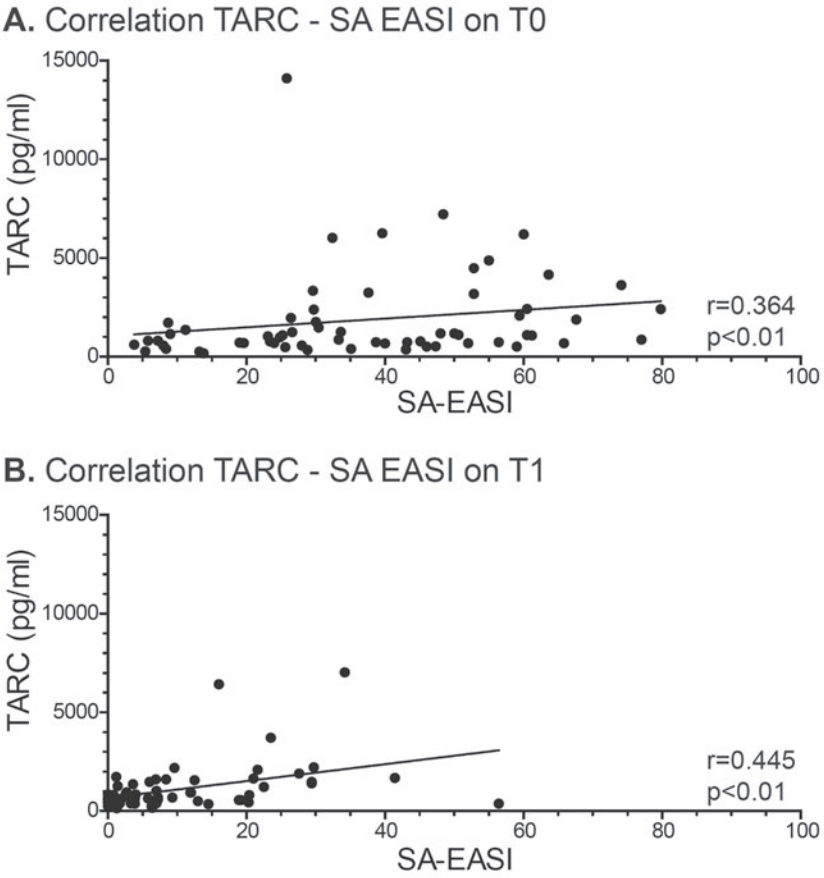
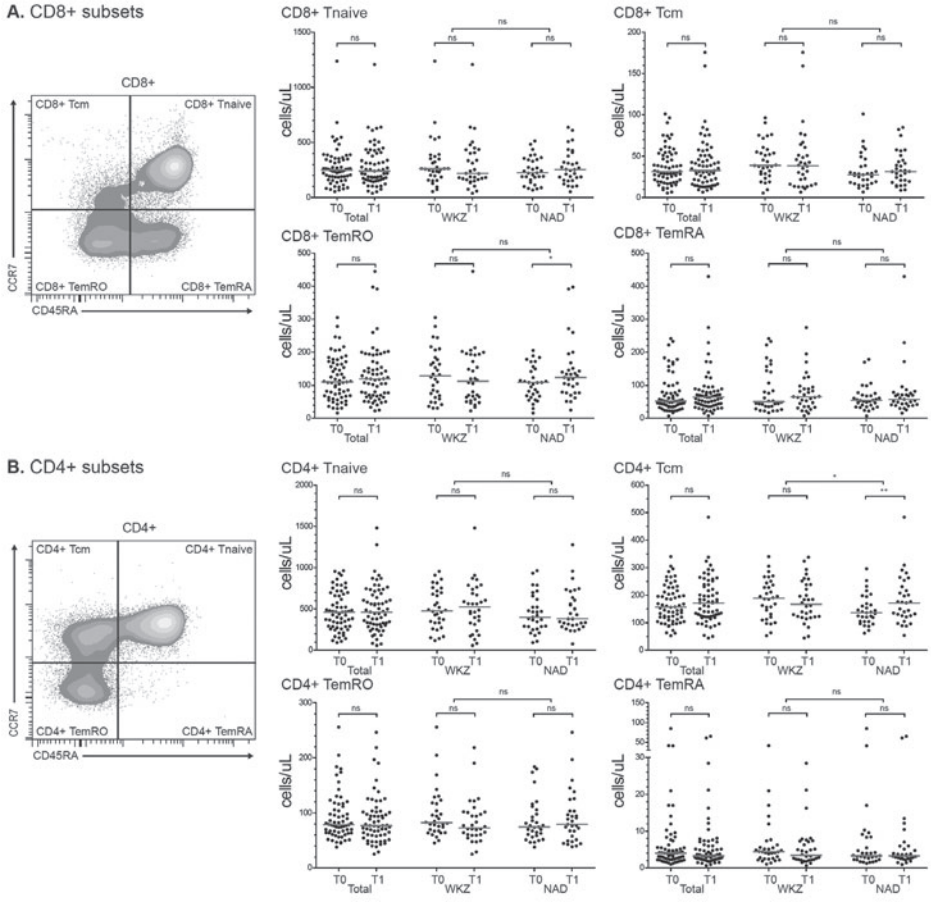
## SUPPLEMENTAL DATA



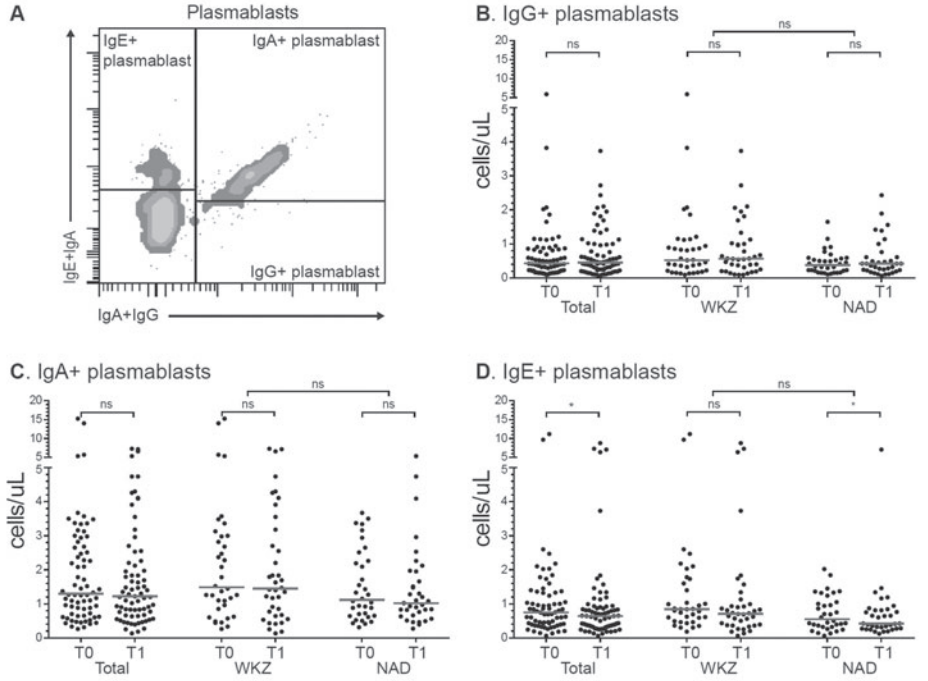

**Supplemental Figure 1.** Flowchart of patient selection. 232 patients were screened for eligibility. 84 patients were randomized to one of the treatment arms. Finally, 64 patients were included for analysis; 31 in the NAD group and 33 in the WKZ group.




**Supplemental Figure 2.** Correlation between serum Thymus- and activation- regulated chemokine (TARC) levels and Self-Administered Eczema Area and Severity Index (SA-EASI) scores on T0 (**A**) and T1 (**B**). Each dot represents one individual; black lines indicate linear correlations. Correlation was calculated with Spearman R.




**Supplemental Figure 3.** CD8+ and CD4+ naive and memory T-cell subsets. A. Representative plots of the flow cytometric analysis of CD8+ naive T-cells (CD8+ Tnaive), CD8+ central memory T-cells (CD8+ Tcm), CD8+ effector memory CD45RA- T-cells (CD8+TemRO) and CD8+ effector memory CD45RA+ T-cells (CD8+TemRA). B. Representative plots of the flow cytometric analysis and absolute counts of CD4+ Tnaive, CD4+ Tcm, CD4+TemRO and CD4+TemRA. Each dot represents one individual, and red lines median values. Statistical analysis between the groups was performed with the Wilcoxon signed rank test, analysis between the groups was performed with analysis of covariance. *P<0.05, **P<0.01




**Supplemental Figure 4.** Plasmablast subsets. A. Representative plots of the flow cytometric analysis of plasmablasts B. IgG+ plasmablasts C. IgA+ plasmablasts D. IgE+ plasmablasts Statistical analysis between the groups was performed with the Wilcoxon signed rank test, analysis between the groups was performed with analysis of covariance. *P<0.05

# CHAPTER 6

# Quick and lasting rise in IgG4+ memory B-cell frequencies following 4-month sublingual immunotherapy regimens for rye grass allergy

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*Manuscript in preperation*

## ABSTRACT

*Background* Treatment for atopic rhinitis is mostly aimed to relieve symptoms, only allergen specific immunotherapy (SIT) is targeted to resolve allergic sensitization. This results in prolonged desensitization, also when treatment has stopped. The immunomodulatory effects of SIT have been mainly attributed to increased regulatory T-cell function and increased serum IgG4, yet little is known about the effect on the memory B-cell compartment and its relation to various T-cell subsets.

*Methods* We here included 24 patients with atopic rhinoconjunctivits and performed a longitudinal analysis of the peripheral immune compartment before and during SLIT for grass pollen allergy. With flowcytometry on peripheral blood we analyzed the direct effects of a 4-month treatment regimen and the long term effects of two courses of treatment over 2 years on circulating IgE+ and IgG4+ memory B-cells, allergen-specific Ig levels and Th and Treg cell frequencies.

*Results* SLIT induced a direct increase in pollen-specific serum IgG4 accompanied by an increase in the frequency of IgG4+ memory B-cells, whereas no effect was observed on the IgE+ memory B-cell compartment. Furthermore, SLIT resulted in an increase in the frequency of regulatory T-cells. This was associated with clinical improvement of allergic complaints.

*Conclusion* Our data provides evidence for long lasting effects of sublingual immunotherapy on the memory compartment of the immune system. Increased numbers of regulatory T-cells lead to a higher frequency of IgG4+ memory B-cells and a beneficial shift in the IgG4+/IgE+ memory B-cell ratio, reflecting the increased IgG4/IgE antibody fraction in serum, which results in a favorable outcome.

## INTRODUCTION

Rhinoconjunctivitis and other IgE-mediated allergies are an increasing disease burden to the world population [1]. Most therapies intended to treat allergies are directed to relieve symptoms, but allergen-specific immunotherapy (SIT) is one of the few therapies aimed to resolve allergic sensitization. Subcutaneous immunotherapy (SCIT) and sublingual immunotherapy (SLIT) have both been proven to be an effective treatment for grass pollen induced rhinoconjuntivitis [2, 3]. The therapeutic effect is maintained until after the treatment has stopped [4-6], it can prevent the onset of new sensitizations [7], and it has the ability to reduce the development of asthma in patients with allergic rhinitis [8]. The immunomodulatory properties of SIT affect local and systemic immune responses, with an effect on the number and function of effector cells, antigen presenting cells (APCs), T cells and B cells [9, 10].

Allergic patients display sensitization by means of allergen specific IgE bound to effector cells, such as mast cells and basophils [11]. The underlying mechanism is thought to be a shifted T-cell balance towards a T helper 2 (Th2) phenotype, and these cells produce interleukin (IL)-4, IL-5 and IL-13 that direct allergen-specific B-cells to produce IgE [12]. Effective immunotherapy has been shown to reverse the Th2 dominance, and to result in anergy of allergen-specific T-cells [13, 14], induction of regulatory T-cells (Treg) [15-17] and production of blocking antibodies of the IgG isotype [18, 19]. Specifically, TGF-β and IL-10 produced by Tregs are pivotal for the successful immune deviation in immunotherapy [20, 21].

The tolerogenic functions of IL-10 are extensive, but mainly encompass the inhibition of mast cell activity [22], suppression of IL-5 production by Th2 cells [23] and cell death induction in eosinophils [24]. Furthermore, IL-10 in combination with IL-4 and IL-13 directs immunoglobulin class switching of B-cells to IgG4 instead of IgE [25]. Indeed, one of the known effects of SIT is an increase in allergen-specific serum IgG4 and an increased serum IgG4/IgE antibody ratio that is associated with successful outcome [26].

SCIT and SLIT have distinct immunomodulatory capacities that appear related to the different routes of administration. Sublingual administration results in fewer side effects, but shows less profound clinical and immunological efficacy than subcutaneous administration [2, 27]. SLIT treatment results in increased numbers of Foxp3+ Tregs both in the oral epithelium and in peripheral blood [19, 28]. Further systemic alterations are more diverse. Some studies report an initial increase in IgE serum levels, followed by a decrease after 1 month [29]. Furthermore, IgG4 and IgA serum levels are reported to increase directly after the start of therapy [29, 30]. However, other studies detected no systemic alterations with regard to allergen-specific lymphoproliferation, cytokine secretion or Ig serum levels [31, 32].

Since immunotherapy has been attributed to have long lasting beneficial effects, it is of specific interest to understand if this is the result of changes in immunological memory, i.e. memory B and T-cells. We here addressed this in 24 patients with moderate to severe seasonal allergic rhinitis, who were studied longitudinally before and during SLIT for grass

pollen allergy. We analyzed the direct effects of a 4-month treatment regimen and the long term effects of two courses of treatment over 2 years on circulating IgE+ and IgG4+ memory B-cells, allergen-specific Ig levels and Th and Treg cell frequencies.

## METHODS

### Study design

Using an open label longitudinal design, 30 subjects were included for treatment with the 5 grass pollen mix Oralair® sublingual tablets for a period of 4 months. The treatment with Oralair® involved placing the tablet under the tongue until complete dissolution (for at least 2 minutes) and then swallowing it. Treatment started 4 months before the grass pollen season (T1; May) and stopped at the start of the pollen season (T2; September). Grass pollen count data were collected to confirm the duration of the pollen season (School of Botany, University of Melbourne). The total treatment duration was approximately 4 months with a follow-up study visit 8 months after completion of the first year of treatment (T3) and 8 months after completion of the second year of treatment (T4). The treatment regimen was composed of an initiation treatment (first month of treatment, including a 3-day dose escalation) and a continuation treatment (second month onwards) as follows: First month (initiation treatment): day 1 – 1 tablet 100 IR (index of reactivity); day 2 - 2 tablets 100 IR; day 3 to day 30 – 1 tablet 300 IR. Month 2-4 (continuation treatment): day 31 to day 120–1 tablet 300 IR. Blood samples were collected at T1, T2, T3 and T4 (Fig 1).

### Patient samples

Patients were recruited from the Allergy Clinics, The Alfred, Melbourne, Australia and had well-characterized moderate to severe seasonal allergic rhinitis due to ryegrass pollen (RGP) allergy and with positive RGP-specific IgE (CAP-Pharmacia score ≥ 1), with or without mild stable asthma. Patients were stratified for disease severity according to current symptoms and presence of asthma. Exclusion criteria were a co-existing immunodeficiency, previous immunotherapy within the last five years, ongoing immunotherapy with other allergens and treatment with continuous oral corticosteroids. Subjects on treatment with β-blockers were excluded as ß-blockers can impair the response to treatment in the event of anaphylaxis. The use of usual medications for allergic rhinitis was permitted, including antihistamines and topical corticosteroids. Alfred Hospital Research and Ethics Committee approval and written informed consent from each patient were obtained prior to the start of inclusion.

### Quantification of allergen-specific IgE and IgG4

Serum was separated from clotted blood for analysis of total and RGP-specific IgE levels using the Pharmacia CAP system by the Alfred Pathology service and for RGP-specific IgG4 antibodies by in-house ELISA as described previously [33].

### Flowcytometry

Peripheral blood mononuclear cells (PBMCs) were isolated within 24hrs of blood collection by Ficoll-plaque density centrifugation and stored in liquid nitrogen. One million thawed PBMCs were incubated with antibody cocktails against B-cell or T-cell markers for 15 minutes on room temperature in 100µL total volume. Flow cytometric analyses were performed on a 4-laser LSRFortessa (BD Biosciences) and data were analyzed using FacsDiva V8.0 (BD Biosciences).

### Statistical analysis

Differences in kinetics in disease scores, Ig serum values and of B- and T-cell subsets before, during and after treatment were analyzed with the Wilcoxon Signed Rank Test. All analyses were two-tailed and were considered statistically significant if *P*-values were lower than 0.05. Statistical analysis was performed using GraphPad Prism software, version 6 (GraphPad Software, La Jolla, CA).



## RESULTS

### Patient characteristics

Twenty-eight patients were included in the study, with a mean age of 35 and a range between 18-59 years. There was an equal gender distribution between males (n=15) and females (n=13). All patients had detectable RGP-specific IgE levels, with a mean CAP-Pharmacia score of 437U/ml (**Table 1**). Blood samples for flowcytometric analysis were obtained from n=24 patients at T1, n=21 at T2, n=23 at T3 and n=14 at T4.

### SLIT has a positive effect on symptoms and disease scores

To study the clinical effects of SLIT we measured symptom and disease scores. Before the start of treatment patients had a median symptom score of 14 and a median disease score of 8 (out of 10). After the first year of treatment patients experienced fewer symptoms after the hay fever season (median symptom score 4, $p < 0.05$), which was also reflected in a decreased disease score (median 4, $p<0.05$) (Fig 1B and C). After the second year of treatment the symptom score remained low (median 4), whereas the disease score continued to decrease (median 2) (Fig 1B and C). Thus SLIT resulted in beneficial clinical outcomes after 1 and 2 years of treatment.

**Table 1.** Patient characteristics

| Patient | Gender | Age (yrs) | RGP SPT (mm) | Total IgE (U/mL) | Rye RAST | Disease score (VAS scale 1-10) | Follow up T2 | T3 | T4 |
|---|---|---|---|---|---|---|---|---|---|
| 1 | M | 19 | 6 | **1000** | 68,9 | 6,5 | + | + | + |
| 2 | F | 37 | 10 | **328** | 100 | 8,7 | + | + | + |
| 3 | M | 32 | 10 | **409** | 54,4 | 9 | + | + | - |
| 4 | M | 38 | 20 | **298** | 69,1 | 9,8 | + | + | + |
| 5 | M | 21 | 20 | **886** | 100 | 9 | + | + | - |
| 6 | F | 55 | 5 | 6 | 0,43 | 7 | + | + | - |
| 7 | F | 18 | 10 | 14 | 4,8 | 2 | + | + | - |
| 8 | M | 48 | 7 | 38 | 2,72 | 3 | + | + | - |
| 9 | M | 29 | 5 | **191** | 3,95 | 9 | + | + | + |
| 10 | M | 34 | 10 | 35 | 13,6 | 8 | + | + | + |
| 11 | F | 19 | 15 | **3271** | 100 | 2 | + | + | + |
| 12 | F | 34 | 6 | **293** | 100 | 7 | + | + | + |
| 13 | F | 23 | 5 | **336** | 100 | 10 | + | + | - |
| 14 | M | 37 | 7 | **138** | 64,4 | 6 | + | + | + |
| 15 | F | 34 | 7 | **690** | 100 | 10 | + | + | - |
| 16 | F | 29 | 15 | **144** | 100 | 8 | + | + | - |
| 17 | M | 42 | 6 | 64 | 16,7 | 2 | + | + | + |
| 18 | F | 59 | 3 | **1212** | 6,7 | 7 | - | + | + |
| 19 | F | 53 | 10 | **202** | 34,1 | 9,5 | + | + | - |
| 20 | M | 37 | 7 | 35 | 16 | 9 | - | + | + |
| 21 | F | 39 | 6 | **576** | 100 | 5 | + | + | + |
| 22 | M | 25 | 9 | 60 | 10,7 | 7,5 | + | + | - |
| 23 | F | 40 | 10 | **153** | 63,9 | 10 | - | - | + |
| 24 | M | 40 | 12 | **114** | 21,2 | 10 | + | + | + |
| | | | | | | | n=21 | n=23 | n=14 |

RGP, rye grass pollen; SPT, skin prick test; RAST, radioallergosorbent test; VAS, visual analogue scale

## Increased IgG4 serum levels and IgG4+ memory B-cell frequencies after 4 months SLIT

To study the direct effects of SLIT on the immune system, we analyzed immunoglobulin serum levels and B- and T-cell subsets before and directly after the first four months of therapy. We observed that after 4 months of immunotherapy all patients showed an increase in RGP-specific serum IgG4 from a median of 0.37g/L before treatment (T1) to 1.16g/L after treatment (T2) (Fig 2A). This was accompanied by a significant increase in the frequency of

IgG memory B-cells expressing IgG4 (Fig 2B). The increase in IgG4+ memory B-cells was not directly correlated to the increase in RGP-specific serum IgG4 (Fig 2A). SLIT did not change IgE+ memory B-cell frequencies. Still the increase in IgG4+ memory B-cells resulted in a significantly higher IgG4+/IgE+ memory B-cell ratio following 4 months of treatment (Fig 2D). Nearly all other B-cell subsets, including transitional, naive mature, memory B cells and plasma blasts remained unchanged after 4 months of SLIT, except for a decrease of IgA+CD27+ memory B-cells. Thus, 4 months SLIT quite specifically affected allergen-specific IgG4 serum levels and the frequencies of IgG4-expressing memory B cells.

