Tissue-specific Regulation of Immune Responses to Dietary Proteins

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Tissue-specific Regulation of Immune Responses to Dietary Proteins

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General introduction and outline of this thesis

INTRODUCTION

The term oral (or mucosal) tolerance has been defined as the suppression of T- and B-cell responses to an antigen by prior administration of the antigen by the oral route (1). From this definition, it can be inferred that mucosal tolerance is not just the ignorance of antigens that are applied via the mucosa or the absence of an immune response, but quite the contrary, mucosal tolerance refers to a dynamic process that depends on the active suppression of antigen-specific immune responses. Although such induction of immunological tolerance is not exclusive for antigens encountered via the mucosa and can be achieved outside the mucosal environment as well (2-4), it is well established that specific features of mucosal tissues favor the induction of tolerance. Here we discuss the mechanisms that are required for the induction of mucosal tolerance and the unique properties of the mucosal microenvironment that allow for intestinal homeostasis and the development of mucosal tolerance.

Mucosal tolerance

The mucosal surfaces of the gastrointestinal tract have developed as the largest surface area of the body that is in contact with the external environment. Not only do most pathogens enter through this site, the intestinal mucosa is continuously exposed to a large variety of completely harmless antigens, such as dietary proteins and constituents of commensal bacteria. To regulate high antigenic pressure the human gut holds approximately 50×10^9 lymphocytes, a large proportion of all immune cells in the body. These intestinal immune cells reside in the organized gut-associated lymphoid tissue (GALT), are scattered in between intestinal epithelial cells or are found throughout the gut lamina propria, three compartments that mediate distinct immune responses. GALT, which comprises Peyer's patches (PP), lymphoid follicles and the appendix, are inductive sites where naive T and B cells are found. In contrast, cells within the lamina propria and epithelial layer have effector and memory function (5). As such, the GALT and draining mesenteric lymph nodes (MLN) are the primary sites for intestinal adaptive immune regulation (Figure 1.1 A).

Antigen-bearing antigen-presenting cells (APC) that are equipped with a large diversity in pattern recognition receptors efficiently traffic to the areas of naive B or T cells to convey specific costimulatory signals that determine T- or B-cell differentiation and migratory capacity (Figure 1.1 B). A close interaction between innate epithelial cells and APC ensures precise sensing of the nature of an antigen. Upon APC – T-cell interaction in the GALT or MLN, these signals are translated into a tailored differentiation of the adaptive immune response leading to elimination of pathogenic antigens but active tolerogenic responses to harmless antigens. Next to regulating T-cell responses, this regional mucosal compartmentalization is of pivotal importance for the formation of immunoglobulin A (IgA) antibodies that protect against mucosal penetration by commensal microbiota (6).

Intriguingly, once an adaptive tolerogenic T-cell response to a soluble protein antigen has developed, not only does the effector immune response provide tolerance in the intestinal
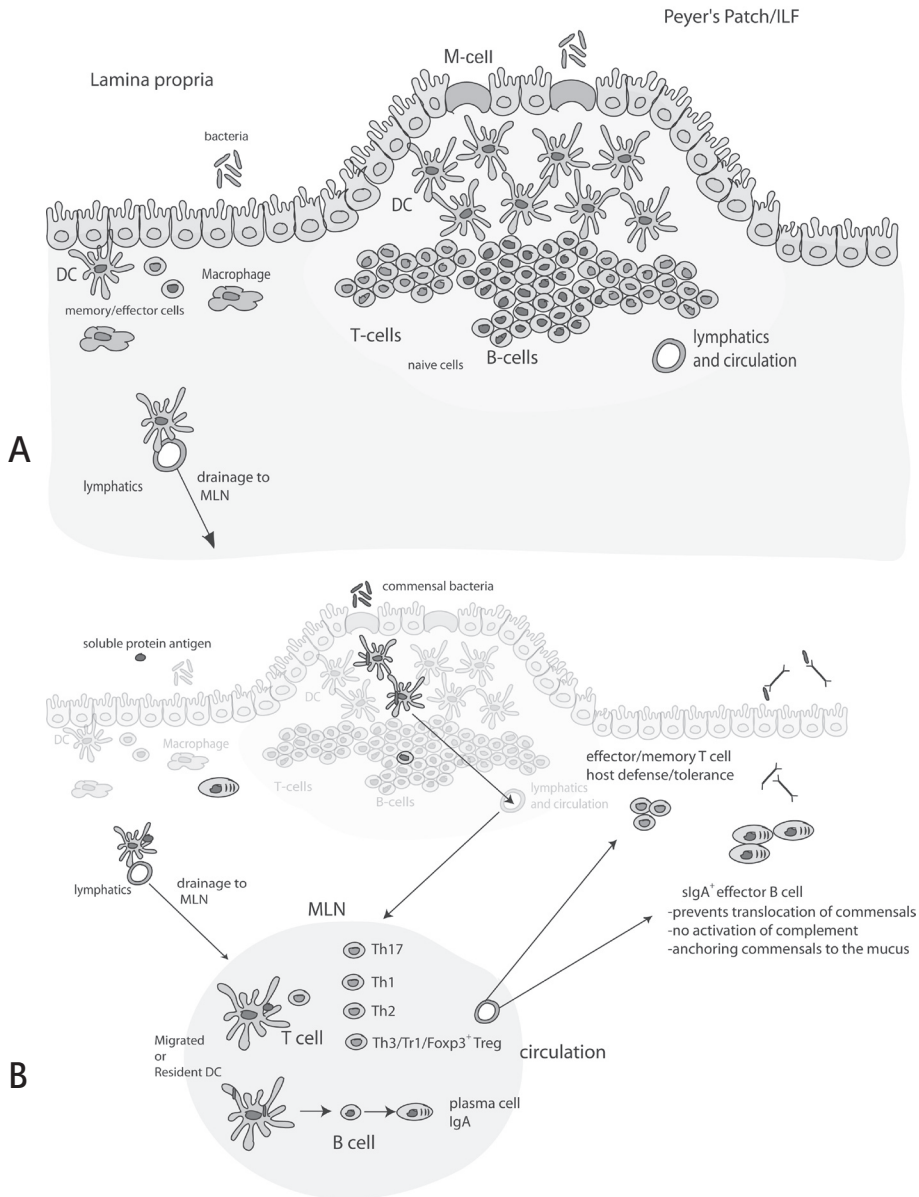
Figure 1.1 Structure of gut-associated lymphoid tissue (GALT). **(A)** GALT is comprised of PP, isolated lymphoid follicles, and the appendix (not shown). These are inductive sites where naive B and T cells differentiate upon antigen recognition. In contrast, cells within the lamina propria have effector or memory function. **(B)** The MLN drain the small intestine. In both PP and MLN antigen-carrying DC traffic to B- and T-cell areas to convey specific costimulatory signals that determine T- or B-cell differentiation and migratory capacity. Upon DC – T-cell interaction in GALT or MLN, these signals are translated into a tailored differentiation of the adaptive immune response leading to Th1, Th2, Th17 or Treg-cell differentiation (Th3, Tr1, Foxp3$^+$ Treg). DC, dendritic cell; IgA, immunoglobulin A; ILF, isolated lymphoid follicle; GALT, gut-associated lymphoid tissue; M cell, microfold cell; MLN, mesenteric lymph nodes; PP, Peyer's patch; Th, T helper cell; Tr1, type 1 regulatory T cell; Treg, regulatory T cell.

tissue but also peripheral immune responses to the same antigen are suppressed (1). Because of this "bystander" peripheral regulation, "oral tolerance" was defined as the suppression of T- and B-cell responses to an antigen by previous administration of the antigen by the oral route (1).

In spite of the robust mechanisms that maintain tolerance, food intolerances are common in genetically susceptible individuals. Many food hypersensitivities are caused by immune responses that are IgE mediated (5, 7). However, also "non-IgE-mediated food hypersensitivities" are distinguished in which the disease-inducing immune mechanisms have not been identified (7). One such non-IgE-mediated food hypersensitivity is coeliac disease (CD). CD patients develop an inflammatory response to the food protein gluten, resulting in severe intestinal pathology (Box 1.1). The induction of mucosal tolerance has been shown to depend on multiple mechanisms including clonal anergy, deletion as well as the generation of antigen-specific regulatory T (Treg) cells (8-12). Originally, high doses of antigen were thought to result in anergy

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Box 1.1 Coeliac disease

Coeliac disease (CD) is one of the most common food intolerances, affecting approximately 1% of the western world (175). In patients with CD, the ingestion of gluten proteins in wheat and similar proteins in barley and rye results in a chronic inflammation in the small intestine, that is characterized by villous atrophy, crypt hyperplasia and increased numbers of infiltrating lymphocytes in both the epithelium and in the lamina propria. The only available treatment for the disease is a life-long gluten-free diet, resulting in a complete restoration of the normal intestinal architecture. Symptoms of CD include diarrhea, abdominal distension, malnutrition, fatigue, weight loss or, in young children, a failure to gain weight. However, symptoms and disease severity vary widely among patients and some patients have a completely asymptomic form of the disease. A small subgroup of CD patients become unresponsive to the gluten-free diet and develop refractory coeliac disease (RCD). RCD is a severe complication of CD and patients with RCD have a high chance of developing an enteropathy associated T-cell lymphoma (176).

Loss of tolerance to gluten proteins in CD patients is associated with an inflammatory gluten-specific CD4^{+} T-cell response. Activated gluten-specific T cells that produce high amounts of IFN-γ can be isolated from the small-intestinal mucosa of CD patients but not of healthy individuals (177-180). These gluten-specific T cells are exclusively restricted to the disease predisposing HLA-DQ2 and HLA-DQ8 molecules (177, 181). More than 90% of the CD patients carry HLA-DQ2, whereas HLA-DQ2-negative patients usually express HLA-DQ8, demonstrating that these molecules play an important role in the presentation of gluten peptides in CD. Efficient binding of gluten peptides requires modification of the peptides by the enzyme tissue transglutaminase (TG2). This enzyme is constitutively expressed in the intestinal lamina propria and transiently activated upon tissue damage (182). TG2 introduces negative charges in certain gluten peptides by converting glutamine residues to glutamate. This process, termed deamidation, increases the affinity of gluten peptides for HLA-DQ2 and HLA-DQ8 molecules (183, 184).