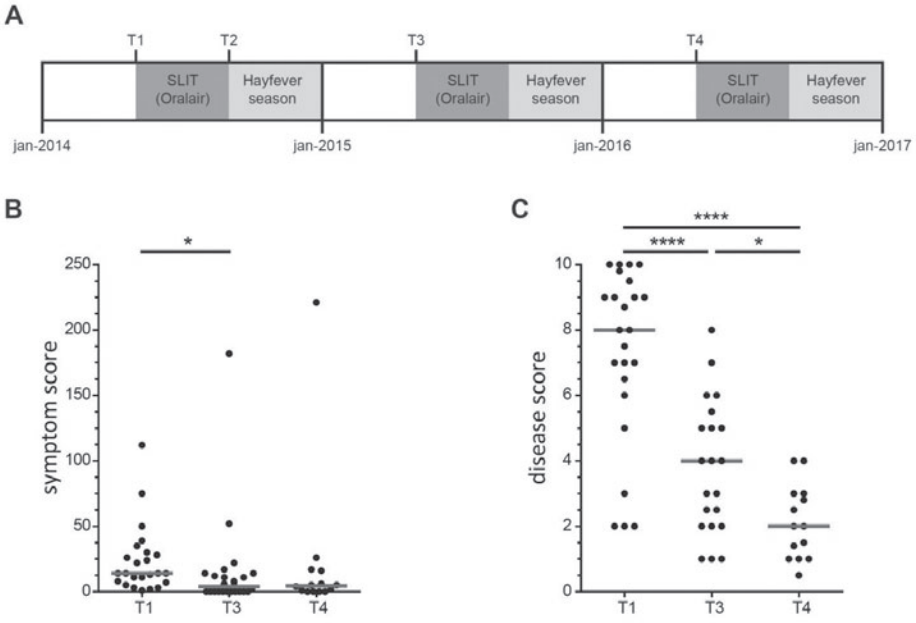


**Figure 1.** Study design and clinical outcomes **A**. Treatment scheme. **B**. Symptom score. **C**. Disease score. T= timepoint. Each dot represents one individual, and red lines median values. Statistical analysis was performed with Mann Whitney *U* test. *P<0.05, ****P<0.0001

Because IgG4 class switch recombination is regulated by Th cells and their cytokines, we subsequently analyzed the patients' T-cell compartments. Within the CD4 and CD8 T-cell subset, naive T-cell frequencies were increased after 4 months of SLIT. This was accompanied by a decrease in the frequency of central memory T-cells (Tcm) within CD4+ T-cells and a decrease in effector memory RO (TemRO) in CD8+ T-cells. Since these are relative changes, the latter two observations may be indirect observations due to the increased numbers of naive T-cells. Within CD4+ T-cells, frequencies of Th1, Th2, Th17 and Tregs did not change after 4 months of SLIT (Fig 2E). Furthermore, within the Treg subset there was no change in ratio of those with a naive over a memory phenotype. However, follicular helper T-cell

(Tfh) frequencies were decreased following SLIT. Thus, the increase in IgG4+ B-cells is not accompanied by direct changes in Th subsets or regulatory T-cells.

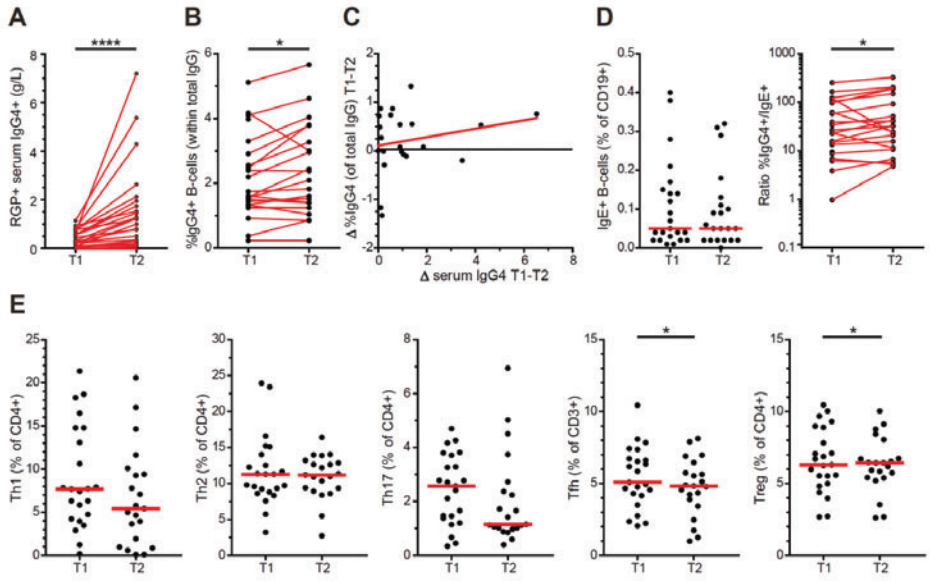


**Figure 2.** 4 months of SLIT affects serum IgG4 levels and numbers of circulating IgG4-expressing memory B cells and Tfh cells. **A** RGP specific serum IgG4 **B**. Relative percentage of IgG4 memory B cells within the IgG memory B-cell compartment **C**.Correlation between increase in serum IgG4 and percentage of IgG4+ memroy B-cells **D.** IgE+ B cells **E.** Thelper and regulatory subsets. Each dot represents one individual; red lines indicate median values; and red line in panel C indicates linear correlations. Statistical analysis was performed with Mann Whitney *U* test. *P<0.05, ****P<0.0001. Correlation was calculated with Spearman R.

## SLIT has persistent long term effects on IgG4 memory B-cells and regulatory T cells.

In addition to direct effects, we studied the long term treatment effects of SLIT, i.e. one year after start of the first treatment course (T3) and 1 year after the second (T4). SLIT resulted in higher RGP specific serum IgG4 levels after the first year of treatment (T3) with a further increase after the second year of treatment (T4) (Fig 3A). The frequencies of IgG4+ memory B-cells were not significantly different between T1 and T3, these were significant between T1 and T4 (Fig 3A). SLIT had no long term effects on IgE+ memory B-cell frequencies nor on total serum IgE levels and IgE Rye RAST (Fig 3B). The remainder of the B-cell compartments remained mostly similar during treatment follow-up, including the IgA+CD27+ memory B-cells which were found to be decreased after 4 months of SLIT. Two courses of SLIT did result in increased frequencies of IgG+CD27- memory B-cells and decreased marginal zone B-cell frequencies.

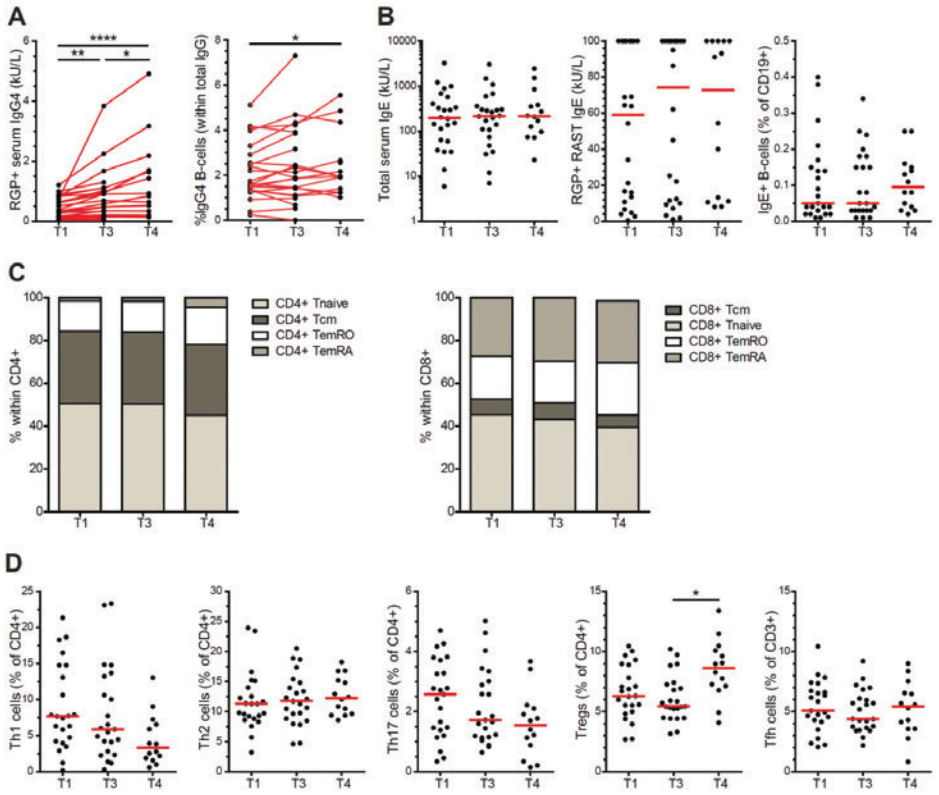


**Figure 3.** Persistent increase in IgG4 and late rise in Tregs after two 4-month SLIT courses. **A.** Serum IgG4 and percentage of IgG4+ memory B-cells. **B.** Serum IgE and percentage of IgE+ memory B-cells. **C.** Naïve and memory CD4+ and CD8+ T-cells. **D.** Thelper and regulatory subsets. Each dot represents one individual; red lines indicate median values.. Statistical analysis was performed with Mann Whitney *U* test. *P<0.05, **P<0.01, ****P<0.0001.

During the two-year follow up, we did not observe changes in naive and memory subsets with the CD4 and CD8 T-cell lineages (Fig 3C). However, within the CD4 T cell lineage, Th1 cell frequencies had decreased after the first year of treatment and continued to decrease after 2 years of treatment (Fig 3D). In contrast, Th2 and Th17 cell frequencies remained stable (Fig 3D). Furthermore, SLIT resulted in a significant increase of regulatory T cells after two years of treatment (Fig 3D). This observation was not specifically attributed to either naive or memory Tregs. The decrease of Tfh cells observed directly after SLIT was not sustained after 2 years of therapy.

In conclusion, low dose allergen exposure during 4 months of SLIT resulted in fast changes in B-cell responses leading to increased serum IgG4 levels and IgG4-expressing memory B cells. These effects were lasting and gradually increased after two rounds of treatment. In contrast,

changes in the T-cell compartment occurred later with decreased Th1 and increased Treg frequencies becoming apparent 2 years after start of treatment.

## DISCUSSION

In the study presented here, we show that sublingual immunotherapy to pollen allergy not only has long term beneficial clinical effects, but also results in sustained systemic effects on the immune system. SLIT induced a direct increase in pollen-specific serum IgG4 accompanied by an increase in the frequency of IgG4+ memory B-cells. Furthermore, SLIT resulted in an increase in the frequency of regulatory T-cells. Both observations were associated with favorable immunological properties by deviating the exaggerated Th2 response from an IgE phenotype towards an IgG4 phenotype.

Currently, SLIT is recommended for courses of 7 months starting 4 months prior to the start of the hay fever season and this has been shown to result in significantly improved symptom and disease scores [2]. Yet long treatment regimens are costly and prone to poor treatment compliance [34]. As patients will be exposed to pollens during hay fever season, we reasoned that 4-month treatment regimens prior to start of hay fever season might suffice to induce clinical effects. Indeed, based on our outcome of symptom and disease scores this approach seemed highly effective. Possibly during season treatment is not necessary for the desired effect, although we did not investigate this with a second treatment group here. Prolonged treatment (duration>12 months) has also shown to have a beneficial effects on symptom and medication scores [35]. The fact that some immunological effects only developed after the second treatment year, or in the case of RGP specific serum IgG4 levels, continued to rise after consecutive treatment, substantiates these observations.

In particular we here observed an increase in RGP specific IgG4. Previously allergen-specific immunotherapy, either SCIT or SLIT, has already been demonstrated to result in increased allergen-specific IgG4 serum levels [36, 37]. Moreover it has been postulated as one of the explanations for the beneficial effects of immunotherapy and has been observed as natural effect in bee-keepers exposed to bee-venom for prolonged periods [38], yet the desensitizing effect in immunotherapy is not entirely clear. Increased allergen specific IgG4 can directly compete for allergen, but it is also thought to have blocking activity on IgE-mediated responses [39]. Furthermore the IgG4 antibody has been attributed to have immune dampening effects, since it can only weakly bind to both C1q and Fcγ receptors [40, 41]. Still, increased RGP specific IgG4 would suggests that the direct competition for allergen is the main mechanism in our study cohort.

The source of increased serum IgG4 levels are IgG4 producing plasma cells. Here we did not investigate numbers of (Ig specific) plasma cells since these mainly reside in tissue, rather than in blood, however we did look at plasma blast numbers, which can be found in blood.

However total plasma blast frequencies were not affected by SLIT. To further characterize plasma blasts for the production of specific Ig's is difficult, since they only display weak membrane immunoglobulin expression and phenotyping can be unreliable. However in the differentiation from naïve B-cell to plasma cell, B-cells can also develop into memory B-cells, which have long lasting memory characteristics and can differentiate into plasma cells in subsequent responses [42].

Indeed we observed that SLIT results in increased frequencies of IgG4+ memory B-cells, whereas there was no effect on frequencies of IgE memory B-cell subsets. The latter observation can explain the absence of a decline in IgE serum levels which has also been observed by others [35, 43, 44]. Since we observed the increase in IgG4+ memory B-cells after 4 months of treatment with SLIT, and before the pollen season, this effect can be directly attributed to the treatment with Oralair. Our observation that frequencies of IgG4+ memory B-cells remain to be increased, can be an explanation for the long lasting effects attributed to immunotherapy [4-6].

In line with previous data, we observed an increase in regulatory T-cells after immunotherapy. The cytokines IL-4 and IL-13 are the most important regulators of immunoglobulin class switch recombination (CSR) to IgE, and these are predominantly produced by Th2 cells. In addition to Th2 cytokines, CSR to IgG4 is also regulated by IL10 which is secreted by Tregs. Previous studies have observed the new generation of allergen-specific Tregs, as well as a clonal expansions of allergen specific Tregs [39, 45]. In our study we could not attribute the increase in Tregs to either naïve or memory Tregs. The underlying mechanism inducing the increase in Tregs possibly originates in the mucosal surface. These are the tissues exposed to the low dose allergen treatment regimen and substantial evidence supports a role for local dendritic cells in the induction of Treg cells [46, 47]. To investigate this mechanism was outside the scope of our study, but a shift towards a local immune response might explain the decreased frequency of Tfh cells observed directly after SLIT.

Surprisingly we did not observe a change in Th2 cell frequency, yet rather a decrease in Th1 cells. Decreases in both subsets have been described as a result of immunotherapy, but solely reduced frequencies of Th1 cells seems to be in contrast with the described skewing if the immune system away from the Th2 phenotype. In some cases the suppression of Th2 cells was found to be transient, yet here patients received a second treatment before the next pollen season. Still, maintained numbers of Th2 cells can be explained by the fact that the Th2 cytokines IL4 and IL13 are required for the CSR towards IgG4 and thus in combination with IL10, can still lead to immune tolerance.

Concluding, our data provides evidence for long lasting effects of sublingual immunotherapy on the memory compartment of the immune system. Increased numbers of regulatory T-cells lead to a higher frequency of IgG4+ memory B-cells and a beneficial shift in the IgG4+/IgE+ memory B-cell ratio, reflecting the increased IgG4/IgE antibody fraction in serum, which results in a favorable outcome.

**ACKNOWLEDGEMETNS**

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# PART IV

## THE ROLE OF IGG4+ B-CELLS IN CHRONIC INFLAMMATORY DISEASE

# CHAPTER 7

# Expansions of blood IgG4+ B-cells, Th2 and regulatory T cells in IgG4-related disease: implications for diagnosis and therapy monitoring

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## ABSTRACT

*Background* IgG4-related disease (IgG4-RD) is a systemic fibro-inflammatory condition affecting various organs and has a diverse clinical presentation. Fibrosis and accumulation of IgG4+ plasma cells in tissue are hallmarks of the disease and IgG4-RD is associated with elevated IgG4 serum levels. However, disease pathogenesis is still unclear and these cellular and molecular parameters are neither sensitive nor specific for diagnosis of IgG4-RD.

*Objective* We here sought to develop a flowcytometric gating strategy to reliably identify blood IgG4+ B-cells to study their cellular and molecular characteristics and investigate their contribution in disease pathogenesis.

*Methods* Sixteen patients with histologically confirmed IgG4-RD, 11 patients with sarcoidosis and 30 healthy individuals were included for 11-color flowcytometric analysis of peripheral blood for IgG4-expressing B cells and T-helper (Th) subsets. In addition, detailed analysis of activation markers and chemokine receptors was performed on IgG4-expressing B cells and IgG4 transcripts were analyzed for somatic hypermutations.

*Results* Cellular and molecular analyses revealed increased numbers of blood IgG4+ memory B-cells in patients with IgG4-RD. These cells showed reduced expression of CD27 and CXCR5 and increased signs of antibody maturation. Furthermore, IgG4-RD patients, but not patients with sarcoidosis, had increased numbers of circulating plasma blasts and $CD21^{low}$ B-cells, as well as Th2 and regulatory T-cells, indicating of a common disease pathogenesis in IgG4-RD.

*Conclusion* These results provide new insights into the dysregulated IgG4 response in patients with IgG4-RD. A specific "peripheral lymphocyte signature" observed in patients with IgG4-RD, could support diagnosis and treatment monitoring.

## INTRODUCTION

IgG4 related disease (IgG4-RD) is defined as a systemic fibro-inflammatory condition that can affect potentially any organ, but predominating the retroperitoneal space, thyroid, pancreas, salivary glands and orbital tissue [1,2]. The pathology is characterized by fibrosis and infiltration of IgG4-producing plasma cells of the affected organs [3,4]. As a result, a tumor-like swelling of the involved organ can occur, which causes organ dysfunction and, if left untreated, can lead to organ failure. Furthermore, AA amyloidosis can develop in IgG4-RD, emphasizing the need for prompt diagnosis and treatment of the disease [5]. Previously, diseases with IgG4+ plasma cell infiltrates were defined predominantly by organ involvement, and only since 2012 these diverse manifestations have been recognized as one disease entity [3,4].

The pathogenesis of IgG4-RD remains poorly understood. It is thought to be triggered by organ damage, resulting from e.g. a bacterial infection with molecular mimicry or from an underlying autoimmune process [1]. In patients with pancreatic involvement, auto-antibodies directed against self-antigens have been observed [6,7]. Importantly, IgG4+ plasma cell infiltrates are not monoclonal [8], and serum IgG4 in patients is polyclonal with reactivity to multiple antigens [9]. The function of IgG4 antibodies remains elusive, because these display weak or negligible binding to both C1q and Fcγ receptors [10,11]. Furthermore, IgG4 molecules have the exclusive ability to exchange Fab-arms, thus creating monovalent bispecific antibodies that can prevent the formation of immune complexes [12]. Therefore, IgG4 has a presumed immune dampening effect, however its role in the pathogenesis of IgG4-RD is controversial [13].

Immunoglobulin class switching of B-cells to IgG4 is regulated by T helper 2 (Th2) cytokines and by IL-10, produced by regulatory T-cells [14,15]. Substantial evidence indicates that indeed Th2 cells and regulatory T-cells (Tregs) are involved in the pathophysiology of IgG4-RD [16-18]. Affected tissues express higher messenger RNA (mRNA) levels of the Th2 cytokines IL-4, IL-5 and IL-13 [16,17]. Furthermore, isolated circulating CD4+ T-cells from patients predominantly produce Th2 cytokines [18,19]. Similarly, IL-10 and tissue growth factor β (TGF-β) transcripts are increased in affected tissues [16], and patients with IgG4-related autoimmune pancreatitis have increased frequencies of circulating Tregs [20,21]. More recently, in affected tissue and blood of IgG4-RD patients clonal expansions of CD4 effector memory T(EM) cells have been identified, which potentially drive fibrosis and IgG4 production [22,23].

Clinically, IgG4-RD manifestations can mimic those of many infectious, inflammatory and malignant disorders [24-30]. Therefore, diagnosis of IgG4-RD can be challenging, often leading to a delay in start of proper treatment. The gold standard for diagnosis is histology [28,31]. Characteristic lymphoplasmacytic infiltrates, rich in IgG4 positive plasma cells, storiform fibrosis (cartwheel arrangement of fibroblasts) and obliterative phlebitis are seen in histological samples [28]. An increase in serum IgG4 supports the diagnosis, but is found in only 50-70% of patients with histological proven IgG4-RD [32,33]. Recently, circulating plasma

blasts have been postulated as a more reliable marker for the disease, irrelevant of IgG4 serum levels [34]. However, the specificity of this marker is limited, because circulating plasma blasts are also increased in active infection, following vaccination and in other chronic diseases, such as systemic lupus erythematosus (SLE) [35-38].

Still the importance of B-cells in the pathophysiology of the disease is illustrated by recent observations demonstrating that B-cell depletion with rituximab is a promising therapy in IgG4-RD [39-42]. As rituximab specifically binds CD20, it does not directly target IgG4+ plasma cells, which do not express CD20. Thus, the therapeutic effect could lie in depletion of memory B cells that are chronically stimulated to generate IgG4+ plasma cells. Furthermore, other chronically activated B cells might be involved in the disease through antigen presentation and cytokine production, such as $CD21^{low}$ B cells that are found to be expanded in diverse chronic inflammatory diseases [43].

Despite previously reported expansions of IgG4+ plasma cells, CD4 Tem, Th2 cells and Treg cells in affected tissue, little is known about the pathogenesis of IgG4-RD. Therefore, we developed a new flow cytometric approach, which enabled us to study IgG4-expressing B cells and their pathogenic contribution to the disease. Our insights into IgG4-expressing B cells in combination with abnormalities in B and T-cell subsets reveal abnormal systemic immune regulation, which are valuable for the improvement of diagnosis and treatment of IgG4-RD.

## METHODS

### Patients.

Patients with IgG4-RD and patients with sarcoidosis were recruited following signed informed consent from the Immunology outpatient clinic at the Erasmus Medical Center Rotterdam and from the Rotterdam Eye Hospital, the Netherlands. All patients were >18 years and were diagnosed based on clinical, serological and histopathological findings. All IgG4-RD patients met the IgG4-RD diagnostic guidelines, including histological confirmation [28], and did not have a known history of an immunodeficiency or any auto-inflammatory disease other than IgG4-RD. All but two patients with sarcoidosis had tissue biopsy confirmed disease with typical presence of non-caseating granulomatous inflammation. In two patients the diagnosis was based on clinical presentation and supportive serological parameters (angiotensin converting enzyme and soluble interleukin-2 receptors) in combination with radiological imaging. Healthy controls were recruited from healthy individuals selected from department staff, and the control group was age- and gender-matched to the patient cohort. None of the healthy individuals showed signs of active inflammatory disease. This study was performed according to the Declaration of Helsinki and was approved by the

Medical Ethics Committee of Erasmus MC (ethics approval numbers MEC-2014-476, MEC-2015-200 and MEC-2017-084).

### Histopathology.

All patients with IgG4-RD were histologically diagnosed. The haematoxylin and eosin stainings were analyzed at the Department of Pathology of the Erasmus Medical Center Rotterdam by a trained pathologist with experience in diagnosing IgG4-RD. The deparaffinized formalin-fixed paraffin embedded sections of the tissue (4 mm thick) were stained using a BenchMark automated immunostainer (Ventana, Tucson, AZ, USA) with the Ultraview Universal diaminobenzidine detection kit (Ventana). Mouse anti-human IgG (clone A57H, 1:200, Dako, Carpinteria, CA, USA) and mouse anti-human IgG4 (clone HP6025, 1:600, Invitrogen Zymed, Camarillo, CA, USA) were used for immunohistochemical staining and were applied to the sections for 32 min. The amount of IgG4+ plasma cells per high-power field (0.28 $mm^2$) and the IgG4/IgG ratio were measured.

### IgG serology.

IgG subclass serum levels were measured by immunonephelometry using a Siemens BN II nepholometer according to manufacturer guidelines. A possible prozone effect for IgG4 levels was excluded through dilution of serum samples until reliable values were obtained [44].

### Flow cytometry of blood samples.

Patients and controls were included over a time period of three years. To ensure consistency in flowcytometry, standardized sample preparation, antibody staining and flowcytometer instrument settings were used [45]. In short, absolute counts of CD3+ T-cells, CD19+ B-cells and CD16+/CD56+ NK-cells were obtained with a diagnostic lyse-no-wash protocol using commercial Trucount tubes (BD Biosciences, San Jose, Calif). For detailed 11-color flow cytometry, red blood cells were lysed with $NH_4Cl$ prior to incubation of 1 million nucleated cells for 15 minutes at room temperature in a total volume of 100µL. After preparation, cells were measured on a 4-laser LSRFortessa flow cytometer (BD Biosciences) using standardized settings. Data were analyzed with FACSDiva software V8.0 (BD Biosciences).

### Molecular analysis of immunoglobulin heavy chain (IGH) gene rearrangements.

RNA was isolated from post-Ficoll mononuclear cells with a GenElute mammalian RNA kit (Sigma-Aldrich, St Louis, Mo) and reverse transcribed to cDNA with random primers (Invitrogen Life technologies). *IGHV* gene rearrangements were amplified in a semi-nested multiplex PCR approach using 4 different L-VH-family forward primers [46] in combination with *IGHG4*-specific (5'GGGCATGATGGGCAYGGGGGACCATA; first round) and *IGHG*-consensus (5'CACGCTGCTGAGGGAGTAG; second round) reverse primers. PCR products were cloned

into a pGEMT easy vector (Promega, Madison WI), amplified by colony PCR, and sequenced on an ABI Prism 3130XL (Applied Biosystems, Foster city, CA). The sequences were analyzed with the IMGT database (www.imgt.org) and BASELINe program (selection.med.yale.edu/baseline) [47, 48].

### Statistical analysis

Frequencies and absolute cell numbers were assumed a non-Gaussian distribution. All results are expressed as median values with interquartile range if applicable. Results were analyzed using the non-parametric Mann-Whitney *U* test. Linear regression was used to study the strength of association between cell-subsets with Spearman r to measure significance. All P-values are two-tailed and were considered statistically significant if values were lower than 0.05. Statistical analysis was performed using GraphPad Prism software, version 6 (GraphPad Software, La Jolla, CA). Principal component analysis (PCA) was performed with Infinicyt software (Cytognos, Salamanca, Spain). Specificity and sensitivity were calculated with SPPS software (IBM SPSS statistics 21.0, Armonk, NY)

## RESULTS

### Patient characteristics.

A total of 16 patients with IgG4-RD were included with a mean age of 56 years (range 18-79 yrs) and a male:female ratio of 2:1 (Table I). All patients were confirmed to have IgG4-RD based on the Boston consensus, with typical histopathologic characteristics and the presence of IgG4-producing plasma cells in affected tissue (Fig 1). Twelve out of 16 patients had increased serum IgG4 levels (mean 5.06g/L; range 0.27-25.25g/L). Four of these patients had an additional increase in serum IgG1 or IgG2 levels, and one patient with normal serum IgG4 had increased levels of IgG1 and IgG3 (see Repository Table E3). Six patients showed signs of active disease based on clinics and increased CRP (C-reactive protein) and/or ESR (erythrocyte sedimentation rate) (Table I).

The majority of IgG4-RD patients were treatment naive (no prior treatment), three patients had received immunomodulatory medication in the past (medication had been stopped for at least 6 months prior to inclusion) and only two patients received low dose prednisone (Table I). Most patients had normal counts of blood leukocytes and lymphocytes (Supplemental Table 1). Increased lymphocytes resulted from high T-cell counts, often in combination with high B- and/or NK cell numbers. One of the two patients treated with prednisone had decreased numbers of B- and T-cells. In the analysis of the distribution of IgG subclasses within the total IgG memory B-cell compartment we analyzed material from 15 of the 16 patients.

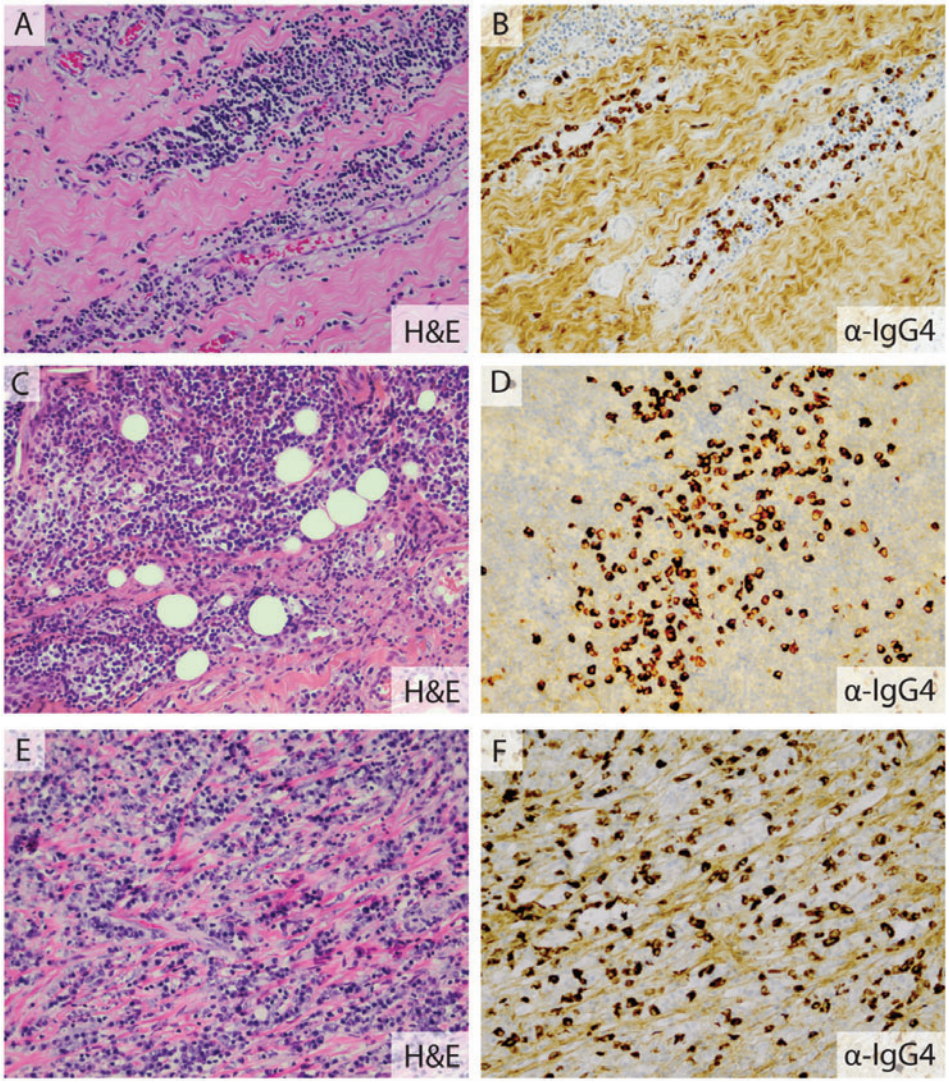


**Figure 1.** Representative hematoxylin and eosin (H&E) and IgG4 stainings of tissue sections of affected organs in patients with IgG4-RD. **A.** and **B.** Pericardial tissue of patient 15 demonstrating lymphoplasmacytoid fibrosis and obliterative phlebitis. **C.** and **D.** Orbital tissue of patient 6 with lymphoid infiltration. **E.** and **F**. Lung tissue of patient 10 with lymphoplasmacellular infiltrates. All images are 200X magnification.

Of the eleven patients with sarcoidosis, one patient used plaquenil. All other patients were therapy naive (Supplemental Table 2). All patients had normal leukocyte counts, although some patients had slightly decreased or slightly increased T-cell or B-cell counts. One patient had an increased IgG4 serum level accompanied with an increased IgG1 serum level. One patient had a slightly decreased IgG4 serum level and from one patient IgG serum

levels were not determined. Five patients showed signs of active disease based on CRP and/or ESR (Supplemental Table 2).

**Table 1. Characteristics of patients with IgG4-RD**

| patient | gender | age (yr) | organs affected | medication at inclusion | time until diagnosis | treatment started after inclusion | CRP (<10mg/L) | ESR (20mm/h) |
|---|---|---|---|---|---|---|---|---|
| 1 | F | 60 | orbita | n.i.m. | 14 years | prednisone, methotrexate, cyclosporine, infliximab | **14** | **40** |
| 2 | F | 67 | orbita | n.i.m. | 8 years | dexamethasone | 1.1 | 1 |
| 3 | M | 63 | orbita, lymph node, lung, prostate | prednisone | 3 years | prednisone, rituximab, methotrexate | 0.9 | 5 |
| 4 | M | 54 | orbita | prednisone | 10 years | prednisone | 1.9 | 9 |
| 5 | F | 53 | orbita | n.i.m. | 3 years | unknown | 1.7 | n.d. |
| 6 | M | 60 | orbita, lymph node | t.n. | 5 years | prednisone, methotrexate | 0.7 | 16 |
| 7 | M | 44 | orbita | t.n. | 12 years | prednisone | 0.4 | 1 |
| 8 | F | 59 | skin | t.n. | 5 months | hydroxochloroquine | 6.0 | 11 |
| 9 | M | 18 | lymph node, lung, cerebra | t.n. | 1 month | dexamethasone, azathioprine | **50** | **107** |
| 10 | F | 62 | pancreas, lymph node, lung | t.n. | 3 months | prednisone | 6 | 2.4 |
| 11 | M | 74 | lymph node, salivary gland, lung | t.n. | 5 months | prednisone, azathioprine | 0.8 | **37** |
| 12 | M | 53 | mesenterium | t.n. | 20 years | prednisone, azathioprine, rituximab | **84** | **119** |
| 13 | M | 62 | thyroid gland | t.n. | 1 year | prednisone | 1.9 | 6 |
| 14 | M | 32 | serous membrane | t.n. | 3 months | prednisone | **18.7** | 8 |
| 15 | M | 79 | lymph node, pancreas | t.n. | 1 year | - | 6.4 | **90** |
| 16 | M | 60 | bile duct, lymph node | t.n. | 2 weeks | prednisone, methotrexate | 4 | 0.7 |

CRP, C-reactive protein; ESR, erythrocyte sedimentation rate; n.i.m., no immunomodulatory medication for at least 6 months prior to inclusion; t.n., treatment naïve; n.d., not determined

## Immunophenotypical analysis of IgG4-expressing B-cells.

The hallmark of IgG4-RD is the accumulation of IgG4-producing plasma cells in affected tissue. To study whether patients had systemic abnormalities in IgG4-expressing B cells, we developed a reliable flow cytometric gating strategy to distinguish cells expressing one of the four IgG subclasses (Fig 2A). Within total CD19+ B cells, CD27+CD38$^{hi}$ plasma blasts were electronically gated and studied for expression of IgG1, 2, 3 and 4. However, surface Ig levels were too low to detect. Therefore, we next focused our attention on CD38$^{dim}$IgM-IgD- memory B cells. These contained presumed memory B cell subsets that expressed either IgG1, IgG2 or IgG3, and within the triple negative fraction, a sizeable IgG4-expressing subset could be identified (Fig 2A).

To confirm the activated nature and memory phenotype of all IgG+ B-cell subsets we studied the expression of B7 family members CD80 and CD86 and the TNF receptor superfamily member transmembrane activator and CAML interactor (TACI). All four IgG-subclass expressing B-cell subsets showed higher expression of the activation markers than naive B-cells (Fig 2B), fitting with an activated memory B-cell phenotype.

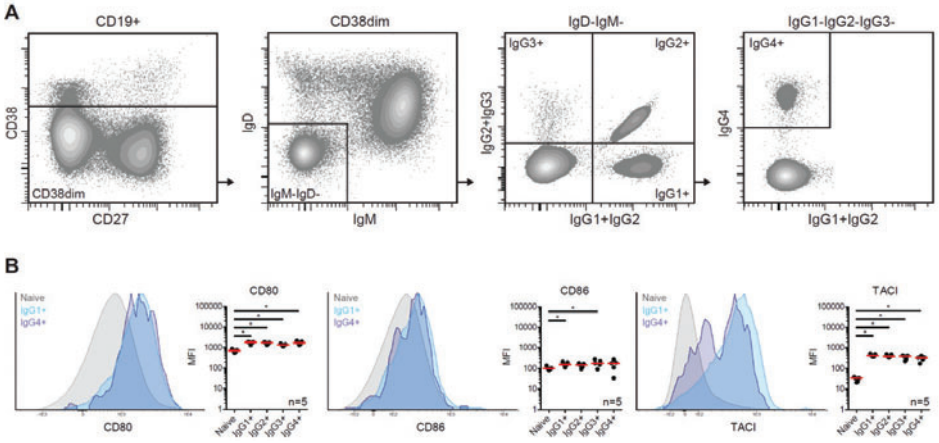


**Figure 2.** Identification and immunophenotyping of IgG subclass expressing memory B-cells. **A**. Stepwise flowcytometric gating strategy to identify IgG1+, IgG2+, IgG3+ and IgG4+ memory B-cells. **B.** Expression of activation and memory markers on B-cell subsets. Shaded grey histograms represent naive B-cells, light blue represent IgG1+ memory B-cells and dark blue represent IgG4+ memory B-cells. Each dot represents one individual, red lines indicate medians. Statistical analysis between the groups was performed with the Mann Whitney U test. *p<0.05

## Increased CD21low B-cells and plasma blasts, but reduced IgM+IgD+ memory B cells in patients with IgG4-RD.

To study systemic abnormalities in IgG4-expressing B cells, we performed extensive immunophenotyping of B-cells in 16 patients with IgG4-RD and compared these with 30 age-matched healthy controls (Fig 3A). The median numbers of transitional and naive

mature B cells were not different between patients and controls. Of the six major memory B cell subsets, only the IgM+IgD+ memory B cells were significantly affected in patients (reduced; $P<0.05$) (Fig 3B). In contrast, the numbers of $CD21^{low}$ B-cells ($CD19+CD38^{dim}CD21^{low}$), and plasma blasts (CD19+CD38+CD27+) were significantly higher in patients with IgG4-RD (both $P<0.05$).

**Patients with IgG4-RD have increased numbers of IgG4+ memory B-cells.**

Despite the normal total numbers of IgG+ memory B-cells, patients with IgG4-RD had significantly higher numbers of IgG4+ memory B-cells than controls ($P<0.01$) (Fig 3C). In addition, patients had reduced numbers of memory B cells expressing IgG1 ($P<0.05$), whereas IgG2 and IgG3 expressing B-cell numbers were similar to controls (Supplemental Figure 1A). When analyzed as fraction of total IgG-expressing memory B cells, the decrease in IgG1 and increase in IgG4 in patients with IgG4-RD became more apparent (both $P<0.0001$). Furthermore, IgG4-RD patients showed a significant increase in the fraction of IgG memory B cells expressing IgG2 ($P<0.01$). To study if there was a direct relation with serum IgG4 levels, we performed linear regression analyses with the percentages and the absolute numbers of IgG4+ memory B-cells (Supplemental Figure 1B). These did not reveal significant correlations, indicating that the memory B cells and serum IgG4 levels were not directly related.

Since both IgG4-expressing B cells and $CD21^{low}$ B cells were significantly increased in IgG4-RD patients, we studied if these were related and investigated $CD21^{low}$ B cells expressing IgG4 (Supplemental Figure 1C). Indeed, patients carried significantly more $CD21^{low}$IgG4+ B cells (Supplemental Figure 1D) and these numbers were directly correlated to the total number of $CD21^{low}$ B cells ($P<0.01$) (Supplemental Figure 1E).

**Cellular and molecular analysis of IgG4-expressing B-cells in patients with IgG4-RD.**

To analyze the nature of the IgG4-expressing B-cell expansion in patients with IgG4-RD, we first studied the expression of CD27 (Fig 4A). In healthy controls, the frequencies of cells that expressed CD27 were highest within the IgG2 and IgG4 subsets, followed by IgG1 and IgG3 (Fig 4B). Thus, CD27 positivity was higher in cells utilizing IgG subclasses encoded by the downstream genes within the *IGH* locus (Cγ2 and Cγ4) (Fig 4C). In patients with IgG4-RD, significantly fewer IgG4+ B-cells expressed CD27 than IgG4+ B-cells from healthy individuals.

To study whether the expanded IgG4-expressing B cells could be normally involved in immune responses, we analyzed the expression levels of cytokine receptors and chemokine receptors involved in germinal center responses. IL21 receptor (IL21R) and IL4R as well as C-C chemokine receptor type 7 (CCR7) expression levels were similar between IgG4+ memory B-cells from healthy individuals and from patients with IgG4-RD (Fig 4D). In contrast, C-X-C receptor type 5 (CXCR5) expression was significantly lower on IgG4+ memory B-cells from patients.

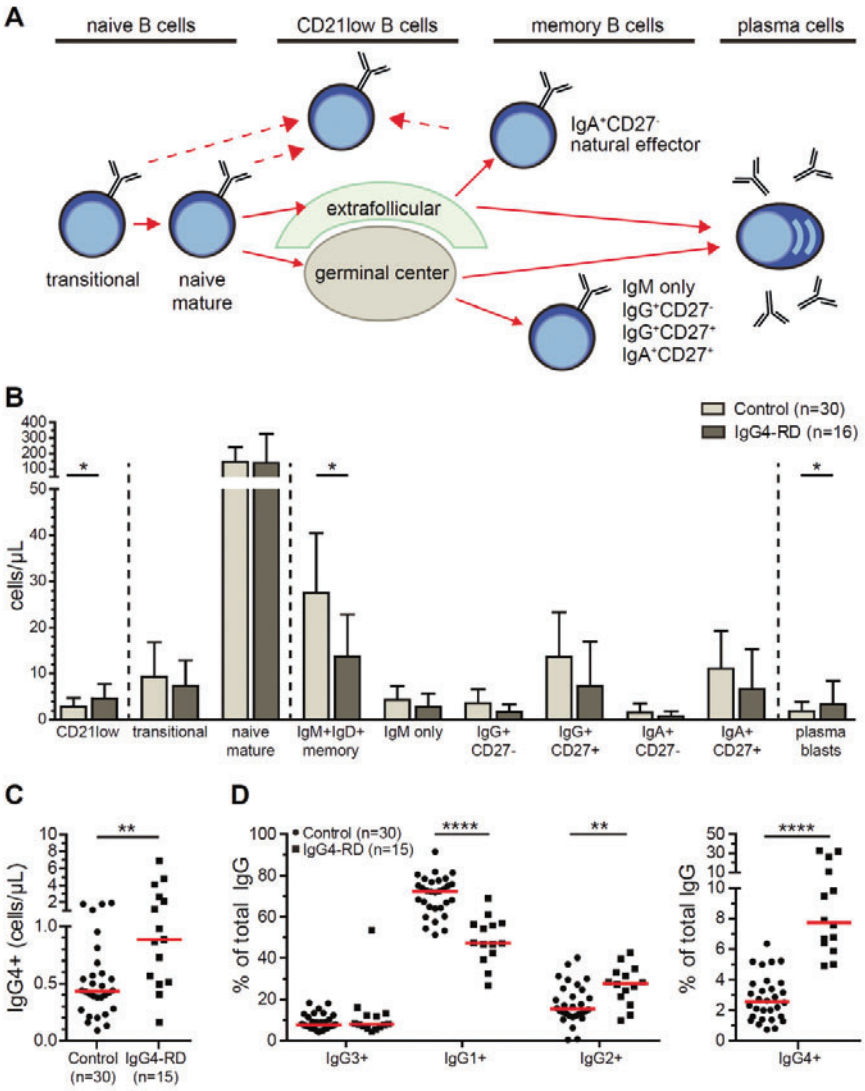


**Figure 3.** Composition of the blood B-cell compartment in healthy individuals and in patients with IgG4-RD. **A.** Model of peripheral B-cell maturation. **B.** Absolute numbers of naive and memory B-cell subsets and plasma cells. Columns indicate median values with interquartile range. **C.** Absolute numbers of IgG4+ memory B-cells. **D.** Distribution of IgG subclass memory B-cells within the total IgG memory B-cell compartment. E. Absolute numbers of $CD21^{low}$IgG4+ memory B-cells. Each dot represents one individual, red lines indicate medians. Statistical analysis between the groups was performed with the Mann Whitney U test. * $p<0.05$, ** $p<0.01$, **** $P<0.0001$

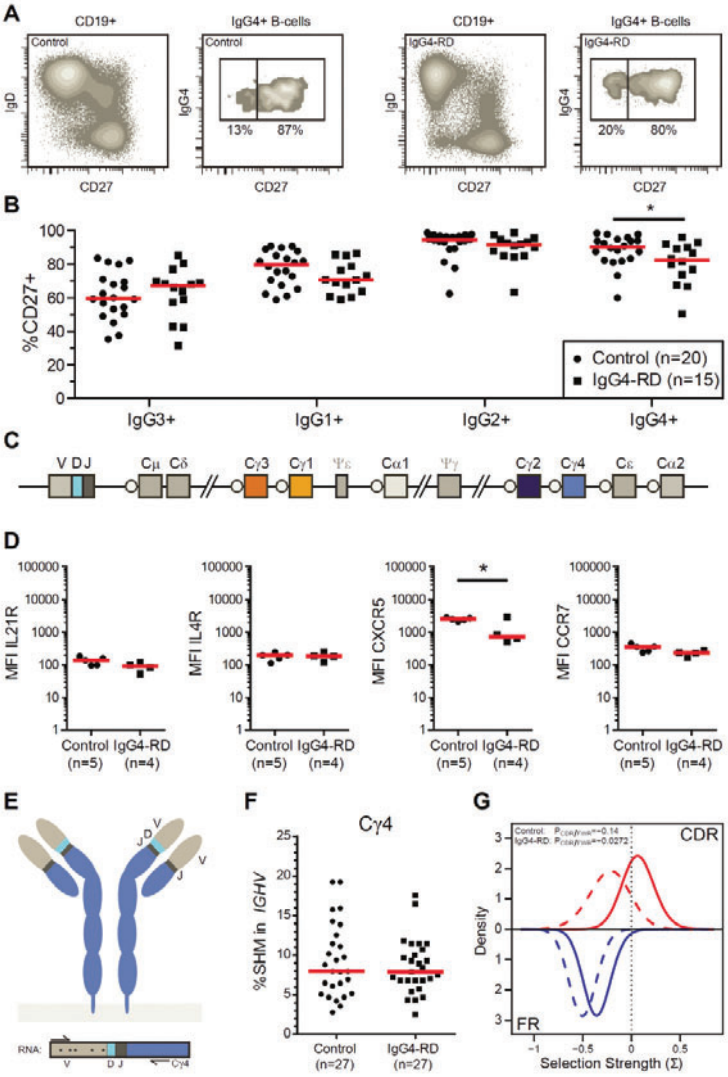


**Figure 4.** Molecular characteristics of IgG4+ B-cells from healthy individuals and patients with IgG4-RD. **A**. Representative flowcytometry plots of CD27 expression on total B cells and IgG4+ B-cells from a healthy individual and from a patient with IgG4-RD **B.** Frequencies of IgG subclass memory B-cells expressing CD27. **C.** Schematic representation of the human *IGH* locus **D**. Expression of cytokine and chemokine receptors on IgG4+ B-cells. **E**. Representation of the membrane B-cell receptor (BCR) with the variable domain of the Ig heavy chain (V, D and J) and Ig light chain (V and J). Asterisks in the RNA represent mutations in the genes encoding the proteins of the BCR. **F.** Frequency of somatic hypermutation (SHM) in rearranged *IGHV* gene. **G.** Selection for replacement mutations in the CDR (red) and FR (blue) regions. Solid lines represent patients; dotted lines represent healthy controls. A selection strength of more than 0 is indicative of positive selection [48].