Despite our understanding about the role of HLA-molecules and gluten-reactive T cells in the pathogenesis of CD, it still unknown why gluten, out of so many food proteins, fails to induce tolerance and triggers an inflammatory immune response in CD patients.

or deletion of antigen-specific T cells, whereas low doses would favour the induction of Treg cells (9, 13). However, it is now recognized that both mechanisms may occur simultaneously and cannot be fully distinguished. For instance, it has been demonstrated that several types of Treg cells have similar characteristics as anergic T cells (14). As a result, the role of Treg cells in mucosal tolerance has been the focus of extensive research in the past decades and it is now widely accepted that suppression by Treg cells is an absolutely essential process for the establishment of mucosal tolerance.

Effector T cells

CD4$^+$ T cells play central roles in the adaptive immune system, helping B cells to produce antibodies, enhancing CD8 T-cell responses, regulating macrophage function and orchestrating immune responses to a wide variety of antigens. Upon activation through T-cell receptor (TCR) signaling and by polarizing cytokines, naive CD4$^+$ T cells may differentiate into at least four different T helper (Th)-cell lineages: Th1, Th2, Th17 and induced Treg cells (reviewed in (15)). The decision to differentiate into one of these three effector T-cell lineages or into induced Treg cells (which are discussed separately below) is profoundly influenced by the cytokines that are present during the initial proliferative phase of T-cell activation. These cytokines induce lineage-specific transcription factors that are the master regulators of Th-cell fate. The cytokine environment that induces the differentiation of Th1 cells, which are characterized by the production of high levels of interferon (IFN)-γ, is characterized by high levels of interleukin (IL)-12 that induce the Th1-lineage transcription factors activator of transcription 4 (Stat4) and T-bet. IL-4-producing Th2 cells differentiate under the influence of high levels of IL-4 and IL-6 and low levels of IL-12. IL-4 signaling activates Stat6, leading to expression of the Th2-lineage transcription factor GATA3.

The differentiation of murine Th17 cells (producing IL-17 and IL-22) is driven by high levels of IL-6 and transforming growth factor (TGF)-β which induce the transcription factors Stat3 and the retinoid related orphan receptor-γt (RORγt). IL-23 has an important role in the expansion and stabilization of established Th17 cells, but may also participate in the differentiation of Th17 cells (16), as was also recently demonstrated by the report that Th17-cell differentiation can occur in the absence of TGF-β under the influence of IL-23, IL-6 and IL-1β (17).

Th1, Th2 and Th17 cells are currently the three well-recognized effector T-cell lineages. Other proposed separate-lineage Th-cell subsets are follicular helper (Tfh) cells, that reside in B-cell follicles and are important for antibody-production and formation of germinal centers (18), and Th9 cells, that are characterized by production of high levels of IL-9 (19, 20). Tfh cells are induced by IL-21 or IL-6 in the absence of TGF-β. Th9 cells require TGF-β and IL-4 during T-cell activation. However, it remains to be elucidated whether the latter Th-cell subsets are true separate lineages with unique essential transcription factors that drive their differentiation. Moreover, it should be noted that Th cells may be much more heterogenous and plastic than originally thought and that Th cells with mixed phenotypes also exist (15).

Regulatory T cells

Treg cells are defined by their functional capacity to suppress an effector T-cell response. Suppression can consist of inhibition of effector-cell proliferation as well as blockade of inflammatory cytokine release. Many different CD4^{+} Treg-cell subsets have been described, all of which are able to inhibit the responses of effector T cells. On the basis of their origin, a distinction between thymus-derived Treg cells and peripherally-induced or adaptive Treg cells can be made. Thymus derived CD4^{+}CD25^{+} forkhead box P3 (Foxp3)[+] Treg cells play a fundamental role in maintaining self-tolerance and preventing auto-immunity (21-24). In addition, thymus-derived Treg cells contribute to tolerance to non-self antigens as was substantiated by their ability to inhibit immune responses directed against commensal bacteria in the intestine and, consequently, to prevent the onset of colitis (25-27). Although these findings indicate that thymus-derived Treg cells contribute to the maintenance of intestinal homeostasis, they are dispensable for oral tolerance to protein antigens. Using mice that contain a monoclonal population of CD4^{+} T cells specific for the model food protein ovalbumin (OVA) and that were crossed on a RAG-1 knockout background, Mucida et al. demonstrated that tolerance to OVA was effectively induced in the absence of thymus-derived Treg cells (28).

In contrast to thymus-derived Treg cells, Treg cells that are induced after protein feed are essential for mucosal tolerance (4, 29-31). Such peripherally-induced or adaptive Treg cells can develop outside the thymus under specific microenvironmental conditions (32). Several subsets of induced Treg cells have been defined, including transforming growth factor (TGF)-β-producing Th3 cells that depend on TGF-β for their regulatory capacity (1, 10), interleukin (IL)-10-producing type 1 regulatory T (Tr1) cells that mediate suppression through IL-10 (33) and induced Foxp3^{+} Treg cells (28, 34). However, these subsets may not be mutually exclusive as for example Th3 and Foxp3^{+} cells have similarity with Tr1 cells by their secretion of IL-10. All subsets have extensively been studied for their role in oral tolerance (see Table 1.1 for a detailed overview of Treg cells studied during oral tolerance). However, as a result of the various experimental settings and the lack of exclusive cell markers identifying the specific Treg-cell subsets, it is difficult to determine what the relative contribution of each cell type is to the induction of mucosal tolerance. Moreover, multiple subsets may act in synergy to achieve suppression of chronic inflammation. As such, orally-induced Foxp3^{+} Treg cells were essential for establishing tolerance to allergic airway inflammation, for suppressing IL-4 production and lymphoid neogenesis in chronic inflammation. However, IL-5 production and eosinophilia were controlled by Foxp3-independent, IFN-γ-dependent mechanisms (30). Crucially, Foxp3-independent regulation may contribute to the conditioning of the tolerogenic microenvironment of the gut and thereby influence the conversion of Foxp3^{+} Treg cells in response to oral proteins. The individual anti-inflammatory mediators that are present in the intestinal environment and how they contribute to the induction of mucosal regulatory T cells will be discussed further down in this introduction.

Table 1.1 Antigen-induced regulatory T cells in oral tolerance

Type	Cytokine	Organ	Model	Reference
TGF-β-producing	**TGF-β**, IL-10, IL-4	MLN	WT mice	(10)
	TGF-β, IL-10, IL-4	Spleen	MBP-TCR Tg mice	(103)
	TGF-β	PP	WT mice	(102)
IL-10-producing	**IL-10**, TGF-β, IL-4	PP	WT mice	(125)
CD25$^-$ (Foxp3?)	?	PP and MLN	DO11.10 transfer	(29)
CD25$^-$ Foxp3$^+$		PP, MLN, Spleen	DO11.10, DO11.10 transfer (OVA/CTB)	(185)
CD25$^+$ (Foxp3?)		PP and MLN	DO11.10 transfer	(4, 29)
	TGF-β, IFN-γ, IL-10	PP	WT mice	(186)
	IL-10, TGF-β	MLN, inguinal LN, Spleen	DO11.10	(187)
	TGF-β	Celiac LN (liver draining) MLN	DO11.10 transfer	(74)
CD25$^+$ Foxp3$^+$		PP, MLN, Spleen	DO11.10, DO11.10 transfer (OVA/CTB)	(185)
CD25$^+$ Foxp3$^+$		MLN+Spleen	T/Bmc RAG1$^{-/-}$	(28)
Foxp3$^+$ (PCR)		PP	DO11.10 transfer	(188)
Foxp3$^+$		PP, MLN, lamina propria	OT-II RAG1$^{-/-}$ transfer	(85)
Foxp3$^+$		MLN	DO11.10 SCID	(113)
Foxp3$^+$		MLN	DO11.10 RAG2$^{-/-}$ transfer	(112)

IFN, interferon; IL, interleukin; LN, lymph nodes; MBP, myelin basic protein; MLN, mesenteric lymph nodes; OVA, ovalbumin; PP, Peyer's patches; TCR, T-cell receptor; Tg, transgenic; TGF, transforming growth factor; WT, wild-type; DO11.10 and OTII mice have a Tg TCR specific for ovalbumin. RAG1/2$^{-/-}$ indicates that the mice are backcrossed on a recombination activation gene deficient background.

Regulation by non-convential lymphoid-cell populations

In addition to the "classical" Treg-cell populations, the intestinal lamina propria is home to several other T-cell populations that have a role in maintaining mucosal homeostasis.

TCRγδ$^+$ T cells

TCRγδ$^+$ T cells are present in large numbers in the intestinal epithelial layer. These cells frequently coexpress the CD8αα receptor but lack expression of the typical TCR coreceptors CD4 or CD8αβ and are likely activated through TCR or natural killer receptor-mediated recognition of stress-induced ligands (35). Numbers of TCRγδ$^+$ intraepithelial lymphocytes (IEL) are increased in patients with CD (36). In contrast to cytotoxic CD8αβ$^+$ IEL, intraepithelial γδ T cells remain

present at high numbers for years after patients are on a gluten-free diet, implying that they do not have a role in the pathogenesis of the disease (37). In contrast, TCRγδ⁺ intraepithelial T cells have been attributed a protective role in the intestinal mucosa, as these cells have been shown to regulate the continuous turnover of intestinal epithelial cells (IEC) (38, 39), contribute to the induction of IgA (40) and produce TGF-β (41). In line with this concept, TCRγδ⁺ IEL are found in greater number in patients with latent CD with normal intestinal histology than in patients with active disease (42). Moreover, TCRδ-deficient mice show an increased susceptibility to chemically-induced colitis (41, 43).