To investigate the nature of the expanded IgG4+ memory B-cells in the pathogenesis of IgG4-RD, we studied somatic hypermutations (SHM) in the *IGHV* regions (Fig 4E). Such mutations are molecular signs of B-cell responses and affinity maturation [49]. In agreement with previous studies, SHM frequencies in IgG4 transcripts of healthy adults were higher than in the more proximal-encoded IgG1 and IgG2[50, 51]. The SHM levels in IgG4 transcripts of patients with IgG4-RD (7.89%) were similarly high as in healthy adults (7.98%; *P*=0.75) (Fig 4F). To study if the mutations were driven by selection for antigen binding, we analyzed the selection for replacement mutations in the complementarity determining regions (CDR) with the Bayesian estimation of Antigen-driven SELectIoN program BASELINe [48]. IgG4 transcripts from healthy controls did not show more replacement mutations than expected by random chance in the CDR regions, i.e. absence of positive selection (Fig 4G). In contrast, CDR regions of IgG4-RD patients did show positive selection for replacement mutations. Both groups showed normal negative selection for replacement mutations in framework (FR) regions that compose the structure of the variable domain. Thus, IgG4+ B-cells in patients with IgG4-RD show increased selection for replacement mutations in CDR regions. Patients and controls displayed high diversity in sequence and length of the CDR3 region, which is encode by the junction of the V, D and J genes. Still, patients showed significantly shorter IGH-CDR3 regions in IgG4 transcripts than healthy individuals. Furthermore, *IGHV* usage seemed to differ between groups with increased usage of IGHV5-51 in patients with IgG4-RD and reduced IGHV3-39 usage, although this did not reach statistical significance (*P*=0.08).

### Increased numbers of Th2 cells and Tregs in IgG4-RD.

The impaired expression levels of CXCR5 and absence of positive selection of SHM in IgG4+ B cells are suggestive of abnormal immune responses in patients with IgG4-RD. IgG4 responses are mediated by IL-4 and IL-13, produced by Th2 cells, and IL-10, produced by Tregs. Therefore, we performed additional studies into Th subsets in our patients (Fig 5A). Flowcytometrc immunophenotyping of their blood T cells demonstrated normal overall numbers of naive, central memory and effector memory CD4 T cells. Still, patients with IgG4-RD had significantly more circulating Th2 cells (CD45RA-CCR6-CXCR3-CCR4+; *P*<0.0001) and Tregs (CD45RA-CD127-CD25+; *P*<0.001) than healthy individuals (Fig 5B). Th1 cells seemed slightly lower, but this was not significant and numbers of follicular helper T cells (Tfh) and Th17 were not different. Linear regression showed a direct correlation between the numbers of IgG4+ memory B-cells and Th2 cells (*P*<0.01), but not for Tregs (*P*=0.24; Fig 5C). $CD21^{low}$IgG4+ B cell numbers were similarly correlated with Th2 cells (*P*<0.05) and not with Treg numbers (*P*=0.49). IgG4 serum levels in patients did not correlate with Th2 nor Treg cell numbers. In contrast to the changes in Th subsets, patients with IgG4-RD had no differences in numbers of CD4+CD45RA+CCR7- TemRA cells, CD4+CD45RA-CCR7- TemRO cells, nor CD27- TemRO cells (CD4 CTL). Finally, naive CD8 T-cell numbers were decreased in IgG4-RD patients, in absence of effector memory expansions.

Skewing of Th subsets towards Th2 is also observed in allergies and frequently accompanied by eosinophilia [52]. However, we did not find increased numbers of eosinophils ($SSC^{high}CD45+CD16^{dim}CD81+$) in patients with IgG4-RD (Supplemental Figure 2), nor any differences in the other granulocyte subsets (neutrophils and basophils). In contrast, the numbers of plasmacytoid dendritic cells ($SSC^{low}CD45^{dim}HLADR+CD123+$), which are potent drivers of Th1 responses [53], were significantly lower in IgG4-RD patients. Thus, the increased numbers of IgG4-expressing B cells in IgG4-RD patients are accompanied by systemic reduction in Th1 immunity and increased Th2 and Treg cells.

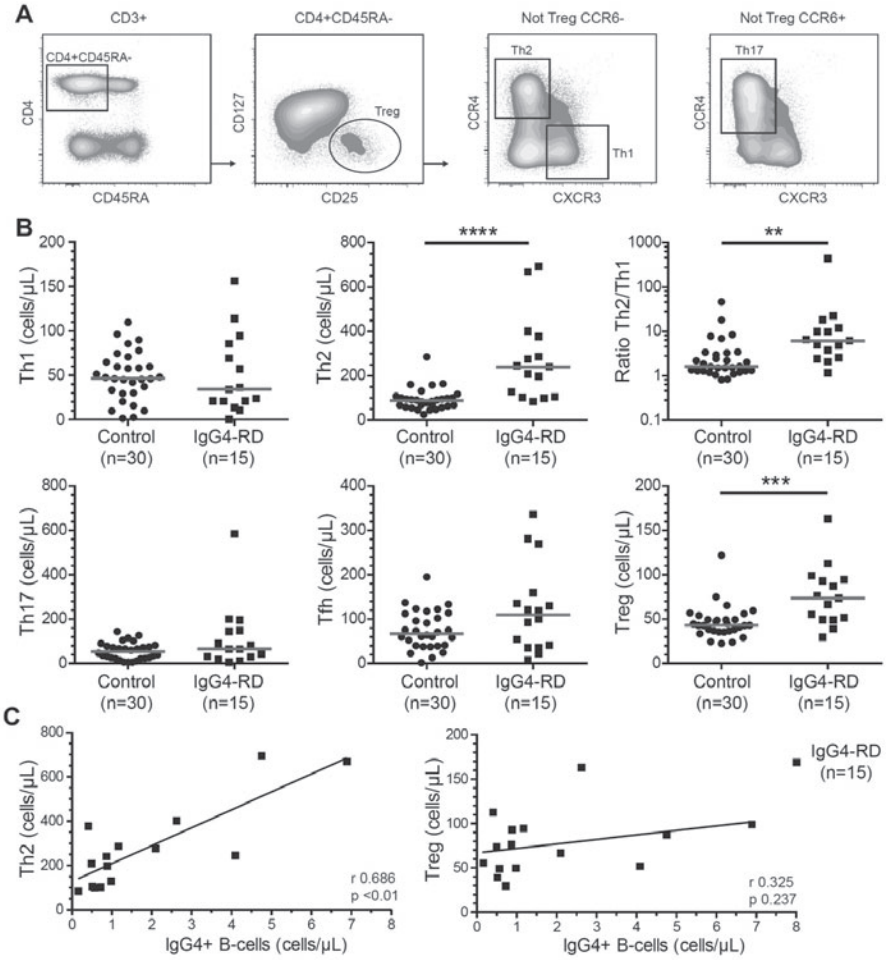


**Figure 5.** Blood T-helper subsets. **A.** Representative plots from flow cytometric analysis. B. Absolute numbers of T-helper 1 (Th1), T-helper 2 (Th2), follicular helper T-cells (Tfh) and regulatory T-cells (Treg). C. Regression analysis of Th2 cells and IgG4+ B-cells and of Treg cells and IgG4+ B-cells. Each dot represents one individual, red lines indicate medians. Statistical analysis between the groups was performed with the Mann Whitney U test. * $p<0.05$, ** $p<0.01$. Correlation was calculated with Spearman R.

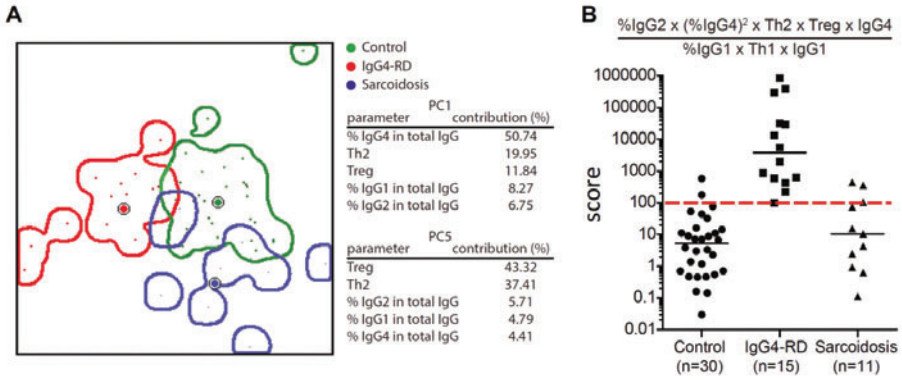


**Figure 6.** Multi-parameter analysis and clustering of individuals based on blood lymphocyte subsets. A. Principal component analysis of peripheral blood lymphocyte subsets from healthy individuals (green), patients with sarcoidosis (blue) and patients with IgG4-RD (red). Each individual is represented by one dot, with indication of group median (large dot) together with 1SD and 2SD. Following automated population separator (APS) based on the absolute counts of various subsets, PC1 and PC5 were selected to visualize separation between healthy individuals, patients with sarcoidosis and patients with IgG4-RD. The contribution of the top 5 components of each PC are listed. B. Scoring of blood samples based on a formula composed of the subsets that were significantly different between healthy individuals and patients with IgG4-RD. The percentage of IgG2+ B-cells and IgG4+ B-cells within total IgG B-cells are multiplied by the absolute numbers of Th2, Treg and IgG4+ B-cells and divided by the percentage of IgG1+ B-cells within total IgG+ B-cells multiplied with the absolute numbers of Th1 and IgG1+ B-cells. Red dotted line indicates a score of 100 considered as cut-off value.

## IgG4+ memory B-cells and other lymphocyte subsets as a diagnostic tool for IgG4-RD.

Despite clinical indications and high serum IgG4 levels, diagnosis of IgG4-RD requires analysis of a tissue biopsy. Obtaining a biopsy can be difficult, is invasive, sample error sensitive and might not always be sufficiently discriminative from other immune-proliferative and auto-inflammatory diseases. A specific marker in blood could be a valuable means to overcome these diagnostic challenges. However, the differences in B-cell and T-cell subsets we identified do not provide a clear discrimination between patients and controls. Therefore, we explored if a combination of measurements could be used as a disease marker. First, we performed a principal component analysis (PCA) on the absolute cell numbers of all B and T-cell subsets from healthy individuals and patients with IgG4-RD and visualized this with the automated population separation (APS) tool of the Infinicyt program [54]. In this analysis we also included a group of patients with treatment naive sarcoidosis, another fibro-inflammatory disease. IgG4-RD resembles many immune proliferative diseases, yet patients with sarcoidosis did not show typical expansions of IgG4+ memory B cells, Tregs and Th2 cells. Of the first 5 principle components, component 1 (PC1) separated patients with IgG4-RD from patients with sarcoidosis and from healthy controls. (Fig 6A). This analysis

indicates the usefulness to combine multiple parameters for optimal discrimination between IgG4-RD patients and controls and potentially from other auto-inflammatory conditions with tissue fibrosis. Therefore, we continued to develop a score based on the B- and T-cell subsets that were most significantly different between IgG4-RD patients and both controls and patients with sarcoidosis (Fig 6B). Application of this scoring method to the 56 samples in our study resulted in 50 samples being correctly assigned as either non-IgG4-RD or IgG4-RD (Fig 6B). The calculated sensitivity for this score was 93.3% (95% confidence interval, 68.05% - 99.83%) and the calculated specificity was 87.8% (95% CI, 73.80% - 95.92%). Thus, IgG4-RD patients carry changes in their blood B- and T-cell subsets that together reflect a unique "lymphocyte signature".

## DISCUSSION

We here demonstrate significant expansions of IgG4-expressing B cells in blood of patients with IgG4-RD. These changes were directly related to the previously described expansion of $CD21^{low}$ B cells and are associated with expansions in plasma blasts, Th2 and Treg cells. Increased numbers of circulating plasma blasts have been reported before in patients with IgG4-RD [8, 34]. This is not a disease-specific effect as similar expansions have been observed in many chronic inflammatory diseases and in individuals with active infection or following vaccination [35-38]. Thus, increased plasma blast numbers mostly reflect active humoral immune responses. Active inflammation is further supported by the increased numbers of $CD21^{low}$ B cells in our patients. These cells have been reported to be increased in multiple states of inflammation [43, 55, 56] and are thought to result from prolonged B-cell activity due to chronic activation by self-antigens [57]. CD21 functions as a co-receptor for the BCR, and downregulation of its expression makes the cells anergic for chronic stimuli [58]. We now demonstrated that the expansion of $CD21^{low}$ B cells is in part due to the expansion of IgG4+ population, which directly links it to the disease. The nature of chronic B-cell stimulation in IgG4-RD remains unclear. It is likely that auto-antigens are involved, as plasma blasts from patients with IgG4-RD produce immunoglobulins that react against human cell lysates [8]. Insights into IgG4-expressing B cells in patients with IgG4-RD are limited, most likely due to the lack of proper reagents to specifically detect and isolate these cells. To overcome this, we developed a flow cytometric approach to stain for IgG4-expressing B cells using newly available reagents and found that frequencies and absolute numbers of IgG4+ memory B-cells were increased in patients with IgG4-RD. However, in contrast to previous observations, these numbers were not correlated to IgG4 serum levels [59]. Absence of correlation is most likely due to the fact that these cells were immunophenotypically memory B-cells [60, 61] rather than IgG4-producing plasma cells. Previously we observed similar

absence of correlation for IgE+ memory B-cell numbers and serum IgE levels in patients with atopic dermatitis [62].

On top of cell numbers, patients' IgG4+ memory B cells were phenotypically different from those of healthy controls. A lower frequency of cells expressed CD27, which is the conventional marker for memory B-cells [63, 64]. Furthermore, expression levels of CXCR5 were lower in patients. These differences could originate from a different maturation pathway for IgG4+ memory B cells in patients. Generation of IgG+ memory B cells in humans is critically dependent on T-cell help [65]. Yet, it is unclear if T-cell help is needed for sequential switching to IgG4 of memory B cells expressing other IgG subclasses. The reduced expression of CXCR5 on IgG4+ B-cells from patients could represent a reduced capacity for homing to B-cell zones in lymph nodes and would suggests that activation and differentiation of IgG4+ B-cells in patients occurs in peripheral tissue, rather than in lymphoid structures [66]. Cytokines specifically inducing CSR to IgG4 have been found in IgG4-RD tissue biopsies [16]. Moreover, expression of activation-induced cytidine deaminase (AID), the enzyme that triggers SHM and which is needed for CSR, was reported to be increased in tissue biopsies of IgG4-RD patients, conforming the ability of local CSR [15].

In addition to differences in IgG4+ memory B cells, we found systemic expansions of Th2 and Treg cells. Ig class switching to IgG4 is mediated by the cytokines IL4 and IL-10, which are predominantly produced by Th2 and Treg, respectively. This is referred to as "the modified T-helper 2 type response" [67], and has been reported in the context of the beneficial effects of immunotherapy in allergy, in which it favors CSR to IgG4 over IgE [68]. Prolonged antigen exposure indeed leads to a serologic shift in the IgG4:IgG1 antibody ratio, as previously illustrated by natural immunization in bee keepers or during subcutaneous immunotherapy with grass pollen [69, 70]. Previous observations in tissue and in blood have also linked the modified Th2 type response to IgG4-RD [16-18, 20, 69]. Our data confirms such a response, with the observation of increased numbers of Th2 cells and Tregs in IgG4-RD and a positive correlation between Th2 cells and IgG4+ B-cells. In contrast to earlier observations we did not find an increase in Tfh cells [71]. Yet, this finding supports the hypothesis that differentiation towards IgG4 in IgG4-RD takes place in peripheral tissue, rather than in follicles in lymphoid structures.

Despite significant differences in numbers of B- and T-cell subsets between patients and controls in the presented study, none of these were sufficiently discriminative to be used as biomarker. This included plasma blasts, which had recently been proposed as a biomarker for IgG4-RD [34]. Importantly, the numbers of plasma blasts in our patients (median 3.36 cells/μL) were in the same range as previously reported (median 4.70 cells/μL) [34], whereas plasma blast numbers were extremely low in controls of that study (0.10 cells/μL) [34]. As plasma blasts in our study (median 1.70 cells/μL) were in the same range as reported by others, it is questionable if this subset is a reliable biomarker [72]. Therefore, we propose to derive a biomarker based on the specific "lymphocyte signature" in peripheral blood of patients with

IgG4-RD. Combined analysis of various subsets has an advantage since it does not rely on one biomarker, rather it reflects the pathophysiology of the disease. Since IgG4-RD can affect various organs, the clinical presentation is diverse, yet PCA of the peripheral lymphocyte compartment illustrates that the underlying immune response is clearly distinct. This is confirmed by the fact that combined analysis with PCA not only distinguishes healthy from disease, but can also discriminate between different fibro-inflammatory conditions (i.e. sarcoidosis from IgG4-RD). Examples of scoring systems in medical practice are ample and systems medicine has been proven of value to study complex diseases and generate predictive models, although some caution is warranted [73, 74].

In conclusion, we here demonstrate that patients with IgG4-RD present with increased numbers of IgG4+ B-cells, $CD21^{low}$ B-cells and plasma blasts in blood. Molecular characteristics reveal that the latter expansion is a result of an ongoing immune response in affected tissue. Furthermore, this is characterized by increased numbers of Th2 cells and regulatory T-cells, known as the modified Th2 type response. This specific "peripheral lymphocyte signature" in patients with IgG4-RD indicates that patients suffering from IgG4-RD have a common disease pathogenesis and in the future, peripheral blood might be exploited as a non-invasive tool to employ for diagnosis and treatment monitoring in patients with IgG4-RD.

## AUTHOR CONTRIBUTIONS

JJH, AFK, JAMvL, PMvH and MCvZ designed research. JJH and AFK performed research. JJH, AFK and MCvZ analyzed data. RMV and DP evaluated and included patients in the study. JJH, AFK and MCvZ wrote the paper, and all authors commented on the paper and approved the final version.

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# SUPPLEMENTAL DATA

**Supplemental Table 1.** Basic immunological characteristics of patients with IgG4-RD

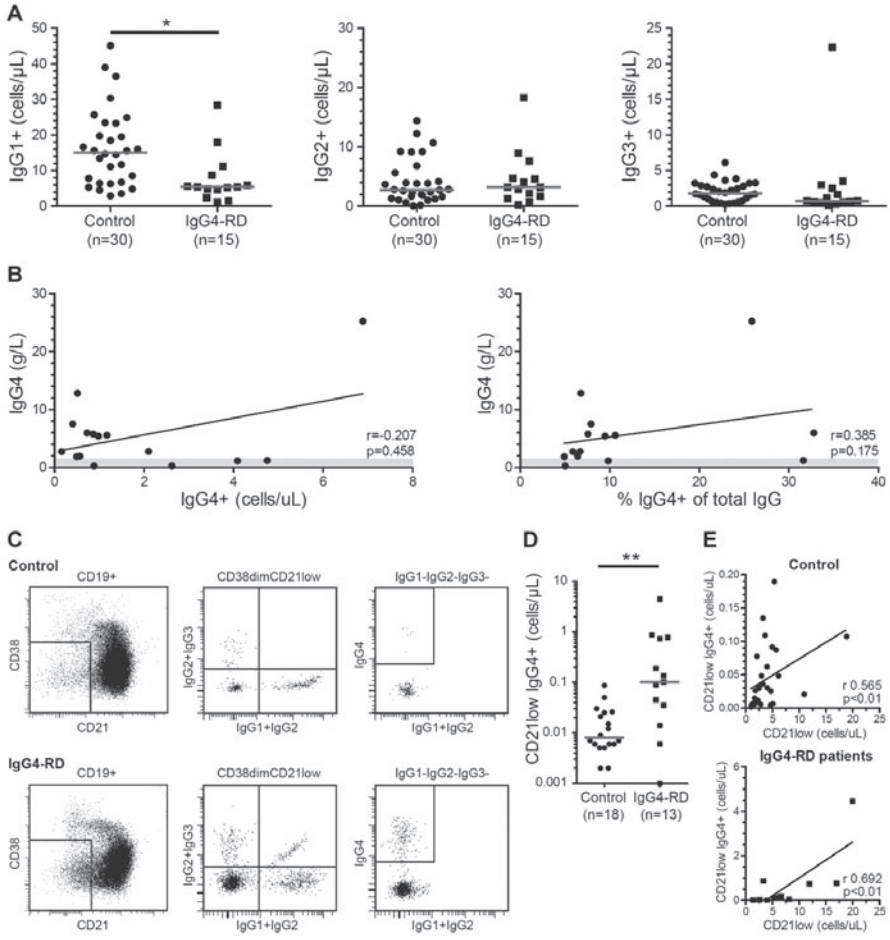
| | serum immunoglobulins (g/L) | | | | blood leukocytes and subsets (cells/µl) | | | | |
|---|---|---|---|---|---|---|---|---|---|
| Patient | IgG1 | IgG2 | IgG3 | IgG4 | leukocytes | lymphocytes | T-cells | B-cells | NK-cells |
| 1 | 8.61 | **6.8** | 0.4 | **2.12** | 6500 | **3480** | **2640** | **620** | 220 |
| 2 | 10.40 | 2.28 | 0.34 | **2.73** | 4500 | 2440 | **1930** | 400 | *70* |
| 3 | 5.24 | 2.36 | 0.38 | **5.96** | 7300 | *790* | *610* | *60* | *90* |
| 4 | 5.37 | 2.13 | 0.35 | 1.22 | 6200 | 1960 | 1640 | 200 | 120 |
| 5 | 4.59 | 3.16 | 0.62 | 0.27 | **12100** | **4470** | **3500** | **490** | **450** |
| 6 | 10.4 | 4.56 | 0.75 | **7.50** | 4800 | 1790 | 1480 | 110 | 180 |
| 7 | 6.49 | 2.49 | 0.32 | **1.85** | 7100 | 1650 | 1160 | 190 | 270 |
| 8 | 6.89 | 3.39 | 0.59 | **1.93** | 6500 | 2040 | 1510 | 270 | 220 |
| 9 | **25.1** | 5.12 | 0.96 | **12.8** | 5800 | 2360 | 1620 | **570** | 130 |
| 10 | 5.27 | 4.31 | 0.35 | 0.27 | 8800 | **2970** | **2140** | **430** | 370 |
| 11 | 10.4 | 2.93 | 0.32 | **5.75** | 7700 | 1280 | 1040 | *60* | 170 |
| 12 | **11.4** | 2.68 | 1.09 | **25.25** | **10200** | **4100** | **3300** | 280 | **440** |
| 13 | 6.85 | 4.66 | 0.23 | **2.76** | 5400 | 2280 | 1440 | 210 | **530** |
| 14 | 9.91 | **7.15** | 0,64 | **5.40** | 7600 | 1530 | 1130 | 110 | 260 |
| 15 | **19.30** | 2.27 | **2.28** | 1.13 | 7000 | 1710 | 1010 | **470** | 150 |
| 16 | 8.87 | 4.36 | 0.36 | **5.55** | 6800 | 1480 | 1150 | *60* | 160 |
| normal range | 4.9-11.4 | 1.5-6.4 | 0.2-1.1 | 0.08-1.4 | 3500-10000 | 1100-2500 | 700-1900 | 100-400 | 100-400 |

Subnormal values are depicted in italics, supranormal values in bold font

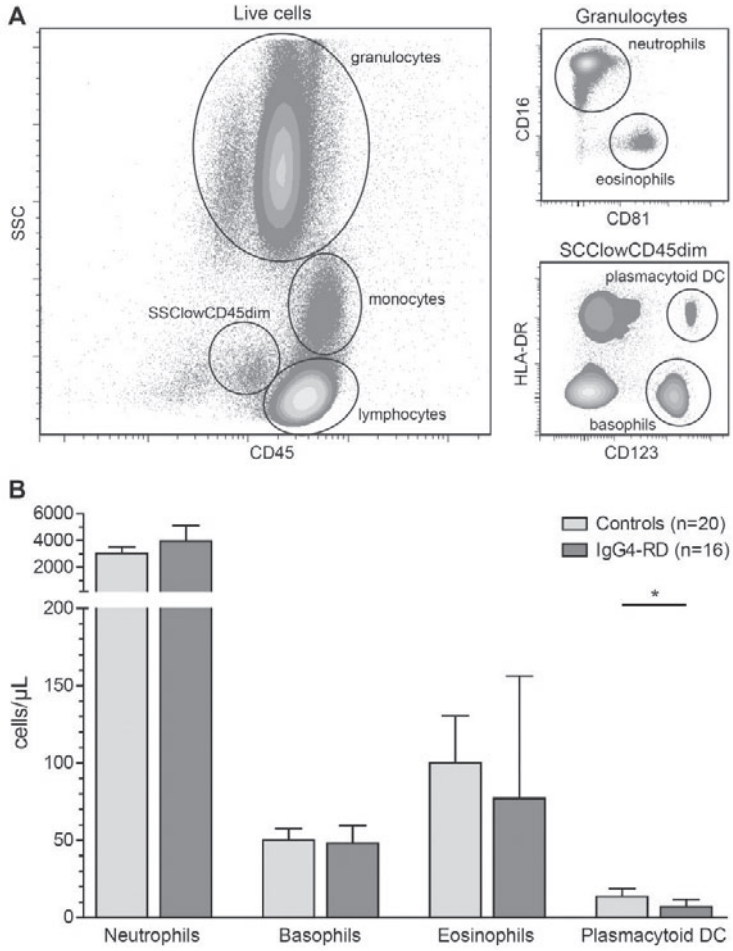
**Supplemental Table 2.** Clinical and immunological characteristics of patients with Sarcoidosis

| patient | gender | age (yr) | organs affected | medication at inclusion | CRP (mg/L) | ESR (mm/h) | immunoglobulins (g/L) | | | | Leukocytes (cells/µL) | T-cells (cells/µL) | B-cell (cells/µL) |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | | | | | | | IgG1 | IgG2 | IgG3 | IgG4 | | | |
| 1 | F | 43 | lung, eye, skin | n.i.m. | 2.6 | 3 | n.d. | n.d. | n.d. | n.d. | 5000 | *430* | 180 |
| 2 | F | 33 | lymph node, eye | n.i.m | **12** | **22** | 5.56 | 3.51 | 0.31 | *0.05* | 4300 | *350* | *80* |
| 3 | M | 67 | ocular | n.i.m | 1.0 | 5 | 5.90 | 2.15 | 0.27 | 0.36 | 8100 | **2180** | 200 |
| 4 | M | 49 | lung, lymph node | n.i.m | 3.9 | **49** | **11.8** | 6.38 | 0.79 | **3.47** | 6300 | 980 | **450** |
| 5 | M | 59 | lymph node | n.i.m | 0.7 | **13** | 9.0 | 4.74 | 0.30 | 1.22 | 8800 | 760 | 330 |
| 6 | M | 44 | lymph node, eye | n.i.m | 0.6 | 7 | 9.32 | 2.60 | *0.13* | 0.82 | 3600 | 930 | 140 |
| 7 | F | 66 | lymph node, skin | n.i.m | 2.4 | 12 | 6.81 | 3.21 | 0.35 | 0.17 | 7200 | 940 | **570** |
| 8 | F | 86 | lymph node, eye | n.i.m | **27** | 12 | 6.56 | 2.27 | 0.31 | 0.28 | 5500 | 830 | *90* |
| 9 | M | 51 | eye, lymph node, kidney | n.i.m | **10** | 12 | 7.12 | 3.88 | 0.47 | 0.23 | 4100 | *430* | 260 |
| 10 | M | 52 | eye, lung | plaquenil | 0.3 | 3 | 7.18 | 2.74 | 0.34 | 0.51 | 3700 | *350* | *60* |
| 11 | F | 58 | lung, eye, skin, joint | n.i.m | n.d. | n.d. | 7.78 | 2.66 | *0.11* | 0.55 | 5700 | 1220 | 380 |
| normal range | | | | | <10mg/L | 20mm/h | 4.9-11.4 | 1.5-6.4 | 0.2-1.1 | 0.08-1.4 | 3500-10000 | 700-1900 | 100-400 |

CRP, C-reactive protein; ESR, erythrocyte sedimentation rate; n.i.m., no immunomodulatory medication, n.d., not determined; Subnormal values are depicted in italics, supranormal values in bold font.




Supplemental **Figure 1.** A. Absolute counts of IgG1+, IgG2+ and IgG3+ memory B-cells. Each dot represents one individual, red lines represent median values. Statistical analysis between the groups was performed with the Mann Whitney U test; *P<0.05. B. Correlation between IgG4 serum values and absolute numbers of IgG4+ memory B-cells and between percentages of IgG4+ B-cells within total IgG+ B-cells. Grey shaded area indicates the reference value for IgG4 serum values. Correlation was calculated with Spearman R. C. Representative plots of the flow cytometric analysis of IgG subclass expressing $CD21^{low}$ B-cells D. Absolute counts of $CD21^{low}$ IgG4+ B-cells. Each dot represents one individual, red lines represent median values. Statistical analysis between the groups was performed with the Mann Whitney U test; *P<0.05, **P<0.01. E. Correlation between $CD21^{low}$ B-cells and IgG4+ $CD21^{low}$ B-cells in healthy controls and in patients with IgG4-RD.




Supplemental **Figure 2. A.** Representative plots of the flow cytometric analysis of granulocyte subsets and plasmacytoid dendritic cells (DC) B. Absolute counts of neutrophils, basophils, eosinophils and plasmacytoid DC. Columns represent median values with interquartile range. Statistical analysis was performed with the Mann Whitney U test, *P<0.05.

# CHAPTER 8

# Local and systemic signs of chronic B-cell responses in IgG4-related disease

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We recently devised a method to examine IgG4+ B cells in blood and applied this to study patients with IgG4-related disease (IgG4-RD) with the implication of a non-invasive test.[1, 2] In our cohort of patients that all met the Boston criteria for IgG4-RD, $IgG4^+$ memory B cell numbers were increased,[2] as well as circulating plasma blasts, $CD21^{low}$ B cells, T-helper (Th)2 and regulatory T cells (Treg) that were reported previously in other cohorts.[3, 4] Together, these abnormalities were regarded as a specific "lymphocyte signature", and we devised a combined score that could discriminate patients from controls with 93.3% sensitivity and 87.8% specificity.[2]

The accumulation of IgG4-expressing B cells and plasma cells is thought to result from enhanced T-cell dependent germinal center responses in lymphoid and non-lymphoid tissue of affected patients. Indeed, of 16 previously reported[2] and 3 newly included patients, 17 (89%) had B-cell follicles in affected tissue (Fig 1A, Table 1, Fig 2). In contrast, we did not identify cellular or molecular signs of enhanced B-cell activation in blood. Rather, IgG4+ memory B cells showed a more anergic phenotype with significantly more of these cells lacking CD21 and CD27 expression, and having reduced expression levels of CXCR5, a chemokine receptor important for homing to B-cell follicles.[2] Moreover, transcripts of these IgG4+ B cells did not show enhanced accumulation of somatic hypermutations (SHM). Additional SHM analysis on transcripts amplified with an IgG consensus primer revealed no increased SHM in the other IgG subclasses either, which is in contrast to patients with sarcoidosis (Fig 1B, Fig 3).[5] Still, the frequency of IgG2 transcripts was significantly increased (Fig 3), fitting with our flowcytometry data[2] and repeated immune responses.[1] This contrasted a previous study, where increased SHM were found in the FR3 and CDR3 regions of IgG4 transcripts. It should be noted that FR3 and CDR3 form less than $1/3^{rd}$ of the variable domain and that mutations in the CDR3 are notoriously difficult to define, because this includes the junctional regions between IGHV, IGHD and IGHJ genes with random nucleotide insertions and deletions from the V(D)J recombination process. Our analysis involved the strongly recommended approach for SHM analysis, which included the whole IGHV gene (>280 nt) including FR1, FR2 and FR3, and the CDR1 and CDR2.[6] Thus, the patients in our original IgG4-RD cohort do show signs of chronic B-cell responses in affected tissue, whereas their circulating B cells have increased anergic properties.

Similar to Akiyama et al,[7] we did not find a significant increase in absolute numbers of circulating follicular helper T (Tfh) cells in our original study,[2] and this remained non-significant upon inclusion of our 3 new patients (Fig 1C). The importance of Tfh subsetting is a relative new finding and was not yet exploited at the start of our original study. Given recent reports that underline the important role of Tfh subsets in IgG4-RD,[7] we here analyzed Tfh1 and Tfh2 numbers in more detail and found the latter to be significantly increased as compared to controls (Fig 1C). However, circulating Tfh2 cell numbers were also significantly increased in patients with sarcoidosis, and are therefore less specific for IgG4-RD as circulating IgG4+ B cells, Treg cells and Th2 cells.

**Table 1.** Histological findings in patients with IgG4-RD

| patient | gender | age (yr) | organs affected | IgG4+ PC/HPF (ratio IgG4/IgG) | B-cell follicles | medication at inclusion | time until diagnosis | serum IgG4 (g/L) |
|---|---|---|---|---|---|---|---|---|
| 1 | F | 60 | orbita | 149 (> 0.4) | + | n.i.m. | 14 years | **2.12** |
| 2 | F | 67 | orbita | >50 (>0.4) | + | n.i.m. | 8 years | **2.73** |
| 3 | M | 63 | orbita, lymph node, lung, prostate | >100 (>0.4) | + | prednisone | 3 years | **5.96** |
| 4 | M | 54 | orbita | 156 (0.90) | + | prednisone | 10 years | 1.22 |
| 5 | F | 53 | orbita | >64 (0.4) | + | n.i.m. | 3 years | 0.27 |
| 6 | M | 60 | orbita, lymph node | 411 (0.67) | + | t.n. | 5 years | **7.50** |
| 7 | M | 44 | orbita | 110 (0.4) | + | t.n. | 12 years | **1.85** |
| 8 | F | 59 | skin | >135 (0.95) | - | t.n. | 5 months | **1.93** |
| 9 | M | 18 | lung, lymph node, cerebra | 150 (>0.4) | + | t.n. | 1 month | **12.8** |
| 10 | F | 62 | pancreas, lymph node, lung | 131 (0.72) | + | t.n. | 3 months | 0.27 |
| 11 | M | 74 | lymph node, salivary gland, lung | 138 (0.7) | + | t.n. | 5 months | **5.75** |
| 12 | M | 53 | mesenterium | 458 (0.5) | + | t.n. | 20 years | **25.25** |
| 13 | M | 62 | thyroid gland | 330 (0.8) | + | t.n. | 1 year | **2.76** |
| 14 | M | 32 | serous membrane | 136 (0.70) | + | t.n. | 3 months | **5.40** |
| 15 | M | 79 | lymph node, pancreas | 1223 (0.9) | + | t.n. | 1 year | 1.13 |
| 16 | M | 60 | bile duct, lymph node | 205 (0.63) | n.d. | t.n. | 2 weeks | **5.55** |
| 17 | M | 48 | mesenterium | 120 (0.56) | + | t.n. | 5 months | **3.01** |
| 18 | M | 60 | orbit, ENT | 207 (0.85) | + | t.n. | 6 years | **15.02** |
| 19 | M | 74 | pancreas, orbit, lymph node | 210 (0.8) | + | t.n. | 3 years | **8.82** |

PC, plasma cells; HPF, high power field; n.i.m., no immunomodulatory medication for at least 6 months prior to inclusion; t.n., treatment naive; n.d., not determined; ENT, ear-nose-throat. IgG4 serum levels above normal range are shown in bold font. Histology analysis was performed on the affected organ listed first.

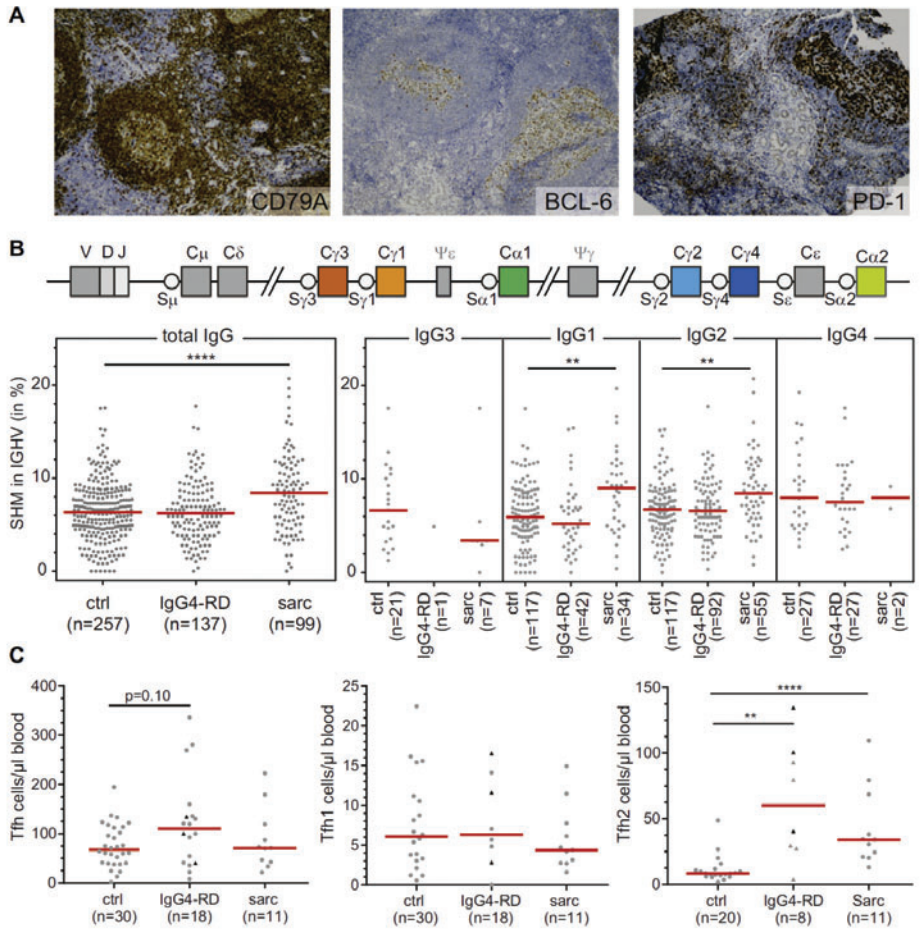


**Figure 1.** B-cell follicles, somatic hypermutations and follicular helper T-cell subsets in patients with IgG4-RD. A. Immunohistochemical analysis of orbital tissue from patient 7 reveals the presence of CD79A positive B-cell follicles containing Bcl-6 positive, PD-1+ B follicular helper T(fh) cells. B. Somatic hypermutations (SHM) in IgG transcripts and IgG subclasses of adult controls, patients with IgG4-RD and patients with sarcoidosis. C. Numbers of Tfh, Tfh1 and Tfh2 cells in blood of controls, patients with IgG4-RD and patients with sarcoidosis. Black triangles denote newly included IgG4-RD patients. Statistics: Mann-Whitney U test, ** p <0.01, **** p <0.0001.

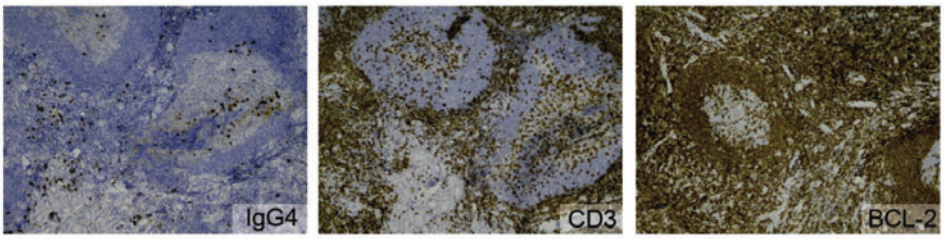


**Figure 2.** Additional immunohistochemical analysis of orbital tissue From IgG4-RD patient 7 with anti-IgG4, CD3, and Bcl-2.

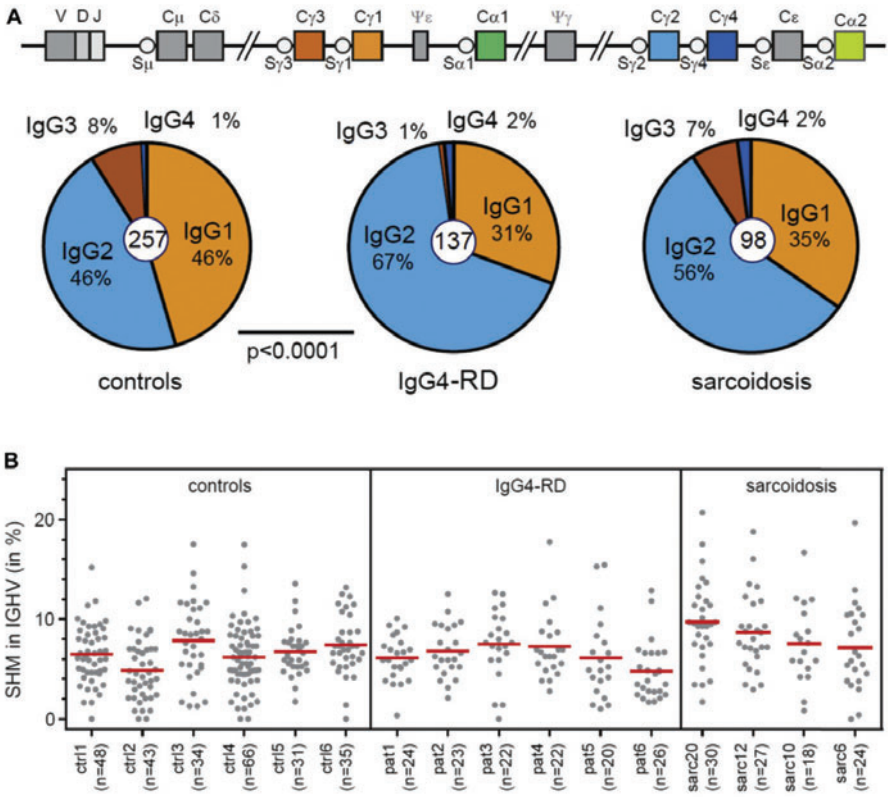


**Figure 3. Somatic hypermutation analysis in IgG transcripts. A.** IgG transcripts were amplified with an IgG consensus primer from 6 IgG4-RD patients and the relative composition of IgG subclasses was compared to previously generated data from controls and from patients with sarcoidosis. Statistics, $\chi 2$ test. **B.** Somatic hypermutation levels in unique transcripts from 6 controls, 6 IgG4-RD patients and 4 patients with sarcoidosis.

## AUTHOR CONTRIBUTIONS

JJH, AFK, JAMvL, WAD, PMvH and MCvZ designed research. JJH and AFK performed research. JJH, AFK and MCvZ analyzed data. RMV and DP evaluated and included patients in the study. JJH, AFK and MCvZ wrote the paper, and all authors commented on the paper and approved the final version.