Invariant natural killer T cells and mucosal-associated invariant T cells

Few non-conventional T-cell subsets populate the intestinal lamina propria, including invariant natural killer T (iNKT) cells and mucosal-associated invariant T cells (MAIT cells or mNKT cells) that interact with the non-classical MHC molecules CD1d and MR1 respectively (44). Despite being rare in the intestine, these cells may have important roles in maintaining mucosal homeostasis. NKT-cell-deficient mice were shown to have an impaired induction of tolerance to orally-administered OVA, suggesting that NKT cells have critical roles in oral tolerance (45). In contrast, some other studies have shown that NKT cells are not required for the establishment of oral tolerance (46), emphasizing that the role of NKT cells in oral tolerance is still controversial and may depend on the experimental settings (44). Similar conflicting results have been obtained in studies investigating the role of NKT cells in the maintenance of tolerance toward the commensal flora. The activation of NKT cells with αGalCer is reported to have some protective effects against chemically-induced colitis and NKT cells can prevent colitis in a T-cell transfer model in a CD1d-dependent manner (47, 48). However, CD1d-deficient mice, iNKT-cell-deficient mice and wild-type mice treated with CD1d-blocking antibodies are all protected from the development of oxazolone-induced colitis and NKT cells are shown to have a pathogenic role in the production of Th2 cytokines involved in mucosal inflammation (49, 50). Taken together, NKT cells may be involved in both pathogenic and regulatory immune responses in the intestines.

Innate lymphoid cells

Innate lymphoid cells are a recently described population of cells that lack markers of mature lymphoid cells yet bear receptors commonly found on lymphoid progenitors. The growing population of mucosal innate lymphoid cells includes already four distinct cell lineages: lymphoid-tissue inducer (LTi) cells, natural killer (NK) cells, NK receptor-expressing LTi-like cells and Th2-like innate cells. These cells are abundantly present in the intestines, secrete large amounts of cytokines and a large proportion expresses the transcription factor RORγt (51-54). Innate lymphoid cells have recently been implicated in the induction of colitis. IL-23 has been shown to drive chronic inflammatory diseases in the intestine in various models (55) and this has now been attributed to previously unrecognized innate lymphocytes that respond to IL-23 (56).

Further research will be needed to establish whether these recently discovered cells contribute to mucosal homeostasis or loss of tolerance to dietary antigens in the small intestine.

Site of mucosal Treg-cell induction

The induction of adaptive mucosal Treg cells depends on the presentation of oral antigens by an APC. Consequently, a crucial issue in the induction of mucosal tolerance is to understand the exact site where naive T cells meet their cognate antigen and differentiate into mucosal Treg cells. Several levels of defence operate in the intestinal tract that prevent intact antigens and microorganisms from entering the body and encountering the mucosal immune system. The first line of defence is formed by IEC, which form a physical barrier that separates the luminal content from the underlying mucosal immune system (immune exclusion). Specialized IEC, such as mucus-secreting goblet cells and antimicrobial-peptide-producing Paneth cells, maintain the mucosal border (57). In addition, antibody-secreting B cells play an important role in maintaining immune exclusion. The GALT contains the vast majority of all plasma cells, most of which produce dimers of secretory IgA antibodies that are transported across the gut-epithelium to the lumen where they bind to dietary antigens and microorganisms in the mucus leading to their elimination (58). However, despite these barrier functions there is extensive crosstalk between luminal antigens and the mucosal immune system. Fed proteins that reach the lumen of the gut can gain access to the mucosal immune system in at least three different ways (Figure 1.1 A).

First, specialized microfold (M) cells can selectively bind antigens from the intestinal lumen. M cells are mainly present in the follicle-associated epithelium lining organized lymphoid follicles, which include PP and isolated lymphoid follicles in the small intestine. Upon internalization by M cells, the intact antigen is effectively transferred to professional APC in the subepithelial dome region of the follicle (59). From there, antigen-loaded APC may present the antigen to naive T cells in the PP or enter the lymphatics to gain access to the gut-draining MLN (60).

Alternatively, luminal proteins can be acquired directly by resident lamina propria dendritic cells (DC) in several ways. It has been reported that M cells exist in the villous epithelium that is not associated with organized lymphoid follicles and in this way provide a mechanism by which lamina propria can sample luminal content (61). In addition, lamina propria DC that are situated underneath the epithelial monolayer can directly gain access to the luminal side of the epithelium, as it has been demonstrated that DC are able to open the tight junctions between adjacent epithelial cells and extend their dendrites through the epithelium to sample luminal content (62, 63). Moreover, DC constitutively endocytose apoptotic epithelial cells, that have the ability to take up intestinal antigens, and transport them to the MLN (64). Thus, under homeostatic conditions, lamina propria DC actively sample antigens from the gut lumen and constitutively migrate from the intestine to the draining MLN.

In addition to active uptake, a fraction of dietary protein antigens may cross the epithelial layer and reach the circulation (65, 66). The majority of antigens that enter the circulation will reach the liver via the portal vein, a route that has previously been associated with oral tolerance induction (67). It has been suggested that the site and mechanism of uptake may in part be dependent on the nature of the antigen (68). It should be noted that many studies are based on the use of ovalbumin. This is of particular importance as the uptake of dietary proteins appears to be widespread throughout the intestinal epithelium while it has been suggested that PP are more involved in the initiation of mucosal immune responses to bacteria (60). The question arises whether these different mechanisms of antigen uptake at different sites all lead to Treg-cell induction.

It was originally assumed that M cells in the PP epithelium provide the main mechanism of entry for most fed antigens. Indeed, antigen-specific Treg cells can be isolated from PP as early as 24h after protein feed, demonstrating that antigen-uptake through PP contributes to the induction of oral tolerance (29). However, at 48h after protein feed antigen-specific Treg cells also differentiate in the MLN (29). Several studies have shown that oral tolerance can be normally induced in the absence of PP, indicating that functional MLN are sufficient for oral tolerance induction to soluble protein antigen (69-72). The presence of mucosa-draining tissue is critical for oral tolerance, as deficiency of both PP and lymph nodes as occurs in lymphotoxin α-deficient mice causes loss of oral tolerance. Reciprocally, treatment of pregnant lymphotoxin α-deficient mice with an agonistic anti-lymphotoxin β-receptor antibody resulted in reconstitution of MLN which was sufficient to restore oral tolerance (70). A more recent study by Worbs et al. (73) demonstrated that surgical removal of the MLN impairs the induction of oral tolerance, emphasizing that intact PP alone are not sufficient to maintain oral tolerance. Using CCR7-deficient mice, which have defective migration of DC from the intestine to the MLN, the authors demonstrate that oral tolerance relies on the uptake of fed antigens by small-intestinal DC and subsequent CCR7-dependent migration to the MLN (73). Moreover, although intestinal antigens have been shown to enter the circulation and reach peripheral lymph nodes (67, 74, 75), transplantation experiments of the small bowel together with the MLN have demonstrated that antigen transport via the circulation is not sufficient to induce proliferation of antigen-specific T cells (73). Taken together, the uptake of oral antigens by lamina propria DC and the subsequent induction of Treg cells in PP and the MLN represent the initial events in the induction of oral tolerance.

Mucosal microenvironment

Although it was long recognized that the mucosal environment is immune privileged by its capacity to induce tolerance, only recently progress has been made in identifying local factors in the mucosal microenvironment that drive this process (Figure 1.2). Resident DC in the intestine have an intrinsic non-inflammatory activational state that preferentially drives tolerogenic T-cell responses. This is best illustrated by the report that Flt3 ligand-induced DC
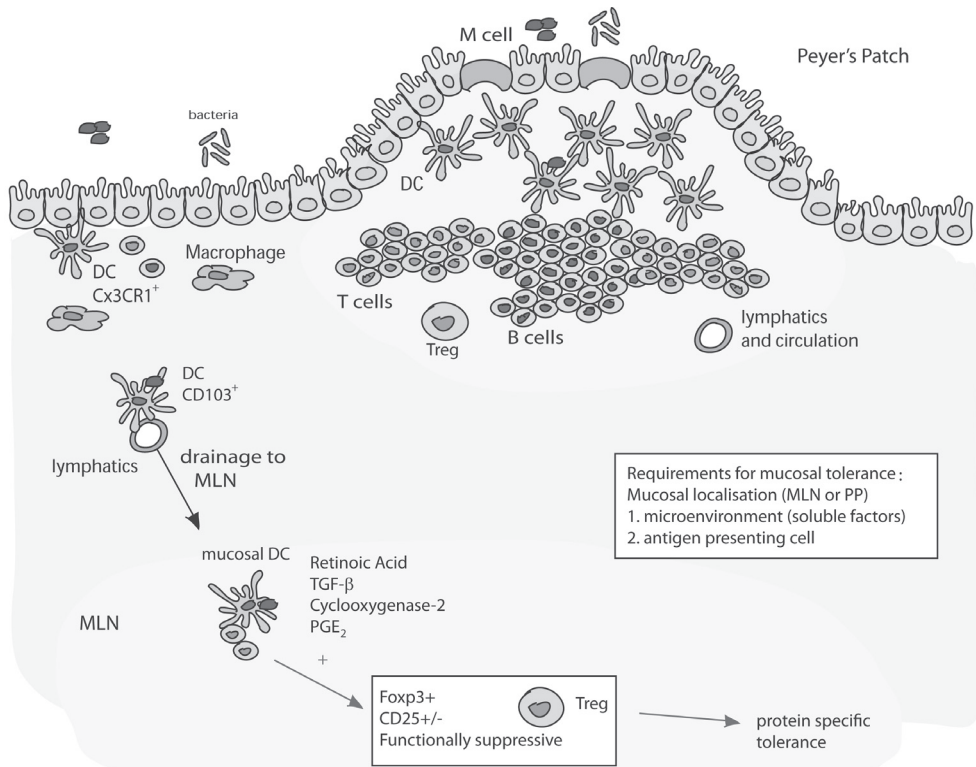
Figure 1.2 Mucosal Treg-cell differentiation is driven by factors within the mucosa-associated lymphoid tissue. Fed proteins that reach the lumen of the small intestine can be taken up by several ways. First, proteins enter by selective uptake through M cells that overlie Peyer's patches. Second, luminal proteins may directly be acquired by DC in the lamina propria outside of the GALT. Third, a fraction of the dietary protein may cross the epithelium and reach the liver via the portal vein (not shown). Both PP and MLN have the capacity to support mucosal Treg-cell differentiation. However, in the absence of PP the presence of MLN is sufficient to sustain oral tolerance. Differentiation of functionally-suppressive Treg cells occurs within 48-72 h post protein feed and requires antigen presentation by specialized DC as well as the presence of mucosal regulatory factors. In particular, CD103+ DC contribute to Treg-cell differentiation by their secretion of retinoic acid. However, also retinoic acid, TGF-β, and COX-2-derived prostaglandins derived from hematopoietic and non-hematopoietic cells contribute to this process. DC, dendritic cell; GALT, gut-associated lymphoid tissue; M cell, microfold cell; MLN, mesenteric lymph nodes; PGE_2, prostaglandin E_2; PP, Peyer's patch; TGF, transforming growth factor; Treg, regulatory T cell.

expansion enhanced oral tolerance, but when activation of the cells was elicited with IL-1, tolerance was lost. In particular, increased levels of co-stimulatory molecule expression CD80 and CD86 were associated with this process (76). This association is corroborated by the lack of functional mucosal Treg-cell induction in FcγRIIB-deficient mice that exhibit enhanced costimulatory molecule expression on DC in mucosa-draining tissue (77).