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# SUPPLEMENTAL DATA

## Methods

*Histopathology*

All patients with IgG4-RD were histologically diagnosed. The amount of IgG4+ plasma cells per hpf (0.28 mm2) and the IgG4/IgG ratio were measured (Table 1), and all patients met the Boston criteria for IgG4-RD. The hematoxylin and eosin stainings were analyzed at the Department of Pathology of the Erasmus MC Rotterdam by a trained pathologist with experience in diagnosing IgG4-RD. The deparaffinized formalin-fixed paraffin–embedded sections of the tissue (4 mm thick) were stained using a BenchMark automated immunostainer (Ventana, Tucson, Ariz) with the Ultraview Universal diaminobenzidine detection kit (Ventana). Mouse antihuman IgG (clone A57H, 1:200; Dako, Carpinteria, Calif) and mouse antihuman IgG4 (clone HP6025, 1:600; Invitrogen Zymed, Camarillo, Calif) were used for immunohistochemical staining and were applied to the sections for 32 minutes. Additional stainings were performed with CD3 (2GV6, ready to use), CD79A (Sp18, ready to use), and Bcl-2 (Sp66, ready to use; all from Ventana) and Bcl-6 (G11GE/A8, 1:10) and PD-1 (Nat105, 1:50; both from Cell Marque, Rocklin, Calif).

*Molecular analysis of IgG transcripts*

RNA was isolated from post-Ficoll mononuclear cells of IgG4-RD patients 5, 7, 8, 10, 11, and 12 with a GenElute mammalian RNA kit (Sigma-Aldrich, St Louis, Mo) and reverse transcribed to cDNA with random primers (Thermo Fischer,Waltham,Mass). Rearranged IgGtranscripts were amplified in a multiplex PCR approach using 4 different IGHV-family leader forward primers in combination with an IGHG-consensus (59CACGCTGCTGAGGGAGTAG) reverse primer. E1 PCR products were cloned into a pGEMTeasy vector (Promega, Madison,Wis), amplified by colony PCR, and sequenced by Micromon facility of Monash University on an Applied Biosystems 3730s DNA Analyzer (Thermo Fisher). Obtained sequences were analyzed using the IMGT database (http://www.imgt.org/IMGT_vquest/vquest) to assign the IGHV, IGHD, and IGHJ genes and alleles, and to identify SHMs. Of each unique clone, the position and frequency of mutations were determined within the entire IGHV gene (FR1-CDR1-FR2-CDR2-FR3). SHMs were determined as variations on the best-matchedV-gene and represented as the percentage ofmutations of the total sequenced V-gene nucleotides. The IgG receptor subclasses were determined using the IGH reference sequence (NG_001019). All results of patients with IgG4-RD were compared with previously generated data sets of controls and of patients with sarcoidosis.

*Flow cytometry*

Stored PBMCs from controls, patients with IgG4-RD (2, 3, 6, 9, 15-19), and patients with sarcoidosis were thawed and prepared for detailed flow cytometric analysis of Th and Tfh

subsets. Two million PBMCs were incubated for 15 minutes at room temperature in a total volume of 100 mL with the following antibodies: CD4-BV510 (RPA-T4), CD25-BV421 (BC96), CD45RA-BV605 (HI100), CD127-APC (A019D5), CCR4-PE-Cy7 (L291H4), CCR6-PerCPCy5.5 (G034E3; all from Biolegend, San Diego, Calif), CD3-BV711 (UCHT1), CD8-APC-H7 (SK1), CCR7-PE-CF594 (150503), CXCR3-PE (1C6/CXCR3), and CXCR5-BB515 (RF8B2; all from BD Biosciences, San Jose, Calif). After preparation, cells were measured on a 4-laser LSRFortessa flow cytometer (BD Biosciences) using standardized settings. Data were analyzed with FACSDiva software V8.0 (BD Biosciences). Tfh cells were defined as CD31CD41CD45RA2CXCR51, and within this subset the Tfh1 cells were defined as being CCR62CXCR31CCR42 and Tfh2 cells as CCR62CXCR32CCR41.

*Statistical analysis*

SHM frequencies and both frequencies and absolute cell numbers were assumed a non-Gaussian distribution. Results were analyzed using the nonparametric Mann-Whitney U test. IgG subclass distributions were analysed with the chi-square test. All P values are 2-tailed and were considered statistically significant if valueswere lower than .05. Statistical analysiswas performed usingGraphPad Prism software, version 7 (GraphPad Software, La Jolla, Calif).

# PART V

# GENERAL DISCUSSION

# CHAPTER 9

# General discussion

Based on sections of:

**Is there a role for IgE in psoriasis?**

Heeringa JJ, van Zelm MC.

*Br J Dermatol. 2016 Jul;175(1):16-7*

**Correspondence: Systemic B-cell abnormalities in patients with atopic dermatitis?**

Heeringa JJ, Hajdarbegovic E, Thio HB, van Zelm MC.

*J Allergy Clin Immunol. 2016 Jul;138(1):317-8.*

## DISCUSSION

Abnormalities in serum antibody levels and specificities are hallmarks of many immune-mediated diseases. Patients with allergic diseases such as asthma, atopic rhinitis, food allergy and atopic dermatitis, have typical increased total and allergen-specific IgE. Successful treatment with immunotherapy in such patients results in rises of total and allergen-specific IgG4. Despite the detailed insights into antibody levels and applications in diagnostic work-up of patients, little is known about the cells that produce IgE and IgG4; i.e. memory B cells and plasma cells. The research presented in this thesis was conducted to expand our knowledge on IgE and IgG4 B-cell biology and to gain new insights into their maturation in healthy individuals and their possible role in the development of immune-mediated disease.

Through the development of a stepwise flow cytometric gating strategy we enabled the identification of IgE+ plasmablasts and two populations of IgE+ memory B-cells. The latter differ in CD27 expression, and detailed molecular characteristics of these subsets showed evidence for a T-cell independent origin of CD27-IgE+ memory B-cells and a T-cell dependent origin of CD27+IgE+ memory B-cells (Chapter II). Adults with atopic dermatitis as well as patients with Netherton disease, who have a reduced epidermal barrier function due to a mutation in the SPINK5 gene, displayed increased numbers of blood CD27-IgE+ memory B-cells (Chapters II, III and IV). Furthermore, children with various forms of allergic disease, i.e. asthma, atopic rhinitis, food allergy and atopic dermatitis, had increased numbers of circulating IgE+ memory B-cells and IgE+ plasmablasts, and the latter were directly correlated to Th2 cell numbers (Chapter III). Outpatient treatment at sea level and inpatient treatment at alpine climate had no effect on blood IgE+ B-cell numbers, but did result in a reduction of total plasmablast numbers, transitional B-cells and regulatory T(reg)-cells. Next to that, therapy in an alpine climate resulted in a significantly greater reduction of disease activity and was accompanied by a reduction of blood eosinophils, and increases in memory B-cells, CD8+ TemRO, CD4+ Tcm and CCR7+ Th2 cells (Chapter V). For several forms of IgE-mediated allergy successful desensitization of patients can be achieved with allergen immunotherapy. Sublingual immunotherapy for grass pollen allergy did not affect IgE+ memory B-cells numbers, but did lead to an increase in IgG4+ memory B-cell numbers. The resulting increase of IgG4+/IgE+ B-cell ratio may provide an explanation for the desensitization and reduction of complaints following treatment (Chapter VI). IgG4 has been associated with successful desensitization in allergic individuals following immunotherapy. However, patients with IgG4-related disease had increased numbers of IgG4+ memory B-cells. Furthermore, patients with IgG4 related disease (IgG4-RD) have increased numbers of Th2 cells and Tregs. Together, these subsets make up a specific "peripheral lymphocyte signature", which can be employed for diagnosis and treatment monitoring (Chapter VII).

## IgE B-cell memory

It has long been an enigma if IgE memory B-cells exist in man or if IgE memory function results from long-lived IgE producing plasma cells in bone marrow and circulating IgG+ memory B-cells which switch to IgE in secondary responses (1, 2). Experimental data from studies conducted in mouse models showed conflicting results and provided evidence for both models (3-6). In our studies presented in Chapter II, we showed that IgE+ B-cells can be reliably identified in peripheral blood of healthy individuals and in that of patients with atopic dermatitis. These cells lacked CD23 expression, excluding the possibility that the IgE positivity was the result of binding of soluble IgE to this low affinity FcεR. Moreover, mature IgE transcripts were detected by PCR-amplification and DNA sequencing from both bulk sorted IgE+ memory B-cells (Chapter II), and single cell sorted IgE+ plasmablasts and IgE+ memory B-cells (Chapter III). Importantly, both subsets showed phenotypic and molecular characteristics of memory B cells, and rapidly differentiated into IgE+ plasma cells upon in vitro stimulation with CD40 and IL-21. Thus in humans, there is a reservoir of circulating IgE+ memory B-cells with the ability to rapidly respond to antigenic stimulation.

Additional molecular characteristics of CD27+IgE+ memory B-cells showed evidence of T-cell dependent (TD) differentiation. In the typical germinal center reaction IL-4, IL-13, IL-21 and CD40 ligation is provided by follicular-helper T(fh)2 cells (7). However, CD27-IgE+ memory B-cells displayed molecular characteristics of T-cell independent (TI) maturation and switch regions showed evidence for more direct switch from naive IgM+ B-cells (Chapter II). TI maturation in CD27-IgE+ B-cells is supported by the fact that these cells could be detected in patients with a CD40 ligand deficiency in which there is an absence of cognate T-cell help due to the lack of direct B and T-cell interaction. Indeed next to T-cells also local basophils can be a source of IL-4. Cysteine proteases induce high IL-4 production by mouse basophils (8) and in vitro stimulation of human basophils with Der p 1 (i.e. a potent house dust mite (HDM) allergen) induces IL-4 secretion (9). The basophil derived IL-4 has been shown to control the function of innate lymphocyte type 2 cells (ILC2s) in lung inflammation (10), but may also provide the essential signals for local B-cells to undergo class switch recombination (CSR) to IgE. Furthermore, several studies have shown that ILC2 cells play an important role in the sensitization to allergen exposure through the production of IL-13 (11). IL-13 is mainly thought to prompt the migration of activated DCs towards draining lymph nodes, yet it may also play an important role in the local differentiation of naive B-cells towards IgE+ B-cells by providing the required signals for IgE CSR. This can be substantiated by studies which show that ILC2 cells facilitate the sensitization to local, but not to systemic allergen exposure (12).

Previous studies have already shown evidence for signs of CSR to IgE in B-cells within the bronchial and nasal mucosa of allergic individuals (13-17). Still, it would be interesting to directly observe IgE+ B-cells in local mucosal tissue and study their reactivity for certain allergens. We have already performed a pilot study to identify IgE+ memory B-cells with flow

cytometry in single-cell suspensions from nasal tissue of patients with nasal polyps (non-allergic) and indeed we could detect CD27-IgE+ memory B-cells and IgE+ plasma cells (Figure 1). Importantly, CD27+IgE+ memory B-cells were also present in single-cell suspensions of nasal tissue. Moreover, CD27-IgE+ memory B-cells could be identified in tonsils of children. Thus, there does not seem to be a clear distinction that CD27-IgE+ memory B-cells only differentiate in local mucosal tissue and that CD27+IgE+ memory B-cells only originate from germinal center reactions. Moreover IgE+ memory B-cells circulate through the periphery, which makes it difficult to exclude that these cells switch locally in the respective tissue. Still future studies performed with immunohistochemistry on tissue biopsies might identify which cells interact in mucosal tissue and play a role in peripheral IgE+ B-cell differentiation.

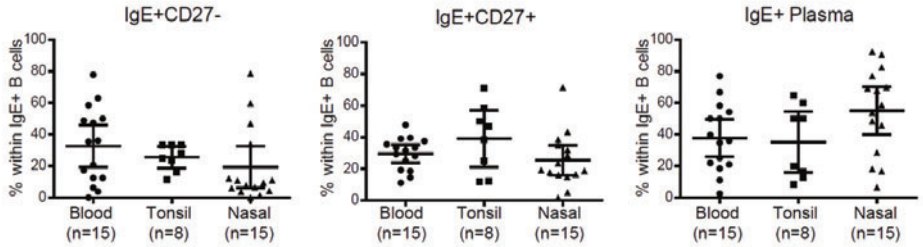


**Figure 1 Frequencies of IgE+ B-cell subsets in human tissues.** Blood and nasal tissue samples are from adults, tonsil samples are from children. All individuals are non-allergic, nasal tissues are from patients with chronic rhinosinusitis. Values are mean frequencies within total IgE+ B-cells from each individual, with 95%CI.

## Disrupted skin barrier affects IgE+ B-cell differentiation through increased allergen exposure

The underlying pathogenesis of IgE-mediated allergic disease is multifactorial, with genetic, environmental and immunological factors contributing to the onset of disease (18-20). Yet, there are monogenetic diseases with allergic manifestations. One of these is Netherton syndrome (NS), a severe genodermatosis typically characterized by chronic skin inflammation (ichtyosis and scaly erythroderma), specific hair shaft defects (trichorrhexis invaginata) and atopic diathesis with elevated IgE serum levels. The disease is caused by mutations in the SPINK5 gene (serine protease inhibitor of kazal type 5) encoding LEKTI (lympho-epithelial kazal type related inhibitor) which is expressed in the stratified epithelium of the skin, the mucosa and in the Hassal corpuscles of the thymus (21, 22). Analogous to patients with AD, patients with Netherton disease displayed increased numbers of CD27-IgE+ memory B-cells in blood, whereas numbers of CD27+IgE+ memory B-cells were normal (Chapter IV). Most probably this is the result of increased allergen infiltration in the underlying epidermis, since the consequence of LEKTI deficiency is a loss of inhibition of serine proteinases. This leads

to an abnormal skin homeostasis and detachment of the stratum corneum, which in turn contributes to a defective skin barrier enabling microbe and allergen penetration. Previously, it has also been suggested that increased thymic stromal lymphopoietin (TSLP) contributes to the atopic diathesis. This hypothesis comes from observations of SPINK5 knock out embryos in mice, which indicate a KLK5-Par2 cascade leading to enhanced production of TSLP, which would in turn lead to increased DC activation and allergen presentation in lymphoid structures. Our observations rather support increased allergen penetration as a main cause of atopic sensitization, because specifically T-cell independent CD27-IgE+ memory B-cell numbers were increased, whereas T-cell dependent CD27+IgE+ memory B-cell numbers were not affected.

### Alternatives to the IL4/IL13-driven pathway of IgE class switching?

Possibly there might also be alternatives to the IL4/IL13–IgE axis. Psoriasis, generally thought to be an auto-inflammatory disease of the skin, is mediated by T-helper (Th)1 and Th17 cells. DNA-LL37 complexes stimulate plasmacytoid (p)DC and myeloid (m)DC (23), which induce Th1 and Th17 cell differentiation through production of interleukin (IL)-12 and IL-23. IL-17A produced by Th17 cells is an important mediator in keratinocyte activation and proliferation (24). The efficacy of antibodies neutralizing IL-12/23 (ustekinumab) and IL-17A highlights the importance of the Th17 axis in psoriasis (25).

Unexpectedly, increased levels of serum Immunoglobulin E (IgE) have been reported in subsets of patients with psoriasis since the early 1980s (26-28). It has remained unclear whether the IgE in psoriasis induces similar responses as in allergic disease and might thereby contribute to disease pathogenesis. Yet, on top of increased serum IgE, lesional skin of patients with psoriasis contains more IgE and FcεRI bearing cells than non-lesional skin (29). These concerned especially mast cells, dermal Langerhans cells, epidermal DCs and macrophages. Following therapy with ustekinumab, the numbers of IgE and FcεRI bearing cells decreased. Although these findings can be merely a bystander effect of inflamed tissue, one could speculate about a Th2 independent pathway driving IgE responses.

Yet indeed, alternative to IL-4 and IL-13, IL-17A can promote IgE production by B-cells (30). Furthermore, autoreactive IgE has been identified in systemic lupus erythematosus (SLE), which can activate pDCs to secrete interferon-α (31). These observations would suggest a role for an IL-17 – IgE – pDC axis that contributes to self-destructive autoimmune responses (Figure 2). Most likely, the IgE response is not the primary mediator of pathogenesis, but is produced after the initial autoreactive IgG (32). Chronic stimulation of autoreactive IgG memory B cells could lead to secondary Ig class switching to IgE. The resulting soluble IgE will then be included in immune complexes and augment pDC responses by involving FcεRI. Current observations support such a mechanism for psoriasis as well (29). However, more functional studies would be needed to substantiate this. In contrast to atopic dermatitis, patients with psoriasis do not have increased numbers of IgE expressing B-cells (Chapter

II) (33). This could be a difference in etiology with most IgE in psoriasis being derived from sequential Ig class switching in the IgG memory reservoir. This could be investigated using in vitro stimulation of IgG memory B cells with Th17 cytokines. On top of total IgE, it would be important to establish if patients with psoriasis also have increased levels of autoreactive IgE, since anti-nuclear antibodies have been reported in patients with psoriasis (34). Finally, with the antigen-specific antibodies, functional studies can be performed to determine the effector cells in the skin. Are these the mast cells and basophils that mediate allergies, or the pDCs that are thought to be the main effector cells in SLE? Such detailed studies could help to unravel pathogenic processes in patients with psoriasis, to identify similarities and differences with other (auto-)immune diseases, and provide new targets for therapeutics.

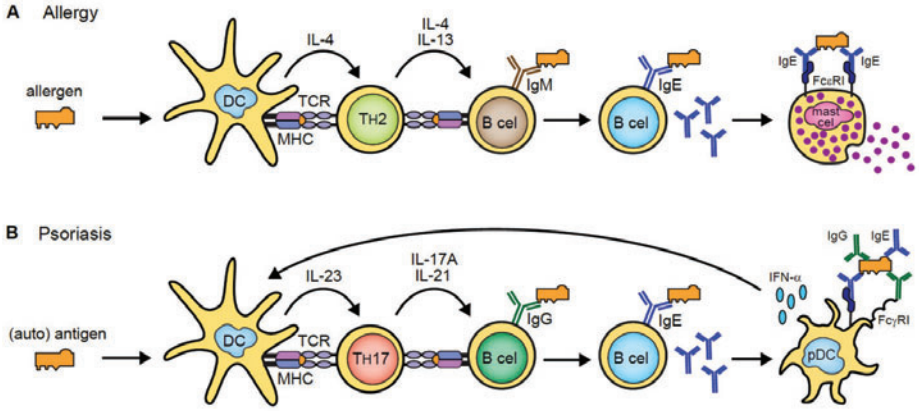


**Figure 2 Models for immunoglobulin (Ig) E responses in allergy and the nonclassical IgE response in psoriasis.** (a) Allergen is taken up by dendritic cells (DC) and presented to naive T-cells, which differentiate into T-helper 2 (TH2) cells. Interleukin (IL)-4 and IL-13 induce class switching in naive B cells to IgE. Allergen-specific soluble IgE produced by plasma cells binds to FceRI on mast cells and basophils. Subsequent allergen exposure leads to cross-linking of cell-bound IgE, resulting in allergic complaints. (b) Self-antigen is taken up by DC and presented to naive Tcells, which differentiate into TH17 cells. IL-17A induces class switching to IgE. Antigen–IgG–IgE complexes can bind to plasmacytoid DC (pDC) and recognition of self-antigen leads to the production of interferon-a, augmenting the psoriatic immune reaction.

## IgE+ B-cells as target for treatment and treatment monitoring of allergic disease

As shown Chapter III, median numbers of IgE+ B-cells in blood of patient groups with various forms of IgE-mediated allergies are significantly increased as compared to healthy children. However, the cell numbers in many patients did not fall outside the range of healthy controls and as a result there is some overlap in values between allergic individuals and non-allergic individuals. Next to that, neither circulating IgE+ plasmablast nor IgE+ memory B-cell numbers correlated with IgE serum levels. Possibly, serum IgE levels are dependent on the production of IgE by tissue residing plasma cells or by the capacity of mast cells, basophils

and eosinophils to bind free IgE. On the other hand, IgE serum levels are also not well correlated to disease severity. Therefore it will be interesting to study the reactivity of IgE+ memory B-cells and investigate if this can be directly correlated to reactivity testing of serum IgE with RAST or ISAC. Strategies to achieve this can be attained through single cell sorting and production of monoclonal antibodies, or through direct detection of allergen specific B-cells with labeled allergen extracts using flow cytometry.

Possibly, allergen specific IgE+ memory B-cells are more specific markers for allergic sensitization and these could be important targets for desensitization treatments possibly leading to a cure for allergic disease. Next to that, these could be used as easy tool for monitoring therapy success. As mentioned previously, memory B-cells can differentiate into plasma cells and thereby sustain the underlying sensitization. Therefore, targeting IgE+ B-cells with anti-IgE treatment might halt the persistent production of allergen-specific IgE. Already omalizumab (an anti-IgE antibody) is being used in patients with severe and difficult to treat asthma and it has shown to diminish the production of IgE (35). Omalizumab can recognize soluble IgE in serum, as well as membrane IgE on B cells. As omalizumab recognizes the domain that binds Fcε receptors, it cannot bind to FcεR-bound IgE and induce activation of target cells. However, as soluble IgE is more numerous than surface IgE on B cells, it is likely that in the majority of patients, the cells are not targeted and omalizumab will not affect IgE B-cell memory. This issue has been circumvented recently by the development of quilizimab. Quilizimab is an antibody directed at the M1' domain of IgE and thereby targets a segment of membrane IgE which is not present in soluble IgE (Figure 3). In mice it has been shown to effectively reduce serum IgE as well as IgE producing plasma cells in vivo (36). However, in patients with chronic spontaneous urticaria and in patients with inadequately controlled allergic asthma, quilizimab did not result in clinically significant improvements (37, 38). Still in both studies quilizimab reduced median serum IgE levels. Probably detection of IgE+ memory B-cells can contribute to patient selection for whom quilizimab would lead to beneficial clinical outcome. Since quilizimab directly targets IgE expressing B-cells, pre-treatment screening and selecting patients with increased IgE-memory B-cell numbers or more specifically, selecting patients with increased allergen specific IgE-memory B-cell numbers, could identify patients benefiting quilizimab therapy. Next to that, monitoring of IgE+ B-cell numbers during therapy could aid in determining therapy efficacy.