However, next to a difference in activational state, particular subsets of mucosal DC exist that can have intrinsic regulatory properties when compared to non-mucosal DC. For example,

DC isolated from MLN and PP very efficiently induce the expression of the gut-homing receptors $\alpha_4\beta_7$ and CCR9 on proliferating T cells *in vitro* (78-81) and stimulate B cells to secrete immunoglobulin A (IgA) (82, 83). Moreover, mucosal DC promote the conversion of naive T cells into Foxp3+ Treg cells (84, 85) and are less able to induce the differentiation of Th17 cells (84). Recent advances in the field of DC development have shown that these intrinsic capacities can be coupled to phenotypically definable CD11c+, CD103+ and CX3CR1+ subpopulations in the intestinal lamina propria, which are derived from preclassical DC and Ly6C+ monocytes, respectively (86) (Figure 1.3). Although CD103+ cells emigrate from the lamina propria to the MLN and have imprinting capacities, CX3CR1+ cells do not emigrate to the MLN or efficiently induce a T-cell response (87). As such, these CX3CR1+ cells share more characteristics with lamina propria macrophages than DC (86). The role of CD103+ DC in mediating oral tolerance is further discusses below (See Retinoic acid).

Accumulating evidence suggests that the function of mucosal DC is conditioned by the local microenvironment (88, 89). As mucosal DC continuously migrate from the lamina propria to the MLN, they could be influenced by local factors at both sites. For the lamina propria, it has been demonstrated that one source of such local factors are IEC. IEC are in close contact

Subtypes of DC within the intestinal lamina propria
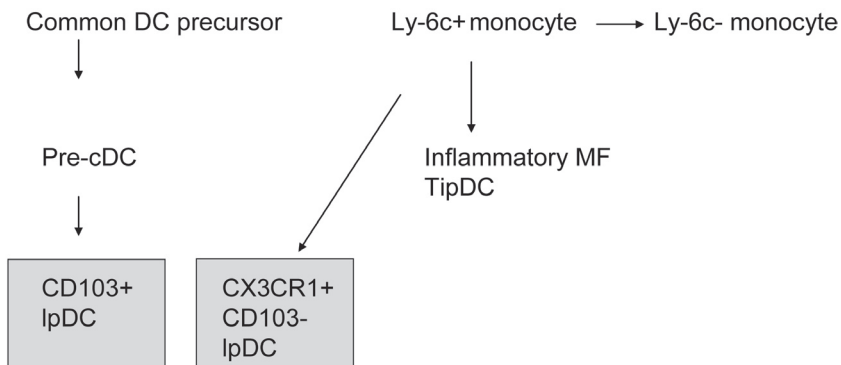
adapted from Science 2010:327, 656-661

Figure 1.3 Subtypes of DC within the small-intestinal lamina propria. Intestinal macrophages and DC all stem from myeloid precursors in the bone marrow. These myeloid precursors give rise to monocytes, some populations of macrophages, and common DC precursors. Two monocyte subsets, Ly-6C+ and Ly-6C-, leave the bone marrow to enter the blood. Common DC precursors give rise to preclassical dendritic cells (pre-cDC). Pre-cDC circulate in blood and populate lymphoid organs and tissues. As such, Pre-cDC may give rise to CD103+ lamina propria DC. Ly-6C+ monocytes can become CX3CR1+ lamina propria DC in non-lymphoid tissues. During inflammation, Ly-6C+ monocytes give rise to monocyte-derived DC, for example, tumor necrosis factor and inducible nitric oxide synthase (iNOS)-producing DC (TipDC), or inflammatory macrophages. DC, dendritic cell; lpDC, lamina propria DC; MF, macrophage.

with lamina propria DC and have been shown to release molecules that condition the DC into a non-inflammatory state, such as the cytokine thymic stromal lymphopoietin (TSLP) (90), TGF-β and the vitamin A-derivative retinoic acid (RA) (91).

The local environment of the mucosa-draining lymph nodes also has unique characteristics that are important for the induction of mucosal tolerance as well as the generation of gut-homing T cells (92, 93). Studies that have focused on the induction of mucosal tolerance via the nasal mucosa have demonstrated that the regional nose-draining lymph nodes are essential for the establishment of tolerance via this route (94). Similar to data obtained from oral tolerance experiments, nasal tolerance was completely abrogated upon surgical removal of the nose-draining lymph nodes (94). Transplantation experiments revealed that only replacement with nose-draining lymph nodes but not with peripheral or gut-draining lymph nodes could restore tolerance, in spite of the fact that all transplanted lymph nodes were fully functional (92, 94). It is currently unclear whether intrinsic capacities of the gut-draining MLN are important for oral tolerance in a similar way. However, evidence that the MLN as well have unique functions that are not shared by peripheral lymph nodes came from a recent study on the imprinting of gut-homing receptors. Hammerschmidt et al. demonstrated that the induction of CCR9 expression on T cells *in vivo* depends on the presence of both resident stromal cells in the MLN as well as steady-state migratory DC (95).

Taken together, these data suggest that both the activational state of resident DC as well as specific subsets of functionally distinct DC maintain mucosal tolerance. However, this occurrence is fully dependent on the various factors derived from local APC, epithelial cells or lymph node stromal cells that together condition the tolerogenic microenvironment of the gut and enforce intestinal homeostasis.

The requirements of the individual regulatory mediators for the development of mucosal tolerance will be discussed separately.

TGF-β

The anti-inflammatory cytokine TGF-β is abundantly expressed by non-hematopoietic as well as hematopoietic cells in the gut-microenvironment and is a critical factor in maintaining intestinal homeostasis. TGF-β has strong immunosuppressive effects on lymphocytes: it inhibits T-cell proliferation in the absence of exogenous IL-2 (96) and suppresses the differentiation of naive T cells into effector Th1 and Th2 cells through direct downregulation of T-bet and GATA3 expression (97-99). In addition to its immunosuppressive properties, TGF-β plays an important role in epithelial cell differentiation and IgA class switching in the intestine (100).

The key role of TGF-β in oral tolerance is well established. Neutralization of TGF-β impairs the development of tolerance to fed proteins (28, 101). In addition, TGF-β-producing Th3 cells can be isolated from the PP and MLN of orally tolerized mice (10, 102, 103). These Th3 cells are antigen-specific and suppress experimental autoimmune encephalomyelitis in a TGF-β-dependent manner (10).

More recently, TGF-β-dependent Treg cells have been identified that express latency-associated peptide (LAP) and TGF-β on the cell surface (104, 105). Suppression by these LAP^{+} Treg cells is cell-cell contact dependent and can be blocked with anti-TGF-β antibodies (104). LAP^{+} Treg cells have been implicated in the suppression of colitis (105). Furthermore, LAP^{+} Treg cells were induced after oral administration of CD3-specific antibodies (106). However, it remains to be determined whether LAP^{+} Treg cells are also induced after protein feed and what the exact relationship is between LAP^{+} Treg cells and Th3 cells.

In addition to functioning as an effector molecule of Th3 cells and LAP^{+} Treg cells, TGF-β can mediate immune tolerance by the induction of both Foxp3^{+} Treg cells. *In vitro* studies have revealed that the activation of naive T cells in the presence of TGF-β induces the *de novo* expression of Foxp3 and the acquisition of suppressive capacity (107–109). Experiments with TGF-β T cell-transgenic mice demonstrated that T cell-derived TGF-β also results in the generation of Foxp3^{+} Treg cells *in vivo* (110). In addition to the induction of Foxp3^{+} Treg cells, TGF-β plays an important role in the maintenance of Foxp3^{+} Treg cells in the periphery (111). Multiple studies have shown that the development of tolerance to oral proteins is associated with the *de novo* induction of Foxp3^{+} Treg cells (28, 85, 112, 113). An important role in this process can be attributed to TGF-β as neutralization of the anti-inflammatory cytokine during induction of oral tolerance resulted in a reduction in Foxp3 expression in MLN and the spleen (28).

The GALT has been recognized as a very efficient site for the conversion of naive T cells into Foxp3^{+} Treg cells (84, 85, 113). Although the efficient induction of Foxp3 may seem evident in a microenvironment that harbors high concentrations of TGF-β, it should be noted that TGF-β is secreted in a biologically inactive form and needs to be activated in order to exert it suppressive activities. Latent TGF-β can be activated by several molecules, including the integrins $α_4β_6$ expressed by IECs and $α_vβ_8$ by DC (114–116). Local mucosal DC are thought to play a key role in maintaining intestinal homeostasis by activating latent TGF-β. It has been demonstrated that mucosal DC are significantly better than splenic DC at inducing the expression of Foxp3 in naive T cells (85, 113). This difference is in part dependent on the ability of mucosal DC to release bioactive TGF-β as mucosal DC lacking the TGF-β-activating integrin $α_vβ_8$ failed to induce the differentiation of Foxp3^{+} cells *in vitro* (114, 116).

In addition to sustaining Treg-cell subsets, TGF-β has also been implicated in the development of effector T-cell subsets in the presence of pro-inflammatory cytokines. Together with IL-6, TGF-β was shown to induce the differentiation of Th17 cells, which express the transcription factor RORγT (117). However, it has recently been demonstrated that Th17-cell differentiation can occur independently of TGF-β as long as Th1- and Th2-cell differentiation mechanisms are absent (118). Thus, TGF-β promotes the generation of Th17 cells indirectly by inhibiting Th1- and Th2-cell differentiation rather than directly inducing Th17. Recently, TGF-β and IL-4 were shown to induce the production of IL-9 in activated T cells and it has been proposed that these IL-9-producing T cells define a distinct lineage of T cells, referred to as Th9 cells (19, 20). However, no specific transcription factor has been identified for this

lineage and it remains to be determined whether TGF-β has a direct stimulatory effect on the differentiation of Th9 cells.

IL-10

IL-10 was originally isolated from mouse Th2 cells and was thought to suppress the differentiation and effector functions of Th1 cells (119). However, IL-10 is now recognized as a cytokine with broad anti-inflammatory properties that is produced by many other cell types including epithelial cells, monocytes, macrophages, DC, NK cells, B cells, CD8^{+} T cells, intraepithelial lymphocytes and different subsets of CD4^{+} T cells, including Th1 cells, Foxp3^{+} Treg cells, Tr1 cells, Th3 cells, Th17 cells and follicular helper T cells (120-123). High numbers of IL-10-producing T cells can be detected in the intestinal microenvironment, especially in PP, small-intestinal intraepithelial lymphocytes and colonic lamina propria lymphocytes (123). Despite its abundant presence in the intestines, the role of IL-10 in oral tolerance is controversial. Several studies have reported that antigen feed results in an increased IL-10 production, mainly in the PP, but also in MLN, the spleen and serum (10, 124-126). It has been suggested that antigen-specific Tr1 cells that depend on IL-10 production are induced in oral tolerance (127). Recently, IL-10-producing gliadin-specific Tr1-cell clones have been isolated from the mucosa of treated CD patients. These Tr1-cell clones inhibited the proliferation of gliadin-specific T-cell clones (128) and these findings may implicate the involvement of IL-10-producing Tr1 cells in response to dietary proteins. However, multiple studies have demonstrated that oral tolerance can be normally induced in the absence of IL-10 (28, 129-131), indicating that, in steady state conditions, IL-10 is not absolutely necessary for the establishment of tolerance to dietary proteins.

Whereas IL-10 seems to be dispensable for the induction of tolerance to dietary proteins, its role in promoting tolerance to intestinal microbiota is well established. IL-10-deficient mice develop spontaneous colitis (132) and reciprocally exogenous IL-10 was shown to restore tolerance in mice with colitis (133). The mechanism by which IL-10 protects from colitis is poorly understood. Whereas in the small intestine both IL-10^{+}Foxp3^{-} Tr1 cells and IL-10^{+}Foxp3^{+} Treg cells are detected, all IL-10-producing T cells in the colonic lamina propria express Foxp3 (134). Specific deletion of IL-10 in Foxp3^{+} cells resulted in the development of spontaneous colitis, demonstrating that IL-10 is required for the suppressive function of Foxp3^{+} Treg cells in the colon (135). However, in a different model it has recently been demonstrated that IL-10 derived from lamina propria macrophages is crucial for the prevention of colitis and may function by supporting the function of Foxp3^{+} Treg cells in the inflamed intestine (136). Thus, it is likely that multiple cell populations contribute to homeostasis in the large intestine through their production of IL-10.

Although it is well recognized that the intestines are a preferential site for the generation of IL-10-producing T cells, the local factors that mediate the differentiation of these cells remain largely unclear. An important role is attributed to tolerogenic APC in mucosal tissues, as MLN-derived CD8α^{+} plasmacytoid DC (137), CD11b^{+} DC from PP (138) and CD11b^{+}F4/80^{+}

lamina propria macrophages (139) have been shown to efficiently induce the expression of IL-10 in responding T cells. The ability of mucosal APC to induce IL-10-producing T cells has been associated with their own secretion of IL-10 (138-141), which in turn may be dependent on conditioning by the tolerogenic environment of the gut (90, 142, 143). However, recently several very different pathways have been reported to lead to IL-10-producing T cells. Importantly IL-10 production is not only restricted to the differentiation of IL-10-producing Tr1 cells from naive T cells, as IL-10-producing T cells with suppressive activity can also derive from effector T cells. This adds to the complexity of studying these cells because the suppressive capacity of IL-10-secreting effector T cells may thus be very transient. Particular levels of antigen encounter may account for this effector T cell-derived IL-10, as high and sustained levels of antigen induce Th1-derived IL-10 through stimulation with IL-12 (144). It has recently been demonstrated that increased frequencies of IL-10- and IL-27-producing DCs that induce IL-10-secreting T cells occur during oral tolerance induction (145). Also IL-27, a member of the IL-12 cytokine family that is produced by APC, is a potent inducer of IL-10 expression in both naive and activated T cells (146-149). However, unlike IL-10-deficient mice, IL-27-receptor-deficient mice do not develop spontaneous colitis (150) indicating that redundant mechanisms for the regulation of IL-10 exist in the intestine. The IL-27-mediated induction of IL-10 may be of particular relevance for the intestinal tissue as it is enhanced by the addition of TGF-β (146). This effect is mediated through induction of the transcription factor c-Maf and subsequent upregulation of IL-21 and ICOS expression (151). IL-21 is an important growth factor for the IL-27-driven differentiation of IL-10-producing Tr1 cells *in vitro* and is particularly efficient in inducing IL-10 expression in already differentiated effector T-cell populations (151, 152). In agreement, the fraction of IL-10-producing T cells is greatly reduced in IL-21-receptor-deficient mice (151).

Further clarification of the mechanisms that contribute to the induction of IL-10-producing Tr1 cells under homeostatic conditions in the small intestine requires further investigation.

Retinoic acid

Another key factor that contributes to the tolerogenic mucosal microenvironment is RA, a metabolite of vitamin A. RA is metabolized from the dietary vitamin A in a two-step reaction involving alcohol dehydrogenase (ADH) and retinal dehydrogenase (RALDH) enzymes. The beneficial effect of vitamin A on mucosal immune responses has been recognized as early as 1928 when vitamin A was termed "the anti-infective vitamin" (153). Vitamin A deficiency is a cause of increased inflammation. Prolonged insufficiency compromises epithelial barrier function and causes a loss of mucus-producing goblet cells, resulting in an increased severity of disease during infections (153). Despite these defects, vitamin A-deficient mice do not show significant inflammation in the small intestine and oral tolerance is normally developed in these mice (154).

Recently, RA is identified as a cofactor that enhances TGF-β-mediated induction of Foxp3 by mucosal DC (84, 85, 113, 155). DC from the MLN and the small-intestinal lamina propria have a significantly greater capacity to induce Foxp3 expression than non-mucosal DC, a quality that is associated with their ability to catalyze retinal into RA (85, 113). In addition to the induction of Foxp3, the induction of the gut-homing receptors $\alpha_4\beta_7$ and CCR9 (156) as well as the stimulation of intestinal B cells to secrete IgA (82) is also dependent on the ability of mucosal DC to convert of vitamin A into RA.

Following up on these findings, it was shown that mucosal DC that express CD103 can induce Foxp3+ Treg cells in the absence of exogenous TGF-β and RA (113) (Figure 1.4). In contrast, CD103- mucosal DC were not able to induce Foxp3 expression in the absence of exogenous TGF-β and, when TGF-β was provided, the induction was much weaker than observed with CD103+ DC (113). This difference in function was explained by the observation that CD103+ DC from MLN express higher levels of Aldh1a2, the gene encoding RALDH2, than CD103- MLN-DC (113).

The exact mechanisms by which RA enhances the capacity of TGF-β to convert naive T cells into Foxp3+ Treg cells remain largely unclear. Recent progress has clarified that RA elicits its effects in multiple ways and can affect naive T cells both directly and indirectly. An important
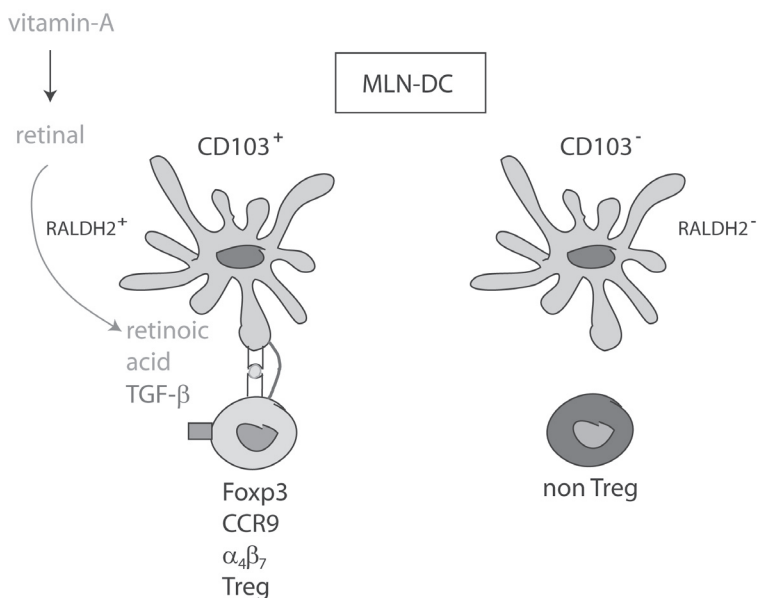
Figure 1.4 Functional characteristics of CD103+ MLN-DC. MLN CD103+ DC have distinct characteristics from CD103- MLN-DC. The majority of CD103+ MLN-DC are thought to be a tissue-derived migratory population that plays a central role in presenting orally-derived soluble antigen to CD8+ and CD4+ T cells. CD103+ DC contain the enzyme RALDH2, which converts retinal into retinoic acid (RA). RA acts on T cells by inducing the mucosal homing receptors $\alpha_4\beta_7$ and CCR9. Moreover, in the presence of TGF-β, RA promotes conversion of naive T cells into Foxp3+ Treg cells. In contrast, CD103- MLN DC, which appear to derive from blood precursors do not have this same capacity. DC, dendritic cell; MLN, mesenteric lymph nodes; RA, retinoic acid; RALDH, retinal dehydrogenase; TGF, transforming growth factor; Treg, regulatory T cell.

strategy by which RA enhances the differentiation of Treg cells is by interference with the function of proinflammatory cytokines, which are known to suppress the induction of Foxp3 in naive T cells (157). Not only does RA inhibit the production of inflammatory cytokines by effector or memory T cells (158-162), it also interferes with the negative effects of those cytokines on naive T cells (84, 162-165). Mechanisms by which RA protects naive T cells from this suppression by cytokines involve the downregulation of IL-6 receptor (164) and regulation of the Foxp3 promoter region by RA receptor α (165). Besides these cytokine-dependent mechanisms, RA can enhance the conversion of naive T cells into Foxp3+ Treg cells independently of secreted inhibitory cytokines (163). It has been demonstrated that RA counteracts the inhibitory effects of costimulation on Foxp3+ Treg-cell differentiation (155, 166). Another suggestion of how RA stimulates Treg-cell differentiation is via its capacity to enhance TGF-β signalling, as RA increases the expression of the TGF-β-dependent Smad3 (164). However, it has recently been demonstrated that despite the increase in Smad3 expression, the enhancement of Foxp3 expression by RA was largely Smad independent (163).