### The effect of different strategies treatments on peripheral B- and T-cell subsets

Most treatments for allergic disease are aimed at allergen avoidance or symptom relieve. In patients with food allergies this can be difficult but is often still feasible, whereas in patients with allergic asthma or atopic rhinitis this is often impossible. Local corticosteroid therapy to suppress immune mediated complaints or anti-histamines to specifically target mast cell and basophil mediators are then the first choice of therapy. Although these therapies can

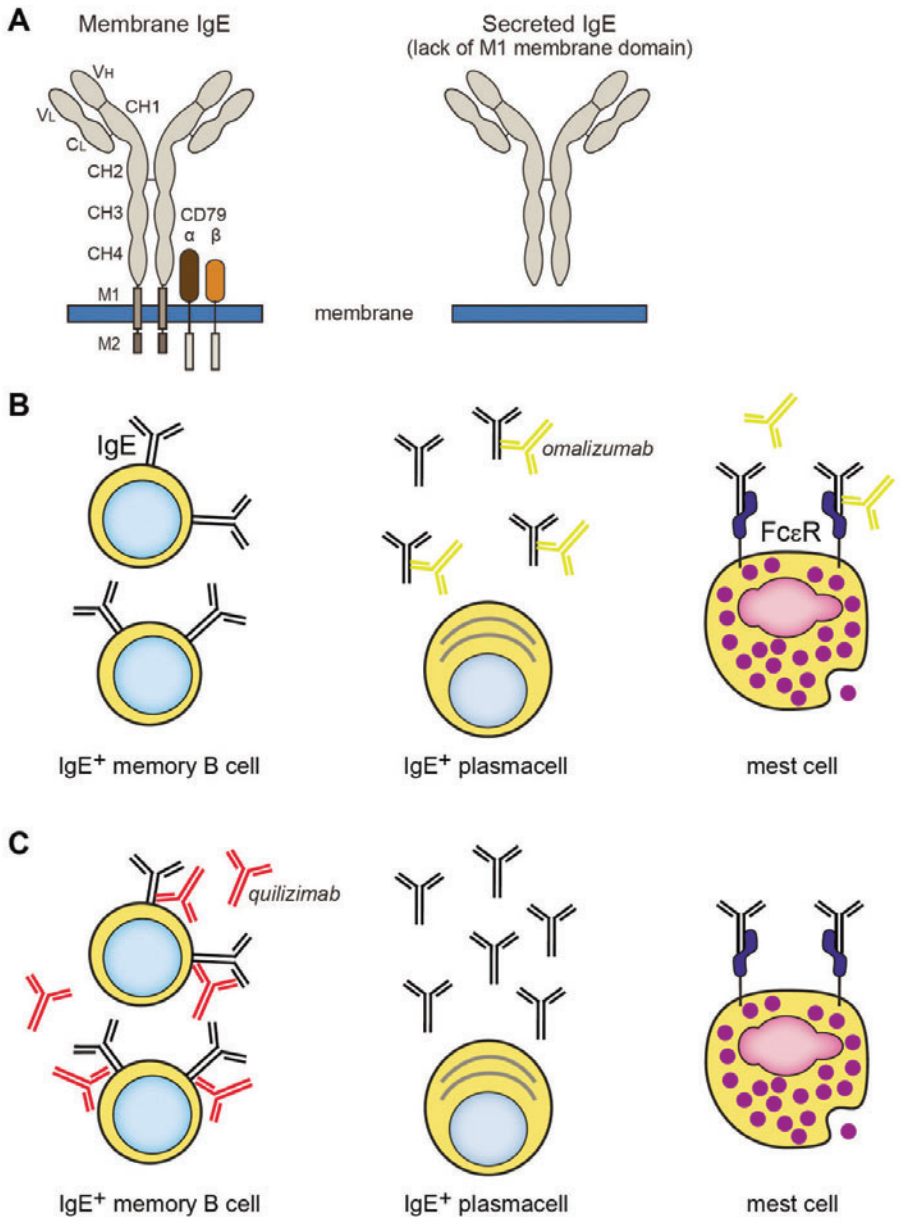


**Figure 3 Mechanism of anti IgE treatment. A.** IgE is composed of various variable and constant domains (CH1-CH4). Next to that membrane IgE also consists of the (trans)membrane domains M1 and M2 and is associated with the transmembrane signaling molecules CD79a and CD79b. **B.** The anti-IgE antibody omalizumab binds to the CH3 domain of IgE, which is present on soluble and membrane-expressed IgE, but which is masked by Fcε receptors on cell-bound IgE. **C.** The anti-IgE antibody quilizimab binds the M1' domain of surface IgE and therefore has the potential to target IgE expressing (memory) B-cells.

have good clinical outcomes, they are targeted at symptom repression and not at curation of the underlying allergy. Still, little is known about the effect of treatment on the peripheral immune compartment in patients with allergic disease whereas this could be important for prediction of treatment outcome. In the studies presented in Chapters V and VI we describe the effects on the peripheral immune system induced by multidisciplinary treatment at sea level and at alpine climate in children with atopic dermatitis and the effects induced by sublingual immunotherapy (SLIT) in adults with atopic rhinitis.

Notably, a profound difference observed between these studies was the effect on regulatory T-cells. This can be explained by the nature of the studied interventions, since the treatments performed in Chapter V were aimed at disease control rather than curation, whereas the treatment of SLIT described in Chapter VI, was intended to specifically resolve the allergic sensitization. In the case of symptomatic treatment (partially through allergen avoidance) disease control is reflected by a reduction of inflammation. Since in a physiological state Tregs suppress inflammation, this can be immunologically translated in a decrease of Tregs upon symptomatic treatment. On the other hand, treatment of disease through disease reversion, such as intended by SLIT, is reflected by skewing of the underlying inflammatory response. This can explain the decrease in Tregs after multidisciplinary treatment at sea level or at alpine climate and the increase of Tregs after SLIT. As a result of increased Treg cell numbers, IgG4 serum values also increase after SLIT. Consequently it is not surprising that the initial observed reduction in disease severity after climate treatment was not maintained after treatment cessation, whereas disease and symptom scores after immunotherapy tended to keep improving.

Still, studies presented in Chapter V and Chapter VI were focused at analyzing peripheral blood, whereas allergic reactions are often experienced at local sites, i.e. the skin and the mucosa of the upper airways. Therefore in the future, it is also necessary to study the immunological changes in these tissues in addition to peripheral blood. Or to specifically analyze tissue homing markers on the relevant immune subsets in blood. For example, are the increased numbers of CCR7+ Th2 cells which were observed after 6 weeks of alpine climate therapy expansions from blood or rather a shift from tissue (i.e. skin) towards blood?



### Deviation of the allergic response through immunotherapy

As mentioned before, we show in Chapter VI that grass pollen SLIT affects the underlying allergic response and that it leads to increased IgG4+ B-cell formation. IgG4 is proclaimed to have an immune dampening effect and thus leads an increased IgG4+/IgE+ B-cell ratio to a desensitizing effect which indeed, in our study, was accompanied by reduced allergic symptoms during the hay fever season. Based on our results it seems most probable that increased IgG4+ B-cells numbers are the result of the existing Th2 skewing, with production of IL4 and IL13, in combination with increased IL10 production from Tregs. Still it would be interesting to investigate how increased IgG4 production originates.

Differentiation of IgG4+ B-cells from (IgM+) naïve B-cells can be the result of direct class-switch recombination (CSR) from IGHM or from indirect CSR through an IGHG (IgG+) or IGHA (IgA+) intermediate. Because the constant domain encoding IgE (i.e. IGHE) is located downstream of IGHG4, and since in the process of CSR the intermediate DNA is excised from the genome, CSR from IgE+ B-cells towards IgG4+ B-cells is not possible. It is therefore not surprising that SLIT has no effect on the numbers of IgE+ memory B-cells. The only constant domain located more downstream of IGHE is IGHA2, encoding IgA2+ B-cells. In our study we did not discriminate between IgA1+ or IgA2+ B-cells, but in future studies it might be interesting to investigate if immunotherapy induces an increase in IgA2+ B-cells. Previous studies have already shown an increase of allergen specific serum IgA2 after grass pollen immunotherapy.(39, 40)

Furthermore it would be interesting to sequence the switch-regions from sorted IgG4+ B-cells after immunotherapy. These regions, just upstream of each IGH constant domain, contain the remnants of the intermediate switch regions and can indicate whether the cell underwent direct or indirect CSR, perhaps revealing more about the exact mechanism of immunotherapy. Based on data from a recently published re-entry model (41), one would expect that allergen specific IgE+ B-cells would engage in secondary immune response after sequential exposures to allergen, as is the case in immunotherapy. Yet also in the natural environment, during hay fever season, allergic patients are continuously exposed against their cognate allergen, and then desensitization does not occur. This confirms that indeed route of allergen exposure and allergen doses are also important factors in the process of deviating the allergic immune response.

Next to that, it is not entirely clear how the IgG4 antibody induces the desensitizing effect. Increased allergen specific IgG4 can directly compete for allergen, reducing the allergen load, but it is also thought to inhibit IgE-mediated responses through FcγRIIb binding, which is expressed on mast cells and basophils.(42, 43) Ligation or co-ligation of FcγRIIb downregulates the FcεRI mediated mast cell degranulation.(44-46) We observed an increase in rye grass pollen (RGP)-specific IgG4 which would suggests that the direct competition for allergen is the main mechanism in our study cohort.

### IgG4+ B-cells: a cell with two faces?

As mentioned before, IgG4 has been attributed to have immune dampening or regulating properties since it only has weak or negligible binding to both C1q and Fcγ receptors. Still in **Chapters VII and VIII** we showed that patients with IgG4-RD, a disease characterized by fibrosis and infiltration of IgG4-producing plasma cells of the affected organs, have elevated numbers of circulating IgG4+ B-cells. The debate remains if IgG4 in IgG4-RD is disease causing or a bystander effect of ongoing (uncontrolled) inflammation. We show that drivers of IgG4 B-cell development, i.e. Th2 cells and Tregs, are also increased, therefore IgG4+ B-cells do not necessarily need to be intrinsically aberrant to undergo (excessive)

proliferation. Moreover, no genetic mutations have been reported which would confirm fundamental defects in IgG4 biology. In principle, IgG4 has the most affinity for the inhibiting FcγRIIb, yet cross-linking of IgG4 might induce stimulating effects on the target cell. Next to that, in other diseases IgG4 has been specifically linked to disease pathogenesis. (47, 48). Nonetheless it would be interesting to sort IgG4+ B-cells from patients and study their replication history via KREC. This would give an indication if indeed extensive replication and thereby possible ongoing inflammation is the cause of increased IgG4. Also the generation of monoclonal antibodies of single cell sorted IgG4+ B-cells and subsequent reactivity testing could learn us more about underlying disease pathogenesis and specific triggers for B-cell stimulation. Previous studies showed that expanded plasmablast numbers in IgG4-RD, which were largely IgG4+, are mainly oligoclonal, and that re-emerging plasmablasts after rituximab therapy were clonally distinct from those prior to treatment (49). This would indicate that not a specific antigen is responsible for IgG4+ B-cell expansions but rather the underlying stimuli.

## CONCLUDING REMARKS

Based on current knowledge from literature and studies presented in this thesis, induction of allergic disease is initiated by increased allergen exposure in mucosal or epithelial tissue. This triggers the release of cytokines which favors IgE B-cell development and allergen specific IgE synthesis. It is very well conceivable that in each allergic individual different underlying mechanisms are causative for the onset of disease, e.g. a slightly reduced barrier function in one individual, a genetic tendency for Th2 skewing in another individual or high exposures to allergens in a third individual. Still the identification, characterization and observation of active contribution to disease of IgE+ B-cells in allergic individuals opens new possibilities for disease monitoring and, more important, the development of treatment strategies aimed at treating allergic sensitization.

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# PART VI

## ADDENDUM

## ABBREVIATIONS

| | |
|---|---|
| AD | atopic dermatitis |
| AID | activation induced cytidine deaminase |
| APC | antigen presenting cell |
| APRIL | proliferation inducing ligand |
| BAFF | B-cell activating factor |
| BCR | B-cell receptor |
| BM | bone marrow |
| BSA | body surface area |
| CD | cluster of differentiation |
| CDR | complementarity determining regions |
| CLA | cutaneous lymphocyte antigen |
| CRP | C-reactive protein |
| CSR | class switch recombination |
| CXCR | C-X-C chemokine receptor |
| ELA2 | elastase 2 |
| ESID | European Society for Immunodeficiencies |
| ESR | erythrocyte sedimentation rate |
| FA | food allergy |
| FeNo | fractional exhaled nitric oxide |
| FLG | filaggrin gene |
| FWR | frame work region |
| GC | germinal center |
| GWAS | genome wide association studies |
| Hep | histamine equivalent prick |
| ICAM1 | intercellular adhesion molecule 1 |
| Ig | immunoglobulin |
| IGA-NS | investigator's global assessment for Netherton syndrome |
| IgG4-RD | IgG4 related disease |
| IgκREHMA | Igκ restriction enzyme hot-spot mutation assay |
| IL | interleukin |
| ILC | innate lymphoid cell |
| ISAC | immune solid-phase allergy chip |
| IQR | interquartile range |
| KLK5 | kallikrein-related peptidase 5 |
| KREC | Kappa-deleting Recombination Excision Circle |
| LEKTI | lympho-epithelial kazal type related inhibitor |
| MHC | Major histocompatibility complex |

| | |
|---|---|
| NK | natural killer |
| NS | Netherton syndrome |
| PAR2 | protease-activated receptor 2 |
| PBMC | peripheral blood mononuclear cells |
| PCA | principal component analysis |
| PCR | polymerase chain reaction |
| pDC | plasmacytoid dendritic cell |
| RGP | rye grass pollen |
| SA-EASI | self-administerd eczema area and severity index |
| SCIT | subcutaneous immunotherapy |
| SHM | somatic hypermutation |
| SIT | specific immune therapy |
| SLE | systemic lupus erythematosus |
| SLIT | sublingual immunotherapy |
| SNPs | single-nucleotide polymorphisms |
| SPINK5 | serine protease inhibitor of kazal type 5 |
| STAT | signal transducer and activator of transcription |
| TACI | transmembrane activator and CAML interactor |
| TD | T-cell dependent |
| TFH | T-follicular helper |
| TH | T helper |
| TI | T-cell independent |
| TLR | toll-like receptor |
| TLSS-NS | total lesional sign score for Netherton syndrome |
| TNF | tumor necrosis factor |
| TSLP | thymic stromal lymphopoietin |

## SUMMARY

Allergies such as asthma, rhinitis, atopic dermatitis and food allergy are common diseases in both children and adolescents, but also affect many adults. The disease presentation can range from mild complaints such as itch and sneezing to life-threatening episodes demonstrated by anaphylactic shock. Immunoglobulin (Ig)E plays an important role in allergic disease. IgE is the antibody which can bind to non-pathogenic allergens such as pollens, house dust mite or peanut epitopes and thereby elicits the allergic reaction. Although serum IgE has been widely used in the diagnosis of allergic disease, still very little is known about the cells producing IgE as these are scarce and difficult to detect. A small subset of B-lymphocytes, i.e. memory B cells, express surface IgE and terminally differentiated B-cells, plasma cells, can secrete IgE. In this thesis, I developed new assays to examine IgE+ B cells and plasma cells in cellular and molecular detail, and assessed how these were different between healthy individuals and patients with allergic disease. Moreover, I adapted these assays to assess IgG4-expressing B cells, as this IgG4 isotype is associated with amelioration of allergic disease following immunotherapy, while also a being a major disease marker in patients with the chronic inflammatory condition IgG4-related disease (IgG4-RD).

In our first study, presented in Chapter 2, we developed a novel flowcytometric gating strategy enabling the identification of IgE+ B-cells in peripheral blood. We show that based on immunophenotypic properties these cells could be characterized as IgE+ memory B-cells, which either express the classical memory surface molecule CD27, or lack CD27 expression. Cell sorting of both IgE+ memory B-cells subsets and subsequent analysis of their molecular characteristics (i.e. proliferation history, somatic hypermutation status and Ig switch region properties), revealed that CD27+IgE+ memory B-cells displayed characteristics of a T-cell dependent maturation, whereas CD27-IgE+ memory B-cells seemed to differentiate through a T-cell independent pathway. This was confirmed in patients with a CD40L mutation, in whom cognate T and B-cell interactions are defective, as they carried CD27-IgE+ memory B-cells. Finally, adults with atopic dermatitis had increased numbers of peripheral blood CD27-IgE+ memory B-cells compared to healthy individuals and compared to patients with psoriasis, whereas numbers of CD27+IgE+ memory B-cells were comparable between these groups. This resulted in the conclusion that T-cell independent, possibly local development of IgE is enhanced in allergic patients.

To further investigate cellular characteristics of IgE+ B-cells in allergic diseases we performed a cross-sectional study in patients with different allergic diseases, which is presented in Chapter 3. Here we collected blood of children with asthma, food allergy and atopic dermatitis and determined numbers of IgE+ B-cells and of other cells which play an important role in the allergic reaction, such as Th2 cells and eosinophils. The results from this study confirmed our previous results that CD27-IgE+ memory B-cell numbers

are increased in patients with allergic disease as compared to non-allergic individuals. In contrast to previous results in adults, allergic children also had increased numbers of CD27+IgE+ memory B-cells. Moreover numbers of CD27+IgE+ memory B-cells were directly correlated to Th2 cell numbers, which illustrates the T-cell dependent differentiation pathway of these cells.

To further investigate a possible enhanced differentiation of IgE+ B-cells in local tissue, we investigated the lymphocyte compartment of patients with increased allergen exposure as the result of a disease with an impaired barrier function. Therefore, in Chapter 4, we studied IgE+ B-cells in patients with Netherton syndrome, which is characterized by mutations in the *SPINK5* gene and results in a reduced skin barrier function. Conform adults with atopic dermatitis, these patients had increased numbers of CD27-IgE+ memory B-cells in peripheral blood, but normal numbers of CD27+IgE+ memory B-cells as compared to healthy individuals. As a result almost all patients had increased IgE serum levels and all patients had complaints of atopic manifestations. Thus increased allergen exposure as a result of barrier defects in local tissue, seems to specifically enhance the differentiation of CD27-IgE+ memory B-cells, leading to allergic sensitization. This observation emphasizes the importance of an intact (mucosal) barrier.

Next we wanted to study the effect of treatment on the lymphocyte compartment and specifically on IgE+ memory B-cells. In Chapter 5 we studied the effect of different treatment strategies on the peripheral immune compartment in patients with allergic disease. Children with moderate to severe atopic dermatitis were randomized to either an intensive outpatient treatment in the Netherlands at sea level, or were assigned to an in-patient treatment in an alpine climate environment in Davos, Switzerland. Both protocols consisted of a 6-week multidisciplinary treatment with blood withdrawal before and directly after the 6-week treatment period. The results showed a clinical benefit after both treatments with reduced disease activity, objectified by allergic serum markers such as serum TARC and eosinophil numbers. However, in-patient treatment in an alpine climate setting resulted in significant better outcomes directly after the treatment period. Although numbers of various peripheral immune subsets changed after treatment, IgE+ B-cell numbers did not change. Possibly this indicates why the beneficial treatment effect did not sustain until 6 months after the treatments had finished. Still, both treatment protocols resulted in a reduction of circulating memory Tregs, transitional B-cells and plasma blast numbers.

A different treatment approach for allergic disease is desensitization through immunotherapy. With this treatment, low doses of the relevant allergen are exposed to, for example, mucosal tissue of the mouth. In Chapter 6 we studied the effect of desensitization through sublingual immunotherapy (SLIT) on circulating IgE+ B-cells and other B- and T-cell subsets. In our study the clinical benefit of SLIT is confirmed. Additionally, our data showed that IgE+ B-cell numbers were not affected, but that IgG4+ B-cell numbers increased and that through this, the IgE+/IgG4+ ratio decreases. This treatment effect may account for the

beneficial clinical effects we observed, since IgG4 is in principal thought to have immune dampening properties. Moreover, since these cells are memory cells, the favorable treatment outcomes may last for a longer period, which were also observed in our study.

As mentioned above, IgG4 is mainly presumed to have immune dampening effects. However increased IgG4 serum levels and tissue infiltration of IgG4 producing plasma cells are also seen in IgG4-RD, a disease further characterized by fibrosis and dysfunction of the affected organ. In Chapter 7 we further investigated this fibro-inflammatory condition and investigated the contribution of IgG4+ B-cells to disease pathogenesis. Our data showed increased numbers of blood IgG4+ memory B-cells in patients with IgG4-RD, with reduced expression of CD27 and CXCR5 and increased signs of antibody maturation. Furthermore, IgG4-RD patients had increased numbers of circulating plasma blasts and $CD21^{low}$ B-cells, as well as increased numbers of Th2 and regulatory T-cells. This specific "peripheral blood lymphocyte signature" we observed, may also be exploited to further support the diagnosis of IgG4-RD, which is now always dependent on an invasive tissue biopsy.

In addition to the previous study, we demonstrate in Chapter 8 that patients with IgG4-RD had B-cell follicles in affected tissue and active responses are reflected by increased blood Tfh2 cell numbers. However patients do not show cellular or molecular signs of enhanced B-cell activation in blood. Rather, IgG4+ memory B cells display a more anergic phenotype. Furthermore, transcripts of IgG4+ B cells do not show enhanced accumulation of somatic hypermutations. Therefore, the exact role of IgG4+ memory B-cells in IgG4-RD remains to be clarified.

In conclusion the results of the studies presented in this thesis demonstrate that IgE producing B-cells can be identified in peripheral blood and that IgE+ memory B-cells with a T-cell independent maturation pathway are increased in patients with allergic disease. Furthermore, treatment does not directly affect IgE+ memory B-cells but immunotherapy does lead to increased numbers of IgG4+ B-cells resulting in a beneficial IgE/IgG4 ratio and a favorable treatment outcome. IgG4+ memory B-cells however, can also be increased in the context of a chronic inflammatory disease. Whether these cells also directly contribute to the disease pathogenesis is not entirely clear.

# SAMENVATTING

Allergieën zoals astma, hooikoorts, atopische dermatitis (allergisch eczeem) en voedselallergie zijn veelvoorkomende ziekten bij zowel kinderen als adolescenten, maar treffen ook veel volwassenen. De aandoeningen kunnen variëren van milde klachten zoals jeuk en niezen tot levensbedreigende episoden zoals anafylactische shock. Bij allergische aandoeningen speelt Immunoglobuline (Ig) E een belangrijke rol. Immunoglobulines van verschillende klassen zoals IgM, IgG, IgA en IgE zijn antistoffen die door het immuunsysteem geproduceerd worden en in principe gericht zijn tegen bacteriën, virussen of parasieten. Je immuunsysteem kan vervolgens immuniteit ontwikkelen door het aanmaken van zgn. B-geheugencellen die er voor zorgen dat je bij een volgende blootstelling aan hetzelfde desbetreffende virus of bacterie sneller en sterker reageert. Bij een allergie daarentegen, wordt door het immuunsysteem IgE geproduceerd dat kan binden aan onschuldige stoffen zoals pollen, huisstofmijt of pinda en hierdoor kan een allergische reactie ontstaan. De meting van de hoeveelheid vrije, in het bloed circulerende, IgE wordt daarom veel gebruikt bij de diagnose van allergieën. Er is echter nog steeds weinig bekend over de cellen die IgE produceren, vooral omdat deze schaars zijn en moeilijk kunnen worden aangetoond. Deze IgE+ B-cellen bestaan uit B- geheugencellen, die IgE in de vorm van een B-cel receptor tot expressie brengen op hun celmembraan; en uit gedifferentieerde B-cellen, genaamd plasmacellen, die IgE kunnen uitscheiden. In dit proefschrift hebben we nieuwe testen ontwikkeld om deze IgE+ B-geheugencellen en plasmacellen op cellulair en moleculair niveau te onderzoeken en hebben daarmee onderzocht op welke manier deze cellen verschillen tussen gezonde personen en patiënten met een allergie. Daarnaast hebben we deze testen toegepast om B-cellen die IgG4 tot expressie brengen te onderzoeken, omdat het IgG4-isotype (een van de isotypes binnen de IgG klasse) geassocieerd is met verbetering van allergische aandoeningen na immunotherapie. Daarentegen is het ook een belangrijke ziektemarker bij patiënten met de chronisch inflammatoire aandoening, genaamd IgG4-gerelateerde ziekte (IgG4-RD).