In addition to these effects on T cells, RA also influences the phenotype of DC in the mucosal microenvironment (154, 167) and may therefore contribute to the specialized ability of mucosal DC to induce Treg cells. In this light, it must be taken into account that apart from mucosal DC other cells including intestinal epithelial cells (91, 168) and MLN stromal cells (95, 169) have been shown to express vitamin A converting enzymes. This epithelial cell or stromal cell-derived RA might be transferred to DC, as it has been demonstrated that DC can acquire RA from other sources, store it and in this way gain the tolerogenic characteristics of mucosal DC (170). Alternatively, contact between epithelial cells or stromal cells and DC may increase the expression of RALDH enzymes and CD103 in the DC in an RA-dependent manner, resulting in an enhanced ability to induce Foxp3+ Treg-cell differentiation (91).

Reciprocally to its role in Treg-cell induction, RA has been shown to inhibit the generation of Th17 cells, depending on the local cytokine environment. DC stimulated in the presence of TGF-β induce Treg cells and in the presence of TGF-β and the proinflammatory cytokine IL-6 induce Th17 cells (84, 161). However, it was recently suggested that the effect of RA depends on the dose as at a low concentration, RA was shown to stimulate instead of inhibit the differentiation of Th17 cells (171). Taken together, these results demonstrate that RA plays an important role in controlling the balance between effector and regulatory CD4+ T-cell populations in the small intestine.

The ability to enhance the reciprocal TGF-β-mediated induction of Foxp3 and Th17 cells is not restricted to mucosal DC, as it has been demonstrated that lamina propria macrophages can also perform this action (139). The induction Foxp3+ cells by lamina propria macrophages was not only dependent on TGF-β and RA, but also on IL-10, suggesting that TGF-β, RA and IL-10 may all act synergistically in inducing Foxp3+ Treg-cell differentiation (139). These findings are substantiated by a recent study showing that toll-like receptor 2 signalling induces the concurrent expression of RALDH2 and IL-10 in DC (172). Moreover, in a human

study vitamin A supplementation in patients with low vitamin A levels resulted in increased serum levels of IL-10 (173). However, these findings are contradicted in a report by Maynard et al. (174), who demonstrate in their model that RA inhibits the TGF-β-mediated induction of IL-10 in T cells and show that vitamin A-deficient mice harbour increased number of IL-10-producing T cells.

CONCLUDING REMARKS

The immunosuppressive microenvironment of the gut is controlled by a complex network of immunological interactions between epithelial cells, antigen-presenting cells and lymphocytes that allow the induction of oral tolerance to food proteins. Over the last few years, the increased identification of tissue-specific factors in the intestines has emphasized the redundancy in mechanisms that contribute to this local tolerogenic environment. However, despite our increasing knowledge about the mechanisms of mucosal tolerance, it still largely unknown why mucosal tolerance fails in patients who develop CD.

SCOPE AND OUTLINE OF THIS THESIS

The aim of the work presented in this thesis was to provide further insight into the regulatory mechanisms that contribute to the induction of mucosal tolerance. In **chapter 2**, we describe a mechanism through which constitutive expression of cyclooxygenase-2 (COX-2) in mucosal DC controls the induction of mucosal tolerance to fed antigens. We observed that COX-2 expression in MLN-DC contributes to the RA- and TGF-β-dependent induction of Foxp3^{+} Treg cells *in vivo* and *in vitro*. Reciprocally, the inhibition of COX-2 in MLN-DC resulted in the increased differentiation of inflammatory T cells with a Th2-like phenotype.

Whereas the role of intestinal DC in the induction of mucosal tolerance to orally administered antigens is well established, little is known about their role in the regulation of adaptive immune responses in the colon. In **chapter 3**, we describe that mucosal tolerance is induced upon both oral and colonic protein administration. However, it was observed that whereas the CD103^{+} DC-cell subset in the lymph nodes draining the small intestine expressed the vitamin A-metabolizing enzyme RALDH2, CD103^{+} DC in the colon-draining lymph nodes did not express this enzyme and instead were characterized by high levels of IL-10. These data suggest that the mechanisms leading to the establishment of tolerance in the small and large intestine are different. Most likely as a result of these differences, colonic antigen administration resulted in a lower conversion of naive T cells into Foxp3^{+} Treg cells than seen during the induction of oral tolerance.

In **chapter 4**, we used newly-generated transgenic mice expressing HLA-DQ2 and a gluten-specific humanized TCR from a CD patient to be able to study the mechanisms of immune regulation in response to gluten feed *in vivo*. Our findings demonstrate that, in sharp contrast

to the model food protein OVA, gluten induced the differentiation of tolerogenic IFN-γ- and IL-10-producing Tr1-like cells in the spleen.

In **chapter 5**, it was assessed whether T-cell activation under the control of tissue-specific factors in the intestine resulted in the expression of certain tissue-specific cell-surface markers that could be used to distinguish gut-activated T cells from non-mucosal T cells. TGF-β- and RA-induced expression of CD62L^{neg}CD38^{+} on CD4^{+} T cells was found to selectively identify mucosally-activated T cells in mice and humans. In **chapter 6**, we used this newly identified mucosal T-cell phenotype to characterize mucosally-induced and naturally-occurring Treg-cell subsets in pediatric and adult patients with CD and in controls.

Finally, in **chapter 7**, the main results of this thesis are summarized and discussed.

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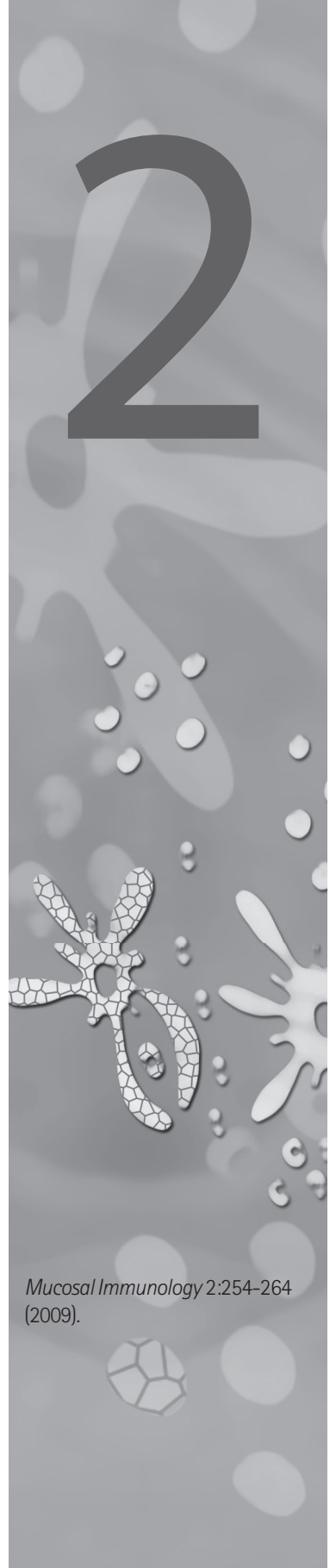
Cyclooxygenase-2 in mucosal DC mediates induction of regulatory T cells in the intestine through suppression of IL-4

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ABSTRACT

Oral intake of protein leads to tolerance through the induction of regulatory T (Treg) cells in the gut-draining mesenteric lymph nodes (MLN). Here we show that inhibition of cyclooxygenase-2 (COX-2) *in vivo* suppressed oral tolerance and was associated with enhanced differentiation of interleukin (IL)-4-producing T cells and reduced forkhead box P3 (Foxp3)$^+$ T-cell differentiation in MLN. As a result the functional suppressive capacity of these differentiated mucosal T cells was lost. IL-4 was causally related to loss of tolerance as treatment of mice with anti–IL-4 antibodies during COX-2 inhibition restored tolerance. Dendritic cells (DC) in the MLN differentially expressed COX-2 and reductionist experiments revealed that selective inhibition of the enzyme in these cells inhibited Foxp3$^+$ Treg-cell differentiation *in vitro*. Importantly, inhibition of COX-2 in MLN-DC caused increased GATA-3 expression and enhanced IL-4 release by T cells, which was directly related to impaired Treg-cell differentiation. These data provide crucial insights into the mechanisms that drive *de novo* Treg-cell induction and tolerance in the intestine.

INTRODUCTION

In the intestine, a tight regulation avoids mounting harmful immune responses to dietary antigens and commensal flora, whereas an efficient inflammatory immune response defends the host against pathogens. One of the crucial steps that underlie the development of mucosal tolerance to harmless antigens and bacterial flora is the induction of adaptive regulatory T (Treg) cells (1-9). Mucosally-induced Treg cells are distinct from thymus-derived naturally-occurring Treg cells as they differentiate from naive T cells in the gut-draining mesenteric lymph nodes (MLN) and Peyer's Patches (PP) (9). Once formed, mucosally-induced Treg cells specifically suppress inflammatory responses to the antigen that was used for tolerization and can transfer tolerance to naive recipients (7-9). Unfortunately, the mechanism that leads to adaptive Treg-cell differentiation in the mucosa draining lymph nodes is largely unclear.

Unique mucosal mediators have been identified that play an essential role in Treg-cell differentiation. One such mediator is transforming growth factor (TGF)-β, which, abundantly expressed in the mucosal environment, can by itself lead to conversion of CD25$^-$ forkhead box P3 (Foxp3)$^-$ cells into CD25$^+$Foxp3$^+$ Treg cells (10). An additional important mucosal factor is retinoic acid (RA) for which recently a synergism with TGF-β in Treg-cell differentiation was demonstrated. Intestinal dendritic cells (DC) drive enhanced TGF-β-dependent Treg-cell differentiation *in vitro* when compared with splenic DC and this exclusive capacity of intestinal DC relies on their ability to convert vitamin A to RA (11-14). The fact that RA from intestinal DC is also involved in the imprinting of gut-tropism on the differentiating Treg cells, enabling them to home to the intestine, underscores the importance of this factor (14-17). It is currently unknown how exactly RA and TGF-β induce mucosal Treg cells.

Another regulatory mediator that is intrinsic to the mucosal environment is cyclooxygenase-2 (COX-2), an enzyme that synthesizes prostaglandins from arachidonic acid. In the intestine COX-2 is constitutively expressed by stromal cells and myeloid cells, which is in contrast to other peripheral sites where it is induced upon inflammation (18). Evidence for a regulatory role for COX-2 in intes-

tinal immune responses was provided when treatment of mice with the COX-2 inhibitor NS-398 led to loss of tolerance to dietary antigen (19, 20). In addition, although mice deficient in COX-2 do not show any spontaneous inflammation in the intestine, they have increased susceptibility to chemically induced colonic enteropathy. This is associated with the inability to produce the arachidonic acid metabolite prostaglandin E_2 (PGE_2) (21). Although these findings identify a role for COX-2 in mucosal tolerance it is unclear whether the enzyme regulates particular adaptive T-cell immune responses. Moreover, the specific immunological events leading to inflammation in the absence of functional COX-2 are unidentified. Therefore, in this study, we determined whether COX-2 is directly involved in adaptive Treg-cell differentiation in the intestine. Moreover, as it has been reported that interleukin (IL)-4 can mediate enteropathy (22) and plays an important role in several models of colitis (23, 24), we aimed to identify whether IL-4 is involved in the inflammatory immune response that occurs in the absence of functional COX-2.

Here we show that inhibition of COX-2 during protein feed caused loss of tolerance induction, which was associated with enhanced T-cell-derived IL-4 production and defective Treg-cell differentiation in the MLN. Crucially, treatment of mice with anti-IL-4 antibodies prior to COX-2 inhibition and protein feed restored oral tolerance. As, predominantly, DC in the MLN expressed COX-2, the role of the enzyme in mucosal T-cell differentiation was further investigated *in vitro*. COX-2 expression in MLN-DC stimulated the TGF-β- and RA-dependent conversion of naive T cells into Foxp3+ Treg cells. Reciprocally, inhibition of COX-2 in MLN-DC increased GATA-3 expression and IL-4 secretion by the differentiating T cells. The differentiation of the IL-4-producing cells in the absence of functional COX-2 was directly related to loss of mucosal tolerance as exogenous IL-4 blocked MLN-DC-driven differentiation of Foxp3+ mucosal Treg cells. These data establish that COX-2 in MLN-DC is required to control the GATA-3/IL-4 axis allowing differentiation of mucosal Treg cells and subsequent tolerance.

METHODS

Mice

Specific pathogen-free BALB/c mice (8-10 weeks) were purchased from Charles River, Maastricht, The Netherlands. DO11.10 transgenic (Tg) mice and DO11.10 Tg x RAG$^{-/-}$ mice, which have a Tg T-cell receptor (TCR) specific for the ovalbumin (OVA) 323-339 peptide, were bred at our own facility. CD2-GATA3 Tg and DO11.10 Tg x CD2-GATA3 Tg mice were generated as described previously (25). All mice were kept under routine animal housing conditions and experiments were approved by the animal experimental committee of the VUMC or the Erasmus MC.

Antibodies and antigen

In all *in vivo* experiments, intact 98% pure OVA (either from Sigma Aldrich, Zwijndrecht, The Netherlands or from Calbiochem, San Diego, CA, USA) was used. In *in vitro* experiments either intact OVA (Calbiochem) or OVA$_{323-339}$ peptide was used. The anti-clonotypic mAb for the DO11.10 Tg

TCR (KJ1.26) was purified from culture supernatant and biotinylated, according to manufacturer's protocol (Molecular Probes, Leiden, The Netherlands). Anti-CD11c (HL3), anti-CD4 (GK1.5), anti-CD3 (145-2C11), anti-CD25 (PC61), anti-CD45RB (16A), anti-B220 (RA3-6B2), anti-$\alpha_4\beta_7$ (DATK32), anti-MHCII (M5/114), anti-CD8α (53-6.7), anti-Gr1 (RB6-8C5) and appropriate isotype control antibodies were purchased from BD-Pharmingen (Woerden, The Netherlands). Conjugates that were used for flow cytometry were, streptavidin-CyChrome, streptavidin-PerCP, streptavidin-APC (BD-Pharmingen) and anti-rat-PE (Jackson laboratories, West Baltimore, USA).

Adoptive transfer and CFSE labeling

Lymph nodes and spleens were isolated from DO11.10 Tg mice and single-cell suspensions were prepared, enriched for CD4$^+$ T cells as described previously (8, 9), and labeled with 5,6-carboxy-succinimidyl-fluoresceine-ester (CFSE) (Molecular Probes) to be able to follow their division profiles *in vivo*. Each mouse received 1 x 10^7 CD4$^+$KJ1.26$^+$ cells in 100 μl saline by intravenous injection.

Delayed-type hypersensitivity response

Mice were injected intraperitoneally 6 times at 8 h intervals with 1 mg kg^{-1} of the freshly dissolved selective COX-2 inhibitor NS-398 (Alexis, Breda, The Netherlands) in saline as described by Newberry et al. (19). Control groups were injected with saline alone or vehicle consisting of a 1% DMSO solution in saline. The first injection of NS-398 was given 5 h prior to intragastric (i.g.) administration of 25 mg OVA in 200 μl saline (Sigma Aldrich). Five days after i.g. OVA administration, mice were sensitized subcutaneously in the tail base with 100 μg OVA in 50 μl of a 1:1 incomplete Freund's adjuvant (IFA):saline solution (Difco, BD. Alphen a/d Rijn, The Netherlands). At day 11, mice were challenged with 10 μg OVA in 10 μl saline in both ears, after 24 h increases in ear-thickness were determined and compared to values prior to challenge (7-9).

Transfer to assess regulatory function of T cells

Mice were adoptively transferred with DO11.10 Tg cells and treated with NS-398 or vehicle prior and after tolerization with 70 mg OVA 1 day later (9). MLN and PP were isolated 48 h after OVA administration, CD4$^+$ cells were enriched, and 5 x 10^5 cells were transferred to naive BALB/c mice. Enriched CD4$^+$ T cells were routinely between 80-90% pure. One day after transfer the mice were sensitized followed by a delayed-type hypersensitivity (DTH) challenge in the ears 5 days later.

TGF-β-induced *in vitro* Treg-cell differentiation

Bone-marrow-derived DC were generated as described earlier (26). To obtain DC from MLN, tissue was digested with Liberase Blenzyme 3 (Roche, Woerden, The Netherlands) in the presence of DNAse I (Roche) during 30-35 min in a 37 °C incubator. CD11c$^+$ cells were isolated using

anti-CD11c MACS beads (Miltenyi Biotec, Bergisch Gladbach, Germany). Purity of the isolated CD11c$^+$ cell subset was 60-80%. DC were loaded with either 0.5 mg ml^{-1} OVA (Calbiochem) for 6 h or with OVA peptide for 2 h. After washing, 2 x 10^4 bone-marrow-derived DC or MLN-DC were incubated with 5 x 10^5 CFSE-labeled CD4$^+$KJ1.26$^+$ x RAG$^{-/-}$ T cells in the presence of 20 ng ml^{-1} rhTGF-β (Preprotech, Rocky Hill, NJ, USA) for 96 h to induce Foxp3$^+$ Treg-cell differentiation. Exogenous recombinant IL-4 (300 ng ml^{-1}, R&D systems), anti-IL-4 (10 μg ml^{-1}; purified from 11B11 hybridoma), isotype control (10 μg ml^{-1}; purified from GL113 hybridoma), NS-398 (75 μM), DMSO (0.09%) or DO11.10 Tg x CD2-GATA3 Tg T cells were used in the culture. In some experiments, DC were stimulated with 10 nM RA (Sigma) during OVA loading and the 96 h culture. To study the role of PGE$_2$ in Treg-cell conversion CD4$^+$KJ1.26$^+$ x RAG$^{-/-}$ T cells were stimulated with 16,16-dimethyl PGE$_2$ (26 μM) (Cayman Chemical, Ann Arbor, MI, USA) 2 h prior to the start of the culture as well as during the 96 h culture. After culture, the cells were analyzed for Foxp3 expression (Clone FJK-16S, e-Bioscience, San Diego, CA, USA), intracellular cytokine production and CFSE content by flow cytometry. In some experiments, CD4$^+$ T cells were isolated from the culture by eliminating DC with a mix of rat antibodies to B220 (clone 6B2), F4/80, CD11b (MAC-1), MAC-2, MHCII (M5/114), CD8 (53.6.72) and anti-rat magnetic beads (Dynal, Oslo, Norway).

Flow cytometry

At 48 and 72 h after i.g. OVA administration, MLN, PP and spleen were isolated and single-cell suspensions were stained for CD4, CD25, CD45RB or $α_4β_7$ and KJ1.26 to determine phenotype and cell division by flow cytometry (FACS Calibur, BD). Cell division was determined based on fluorescence intensity of single CFSE peaks. For further analysis at least 20,000 KJ1.26$^+$ cells were counted. For the analysis of OVA-containing DCs, mice were fed with 5 mg per mouse of OVA-Alexa–488. At 48 h after OVA-Alexa–488 feed, MLN were isolated and single-cell suspensions were stained for CD4, CD19, CD40, CD86, CD8α, CD11b, MHCII and CD11c.

For obtaining purified populations of T cells, B cells, CD45$^-$ cells, DC and DC-cell subsets, cells were isolated from LN as described above. Purified T cells, B cells DC and CD45$^-$ cells were obtained in a single step by flow-cytometric cell sorting on expression of CD3, B220, CD11c, MHCII and CD45. DC subsets were purified by negative enrichment of lymph nodes cells for DC using a mouse DC enrichment kit (Dynal) and subsequent flow-cytometric cell sorting on expression of CD11b, CD45, CD8α and Gr-1.