In onze eerste studie (Hoofdstuk 2) hebben we met behulp van flowcytometrie een nieuwe methode ontwikkeld waarmee IgE+ B-cellen in bloed kunnen worden aangetoond. We hebben laten zien dat deze cellen op basis van uiterlijke eigenschappen kunnen worden onderscheiden in twee verschillende IgE+ B-geheugencelpopulaties, die ofwel het typische geheugenoppervlaktemolecuul CD27 tot expressie brengen, ofwel de CD27-expressie missen. Door middel van het sorteren van beide IgE+ B-geheugencelpopulaties en de daaropvolgende analyse van hun moleculaire eigenschappen op DNA niveau, bleek dat CD27+IgE+ B- geheugencellen kenmerken vertonen van een T-celafhankelijke rijping, terwijl CD27-IgE+ B- geheugencellen zich lijken te ontwikkelen via een T-cel onafhankelijke route. Dit werd bevestigd bij patiënten met een CD40L-mutatie, waardoor deze patiënten geen T- en B-celinteractie kunnen ondergaan, maar waarbij toch CD27-IgE+ geheugen cellen

konden worden aangetoond. Ten slotte hadden volwassenen met atopische dermatitis een verhoogd aantal CD27-IgE+ geheugen B-cellen in hun bloed ten opzichte van gezonde personen terwijl het aantal CD27+IgE+ geheugen B-cellen vergelijkbaar was tussen deze groepen. Dit resulteerde in de conclusie dat de T-cel onafhankelijke, mogelijk lokale, ontwikkeling van IgE is verhoogd bij patiënten met een allergie.

Om de eigenschappen van IgE+ B-cellen bij allergische aandoeningen verder te onderzoeken, hebben we een studie uitgevoerd bij patiënten met verschillende allergische aandoeningen (Hoofdstuk 3). Hiertoe hebben we bloed verzameld van kinderen met astma, voedselallergie en atopische dermatitis en hebben we gekeken naar aantallen van IgE+ B-cellen en andere cellen die een belangrijke rol spelen bij de allergische reactie, zoals T-helper 2 cellen (Th2) en eosinofielen. De resultaten van deze studie bevestigden onze eerdere resultaten dat de aantallen van CD27-IgE+ B-geheugencellen zijn toegenomen bij patiënten met allergische aandoeningen in vergelijking met niet-allergische personen. In tegenstelling tot eerdere resultaten bij volwassenen hadden allergische kinderen ook een verhoogd aantal CD27+IgE+ B-cellen. Bovendien konden de aantallen van CD27+IgE+ B-cellen direct worden gecorreleerd aan Th2-cel aantallen, wat de T-cel-afhankelijke differentiatie van deze cellen ondersteunt.

Om een mogelijk versterkte differentiatie van IgE+ B-cellen in lokaal weefsel verder te onderzoeken, onderzochten we in Hoofdstuk 4 het immuunsysteem van patiënten met het syndroom van Netherton, een zeldzame ziekte die gekenmerkt wordt door mutaties in het *SPINK5*-gen. De aandoening gaat gepaard met huidafwijkingen en een verminderde barrièrefunctie van de huid. Hierdoor zijn patiënten vatbaarder voor infecties en hebben ze vaak een verhoogde allergeenblootstelling. In overeenstemming met volwassenen met atopische dermatitis hadden deze patiënten een verhoogd aantal CD27-IgE+ geheugen B-cellen in het bloed, maar normale aantallen CD27+IgE+ geheugen B-cellen ten opzichte van gezonde personen. Als gevolg hiervan hadden bijna alle patiënten verhoogde serumconcentraties van IgE en hadden alle patiënten klachten van allergieën. Een verhoogde blootstelling aan allergenen als gevolg van barrière-defecten in lokaal weefsel lijkt dus specifiek de differentiatie van CD27-IgE+ B-geheugencellen te versterken, wat leidt tot allergische sensibilisatie. Deze waarneming benadrukt het belang van intacte barrières.

Vervolgens hebben we gekeken naar het effect van behandeling op het immuunsysteem en hebben we specifiek het effect op IgE+ B-geheugencellen bestudeerd. Daarbij hebben we verschillende behandelingsstrategieën onderzocht. In de studie beschreven in Hoofdstuk 5 werden kinderen met matige tot ernstige atopische dermatitis gerandomiseerd voor ofwel een intensieve poliklinische behandeling in Nederland op zeeniveau, ofwel voor een intramurale behandeling in een alpine klimaatomgeving in Davos, Zwitserland. Beide strategieën bestonden uit een 6 weken durende multidisciplinaire behandeling met bloedafname vóór en direct na deze behandelingsperiode. De resultaten toonden een klinisch voordeel van beide behandelingen met verminderde ziekteactiviteit, geobjectiveerd

door allergische serummarkers zoals serum-TARC en eosinofielen. Klinische behandeling in een alpine klimaatomgeving resulteerde daarbij echter tot significant betere resultaten direct na de behandelingsperiode. Hoewel de aantallen van verschillende lymfocytenpopulaties veranderde na de behandeling, veranderden de IgE+ B-cel aantallen niet. Mogelijk geeft dit aan waarom het gunstige behandelingseffect niet standhield tot 6 maanden nadat de behandelingen waren beëindigd. Toch resulteerden beide behandelingsprotocollen in een vermindering van circulerende regulatoire T-cellen, transitionele B-cellen en plasmablasten (onrijpe plasmacellen).

Een andere benadering voor de behandeling van allergische aandoeningen is desensibilisatie door immunotherapie. Met deze behandeling wordt het immuunsysteem blootgesteld aan lage doseringen van het betrokken allergeen. In Hoofdstuk 6 hebben we het effect bestudeerd van desensibilisatie door middel van sublinguale immunotherapie (SLIT), oftewel dagelijks "een tabletje onder de tong", en hebben we gekeken naar de aantallen van circulerende IgE+ B-cellen en andere B- en T-cel populaties. In onze studie werd het klinische voordeel van SLIT bevestigd. Bovendien toonden onze resultaten aan dat de IgE+ B-celaantallen niet werden beïnvloed, maar dat de IgG4+ B-celaantallen toenamen en dat hierdoor de IgG4+/IgE+ verhouding ook toenam. Dit behandeleffect kan verantwoordelijk zijn voor de gunstige klinische effecten die we hebben waargenomen, omdat wordt verondersteld dat IgG4 immuundempende eigenschappen heeft. Omdat deze IgG4+ B-cellen geheugencellen zijn, kunnen de gunstige behandelresultaten een langere periode aanhouden, wat ook in onze studie werd vastgesteld.

Zoals eerder genoemd, wordt over het algemeen aangenomen dat IgG4 immuundempende effecten heeft. Verhoogde IgG4-serumniveaus en weefselinfiltratie van IgG4-producerende plasmacellen worden echter ook gezien bij de IgG4 gerelateerde ziekte (IgG4-RD), een ziekte die verder wordt gekenmerkt door fibrose (verlittekening) en verminderde functie van het aangetaste orgaan. In Hoofdstuk 7 hebben we deze fibro-inflammatoire aandoening verder onderzocht en de bijdrage van IgG4+ B-cellen aan de ziekte pathogenese bestudeerd. Onze resultaten toonden verhoogde aantallen IgG4+ B- geheugencellen in het bloed van patiënten met IgG4-RD, met daarbij verminderde expressie van CD27 en CXCR5 en verhoogde tekenen van antistofrijping (verhoogd aantal mutaties in de genen die voor de B-cel receptor coderen). Bovendien hadden patiënten met IgG4-RD een verhoogd aantal circulerende plasmablasten en $CD21^{low}$ B-cellen, evenals een verhoogd aantal Th2 en regulerende T-cellen. Deze specifieke kenmerken namen wij waar in het bloed en kunnen mogelijk worden gebruikt voor het verder ondersteunen van de IgG4-RD diagnose, die nu nog afhankelijk is van invasieve weefselbiopten.

In aanvulling op de vorige studie, laten we in Hoofdstuk 8 zien dat patiënten met IgG4-RD B-celfollikels hebben in aangetast weefsel en dat actieve reacties worden weerspiegeld door verhoogde folliculaire T-helper-2 cel aantallen in het bloed. Patiënten vertonen echter geen cellulaire of moleculaire kenmerken van versterkte B-celactivering in het bloed.

Integendeel, IgG4+ B- geheugencellen vertonen een meer anergisch (=niet reactief) fenotype. Bovendien vertonen transcripten van IgG4+ B-cellen geen aanwijzingen van antistofrijping. De exacte rol van IgG4+ B- geheugencellen in IgG4-RD zal dan ook nog verder moeten worden opgehelderd.

Concluderend tonen de resultaten van de verschillende studies in dit proefschrift aan dat IgE-producerende B-cellen in bloed kunnen worden geïdentificeerd en dat de aantallen IgE+ B-geheugencellen met een T-celonafhankelijke rijpingsroute zijn toegenomen in patiënten met een allergische aandoening. Bovendien beïnvloedt behandeling niet direct de IgE+ B geheugencel aantallen, maar immunotherapie leidt wel tot verhoogde aantallen IgG4+ B-cellen, wat resulteert in een gunstige IgE/IgG4-verhouding met daarbij een gunstig behandelresultaat. IgG4+ B-geheugencellen kunnen echter ook verhoogd zijn in de context van een chronische ontstekingsziekte. Of deze cellen daarbij ook rechtstreeks bijdragen aan de ziektepathogenese is nog niet helemaal duidelijk.

# PHD PORTFOLIO

| | |
|---|---|
| Name PhD student: | Jorn J. Heeringa, MD |
| Erasmus MC department: | Dept. of Immunology |
| | Dept. of Pediatrics |
| Research school: | Erasmus Postgraduate School of Molecular Medicine |
| PhD period: | January 2012-August 2016 |
| Promotor: | Prof. dr. Jacques J.M. van Dongen, MD, PhD |
| | Prof. dr. Johan C. de Jongste, MD. PhD |
| Co-promotor: | Dr. Menno C. van Zelm, PhD |

## In-depth courses

| | | |
|---|---|---|
| 2012 | Course on Molecular Medicine | 0.7 ECTS |
| 2012 | Advanced course on Molecular Immunology | 3.0 ECTS |
| 2012 | Course on Biomedical Research Techniques XI | 1.5 ECTS |
| 2012 | Basiscursus regelgeving en organisatie voor klinisch onderz. (BROK) | 1.5 ECTS |
| 2014 | Projectmanagement promovendi en postdocs (NIBI) | 1.0 ECTS |
| 2014 | Deel van de Basiskwalificatie Onderwijs (BKO) | |
| 2014 | BKO Teach the teacher 1 | |
| 2014 | BKO Omgaan met groepen | |
| 2014 | Research integrity | 0.3 ECTS |
| 2015 | Photoshop and illustrator workshop | 0.3 ECTS |
| 2015 | English Biomedical Writing and Communication | 3.0 ECTS |

## Teaching activities

| | |
|---|---|
| 2012 – 2015 | Immunology teaching for medical students |
| 2012 | Tutor education medical students |
| 2012 – 2013 | Supervisor of higher laboratory education student |
| 2013 – 2014 | Supervisor of master student molecular medicine |
| 2015 | Supervisor of bachelor student Life Sciences |

## Seminars and symposia

| | |
|---|---|
| 2012 – 2016 | Seminars and mini-symposia at dept. of Immunology Rotterdam |
| 2012 – 2016 | Weekly journal club at dept. of Immunology Rotterdam |

## Presentations and conferences

*Mar 2012 - Annual Molecular Medicine Day, Rotterdam, the Netherlands*
Poster: "Small populations with major implications? Identification of two IgE+ memory B cell subsets in human blood"

*Dec 2012 – Annual Meeting Dutch Society for Immunology (NVVI), Noordwijkerhout, the Netherlands*
Oral: "Human IgE+ plasma cells and memory B-cell subsets are derived from germinal center-dependent and –independent responses"

*Feb 2013 – Keystone Symposium: B-cell development and function, Colorado, USA*
Poster: "Human IgE+ plasma cells and memory B-cell subsets are derived from germinal center-dependent and –independent responses"

*Dec 2013 – Annual Meeting Dutch Society for Immunology (NVVI), Noordwijkerhout, the Netherlands*
Poster: "IgE+ memory B cells in asthma and atopic dermatitis reveal distinct T-cell dependent and independent immune responses"

*Jan 2014 – Wetenschapsdagen Interne Geneeskunde, Antwerpen, Belgium*
Poster: "Human IgE+ plasma cells and memory B-cell subsets are derived from germinal center-dependent and –independent responses"

*Feb 2014 - Annual Molecular Medicine Day, Rotterdam, the Netherlands*
Poster: "IgE+ memory B cells in asthma and atopic dermatitis reveal distinct T-cell dependent and independent immune responses"

*Dec 2014 – Annual Meeting Dutch Society for Immunology (NVVI), Kaatsheuvel, the Netherlands*
Poster: "Differentiation pathways of blood and mucosal IgE+ plasma cells"

*March 2015 - Annual Molecular Medicine Day, Rotterdam, the Netherlands*
Oral: Presentation for best publication award 2015 "Human IgE+ B cells are derived from T cell-dependent and T cell-independent pathways"

*May 2015 – ESF-EMBO Conference on B-cells, St. Feliu de Guixols, Spain*
Oral: "Differentiation pathways of blood and mucosal IgE+ plasma cells"

*Dec 2015 – Australasian Society of Immunology (ASI), Canberra, Australia*
Poster: "IgG4 expressing B-cells: a possible new marker for IgG4-Related Disease"

## Awards

| | |
|---|---|
| 2015 | Best publication award post-graduate school of Molecular Medicine |

## Memberships

| | |
|---|---|
| 2012-2018 | Member of the Dutch society of Immunology (NVVI) |

## Funds

| | |
|---|---|
| 2012 | Sophia Children's Hospital Foundation for medical research (SFK)<br>PhD project grant "The B cell side of allergy and asthma: studying the origin of IgE to understand, classify, and monitor treatment of allergic diseases" |
| 2013 | Erasmus Trustfonds<br>Travel grant for Keystone Symposium: B-cell development and function, Colorado, USA – Feb 2013 |
| 2015 | NVVI Working visit grant<br>Working visit to Monash University, Melbourne, Australia, Nov 2015 - Apr 2016 |

## Extracurricular activities

| | |
|---|---|
| 2012 – 2013 | Member of the festivities committee FC at Dept. of Immunology |

# LIST OF PUBLICATIONS

**Heeringa JJ***, Fieten KB*, Bruins FM, van Hoffen E, Knol EF, Pasmans S, et al. Treatment for moderate to severe atopic dermatitis in alpine and moderate maritime climates differentially affects helper T cells and memory B cells in children. *Clin Exp Allergy* 2018; 48:679-90.

**Heeringa JJ**, Rijvers L, Arends NJ, Driessen GJ, Pasmans SG, van Dongen JJM, et al. IgE-expressing memory B cells and plasmablasts are increased in blood of children with asthma, food allergy, and atopic dermatitis. *Allergy* 2018; 73:1331-6.

Karim AF*, **Heeringa JJ***, van Laar JAM, Verdijk RM, Dik WA, Paridaens AD, et al. Reply. *J Allergy Clin Immunol* 2018; 141:1958-60 e4.

**Heeringa JJ***, Karim AF*, van Laar JAM, Verdijk RM, Paridaens D, van Hagen PM, et al. Expansion of blood IgG4(+) B, TH2, and regulatory T cells in patients with IgG4-related disease. *J Allergy Clin Immunol* 2018; 141:1831-43 e10.

**Heeringa JJ**, van Zelm MC. Is there a pathogenic role for IgE in psoriasis? *Br J Dermatol* 2016; 175:16-7.

**Heeringa JJ**, Hajdarbegovic E, Thio HB, van Zelm MC. Systemic B-cell abnormalities in patients with atopic dermatitis? *J Allergy Clin Immunol* 2016; 138:317-8.

**Heeringa JJ**, van Zelm MC. De bron van specifiek IgE: B-geheugencellen en plasmacellen. *Ned Tijdschr Allergie & Astma* 2015;3:71-77

Berkowska MA*, **Heeringa JJ***, Hajdarbegovic E, van der Burg M, Thio HB, van Hagen PM, et al. Human IgE(+) B cells are derived from T cell-dependent and T cell-independent pathways. *J Allergy Clin Immunol* 2014; 134:688-97 e6.

Labuda LA, Ateba-Ngoa U, Feugap EN, **Heeringa JJ**, van der Vlugt LE, Pires RB, et al. Alterations in peripheral blood B cell subsets and dynamics of B cell responses during human schistosomiasis. *PLoS Negl Trop Dis* 2013; 7:e2094.

# DANKWOORD

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Dan de mensen op het lab! Als eerste de mensen van de BCD groep; **Magda B.** thank you for teaching me the first practical skills considering lab research. I hope it hasn't been to frustrating in the beginning, but I think in the end it was a succes! **Diana, Magda R., Britt** en **Christina**, jullie ook allemaal bedankt voor technische uitleg, hulp met antistoffen, samples opvangen en prepareren als ik er niet was, of mentale ondersteuning (met koffie) voor wat dan ook. Ik ben ontzettend blij dat er altijd een groot samenhorigheidsgevoel is geweest binnen de groep! **Hanna**, jij als kamergenoot hoort hier natuurlijk net zo goed bij! En **Marieke** ook jij schaarde je steeds meer bij de BCD groep. Daarna waren we deels tegelijkertijd in Melbourne, zijn we begonnen met de opleiding Interne geneeskunde en nu promoveren we ook nog op dezelfde dag! Gelukkig allemaal leuke dingen om te delen.

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bei der Verteidigung zur Seite stehst. Dann weiß ich zumindest, dass auf jeden Fall ein echter Wissenschaftler dem PhD-Komitee gegenübersteht.

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Many thanks for a wonderful time also goes out to the Monash lab! All of you welcomed me so kindly and thanks to you I had a great time in Melbourne, including all the cappuccinos in Café 12, beers at College Lawn and burger picnics in Fawkner park. **Jodie, Beatriz, Pei, Dan, Katherine, Maria, Sam, Eliza, Angela, Mahtab, Gavin, Neeru** and all others thank you very much! Hope to see you soon!

Daarnaast is de mentale ondersteuning, of juist afleiding, naast het onderzoek doen net zo belangrijk voor het volbrengen van een succesvolle promotie. Daarvoor wil ik dan ook iedereen van mijn vrienden, collega's en familie bedanken die hier aan hebben bijgedragen.

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## CURRICULUM VITAE

Jorn Jesse Heeringa werd geboren op 27 december 1984 in Karlsruhe, Duitsland. Na 10 jaar in Duitsland te hebben gewoond, verhuisde hij in 1994 naar Brussel, België. Aldaar behaalde hij in 2002 het Europees Baccalaureaat aan de Europese School te Brussel. In hetzelfde jaar startte hij met de studie Economie aan de Erasmus Universiteit te Rotterdam. In 2005 stapte hij over naar de opleiding Geneeskunde waarbij in 2012 het artsexamen met Cum Laude werd behaald. Tijdens de opleiding Geneeskunde heeft hij 6 weken stage gelopen in het UC Davis Medical Center te Sacramento, VS en voor 3 maanden een co-schap Tropengeneeskunde gevolgd in Usangi, Tanzania. Na de studie Geneeskunde startte hij in januari 2012 met zijn promotieonderzoek aan de afdeling Kindergeneeskunde en de afdeling Immunologie van het Erasmus MC, onder supervisie van Prof. dr. Johan C. de Jongste, Prof. dr. Jacques J.M. van Dongen en Dr. Menno C. van Zelm. Gedurende zijn promotieonderzoek heeft hij een half jaar gewerkt bij de afdeling Immunology and Pathology van de Monash University te Melbourne, Australië. Sinds januari 2017 is hij in opleiding tot internist aan het Erasmus MC (opleider Dr. S.C.E. Klein Nagelvoort-Schuit) en heden werkzaam in het Amphia Ziekenhuis te Breda (opleider Dr. J. van Esser). Naast de opleiding interne geneeskunde heeft hij een grote interesse in Expeditie Geneeskunde met inmiddels de eerste ervaring in het begeleiden van een project in Oeganda.

Jorn Jesse Heeringa was born on December 27, 1984 in Karlsruhe, Germany. After living in Germany for 10 years, he moved to Brussels, Belgium in 1994. In 2002 he obtained the European Baccalaureate at the European School in Brussels. In the same year he started with the study Economics at the Erasmus University in Rotterdam. In 2005 he switched to study Medicine and in 2012 the medical exam was obtained with Cum Laude. During his Medicine studies, he did an internship for 6 weeks at the UC Davis Medical Center in Sacramento, USA, and a 3 months rotation in Tropical Medicine at Usangi Hospital in Tanzania. After studying Medicine he started his PhD in January 2012 at the Department of Pediatrics and the Department of Immunology at Erasmus Medical Center, under supervision of Prof. dr. Johan C. de Jongste, Prof. dr. Jacques J.M. van Dongen and Dr. Menno C. van Zelm. During his PhD he worked for six months at the Immunology and Pathology department of the Monash University in Melbourne, Australia. Since January 2017 he has been in training as resident Internal medicine at the Erasmus MC (under supervision of Dr. S. Klein Nagelvoort-Schuit) and now works at the Amphia Hospital in Breda (under supervision of Dr. J. van Esser). In addition to internal medicine, he has a great interest in Expedition Medicine and recently acquired his first experience in guiding a project in Uganda.