Cytokine secretion

The percentage of cytokine-secreting cells, and the amount of cytokine secreted during a 16 h restimulation period, were assessed by isolating MLN and PP at 24, 48 and 72 h after i.g. OVA administration. Cells were incubated at 5 x 10^6 cells per ml with either 0.5 mg ml^{-1} OVA, 100 ng ml^{-1} OVA peptide, or medium as a control. Cytokine concentrations in supernatants were determined by BD cytometric bead array according to manufacturers' instructions (BD-

Pharmingen). Antigen-specific cytokine-secreting cells were determined using the cytokine specific capture assays for IL-4 and IFN-γ according to the instructions of the manufacturer (Miltenyi Biotech).

Real time PCR

Total RNA was purified from DC or CD4$^+$KJ1.26$^+$ T cells from MLN using the Qiagen RNeasy kit (Westburg, Leusden, The Netherlands). One microgram of RNA was reverse transcribed to single-stranded cDNA using a mix of random hexamers (2.5 µM) and oligodT primers (20 nM). The RT reaction was performed in a total volume of 25 µL containing 0.2 mM of each dNTP (Amersham Pharmacia BioTech, Piscataway, NJ), 200 U Moloney murine leukemia virus reverse transcriptase (M-MLV RT; Promega, Madison, WI), and 25 U RNAsin (Promega). Conditions for the RT reaction were 37 °C for 30 min, 42 °C for 15 min, and 94 °C for 5 min. The cDNA was diluted to a final concentration of 8 ng µL^{-1} and stored at -80 °C. Real-time quantitative PCR was performed using an AbiPrismR 7900 Sequence Detection System (PE Applied Biosystems, CA, USA) based on specific primers and general fluorescence detection with SYBR green. Cyclophillin was used to control for sample loading and to allow normalization between samples. The expression levels relative to cyclophillin were calculated following the equation: relative expression level = $2^{-\Delta Ct}$, whereby $\Delta Ct = Ct_{target} - Ct_{cyclo}$. Specific primers were designed across different constant region exons resulting in these primers:

GATA3: Fw: 5' ATG CCT GCG GAC TCT AC 3' Rv: 5' GGT GGT GGT GGT CTG AC 3'
T-BET: Fw: 5' CAG GGA ACC GCT TAT ATG 3' Rv: 5' CTG GCT CTC CAT CAT TCA 3'
COX-2: Fw: 5' ACC CGG ACT GGA TTC TAT 3' Rv: 5' GCT TCC CAG CTT TTG TAA 3'
FOXP3: Fw: 5' ACC TGG GAT CAA TGT GG 3' Rv: 5' TGG CAG TGC TTG AGA AA 3'
CYCLO: Fw: 5' AAC CCC ACC GTG TTC T 3' Rv: 5' CAT TAT GGC GTG TAA AGT CA 3'
IFN-γ: Fw: 5' CAAAAGGATGGTGACATGA 3' Rv: 5' GGGTTGTTGACCTCAAACT 3'
IL-4: Fw: 5' CAAGGTGCTTCGCATATT 3' Rv: 5' GCATGGTGGCTCAGTACTA 3'
IL-17: Fw: 5' CTTGGCGCAAAAGTTGA 3' Rv: 5' TTGCTGGATGAGAACAGAA 3'

Statistics

Statistically significant differences for ear-thickness measurements were determined using a one-way ANOVA followed by a Turkey-Kramer multiple comparisons test to assess differences between individual groups. $P<0.05$ was considered significant. All experiments were performed at least three times unless otherwise indicated.

RESULTS

The COX-2-dependent arachidonic acid metabolism is essential for oral tolerance induction

As the role for COX-2 in mucosal tolerance has not been demonstrated for the classical model of oral tolerance induction in normal BALB/c mice, mice were treated with NS-398, a competitive inhibitor of COX-2 with a 1000-fold higher affinity for COX-2 than COX-1. During the first 48 h after oral OVA ingestion, mice were treated with the inhibitor every 8 hours as described previously by Newberry et al. (18), whereas control mice received vehicle or saline. To assess effects of COX-2 inhibition on oral tolerance induction, the mice were subjected to a DTH response consisting of sensitization with OVA in the tail base and a subsequent challenge with OVA in the ears. Treatment with NS-398 during oral OVA administration reduced oral tolerance induction as evidenced by a pronounced increase in ear-thickness compared to saline treated mice (Figure 2.1 A). NS-398 treatment had no effect on the DTH response in control
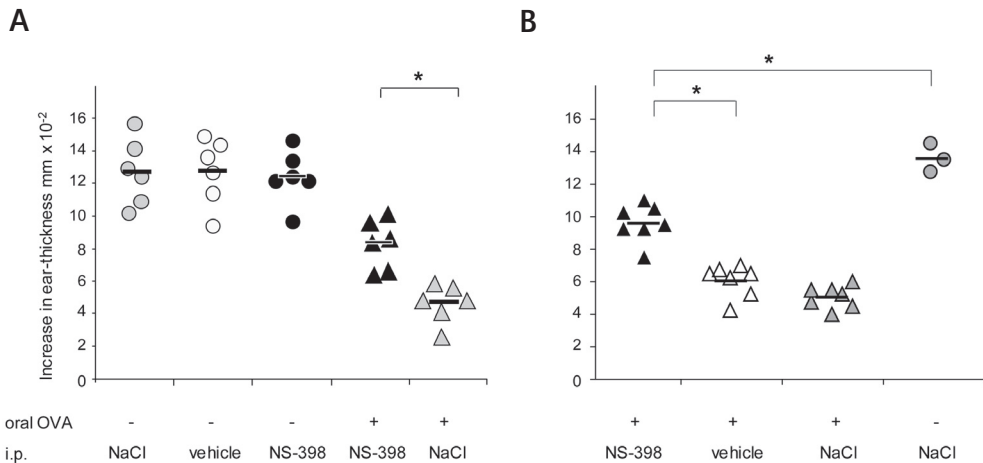
Figure 2.1 COX-2-dependent prostaglandins are essential during oral tolerance induction. **(A)** Effect of COX-2 inhibitor (NS-398) on orally induced suppression of a DTH response in BALB/c mice. Mice were i.p. injected 6 times with 1 mg kg^{-1} of the selective COX-2 inhibitor, NS-398, saline or 1% DMSO at 8 h intervals. The first i.p. injection was given 5 h before tolerance induction via i.g. treatment with 25 mg OVA. Five days after OVA feed, mice were sensitized s.c. in the tailbase with 100 µg OVA in IFA. At day 11, mice were challenged with 10 µg OVA in 10 µl saline in the auricle of both ears and after 24 h increases in ear-thickness were determined and compared to values prior to challenge. * Statistically significant ($P<0.05$). **(B)** Effect of COX-2 inhibitor (NS-398) on orally induced suppression of a DTH response in DO11.10 reconstituted mice. BALB/c mice were injected with 1 x 10^7 CD4$^+$ KJ1-26$^+$ cells i.v.. Starting the next day, mice were treated with NS-398 as described above. The first i.p. injection was 5 h before tolerance induction by a single i.g. dose of 70 mg OVA. Five days after i.g. OVA administration, mice were sensitized s.c. with OVA in IFA, at day 11 mice were challenged with OVA in the ears and 24 h thereafter increases in ear-thickness were determined and compared to values prior to challenge. * Statistically significant ($P<0.001$). COX-2, cyclooxygenase-2; DMSO, dimethyl sulfoxide; DTH, delayed-type hypersensitivity; IFA, incomplete Freud's adjuvant; i.g., intragastric; i.p., intraperitoneal; i.v., intravenous; OVA, ovalbumin; s.c., subcutaneously.

mice. The treatment did not lead to a change in kinetics of tolerance development as at 48 h postchallenge the DTH of NS-398-treated and OVA-fed mice remained elevated (8.4 $\pm$ 0.6 x 10 mm^{-2}) when compared to tolerant controls (4.8 $\pm$ 0.5 x 10 mm^{-2}) and was comparable to NS-398-treated DTH controls (10.5 $\pm$ 1.4 x 10 mm^{-2}). Moreover, COX-2 inhibition during the first 48 h after a single dose of oral OVA ingestion did not result in pathological changes in the small intestine, such as expansion of the numbers of lamina propria lymphocytes, crypt expansion or villus blunting (data not shown).

COX-2-dependent arachidonic acids inhibit differentiation of IL-4-producing cells

To elucidate whether COX-2 regulates T-cell differentiation in the MLN and PP during tolerance induction, mice were reconstituted with OVA-specific T cells by transfer of OVA-specific TCR Tg DO11.10 cells. To ensure that OVA feeding to recipients of TCR Tg cells induces suppression of DTH response and to confirm that COX-2 inhibited this phenomenon, mice reconstituted with DO11.10 cells were treated with NS-398 during OVA feed and subjected to a DTH response. As seen in Figure 2.1 B, oral OVA treatment of recipients of DO11.10 cells induces suppression of a subsequent DTH response and NS-398 treatment effectively reduced oral tolerance induction. To study T-cell differentiation in the MLN and PP during tolerance induction, BALB/c mice received naive CFSE-labeled OVA-specific CD4$^+$KJ1.26$^+$ T cells, were fed OVA, and 48 h and 72 h later, division of CD4$^+$KJ1.26$^+$ cells in the MLN and the PP was assessed.

The division profile of OVA-specific T cells in MLN and PP at 48 h and 72 h after OVA feed was comparable in vehicle and NS-398-treated mice (Figure 2.2 A, B and supplementary Figure 2.1 A). Moreover, total numbers of CD4$^+$KJ1.26$^+$ cells in MLN were comparable between vehicle and NS-398-treated mice (MLN of NS-398-treated mice 6.6 x 10^5 $\pm$ 2.6 x 10^5 KJ1.26$^+$ cells, compared to 6.6 x 10^5 $\pm$ 2.4 x 10^5 in vehicle-treated mice), excluding that failure of tolerance induction in NS-398-treated mice was due to abortive T-cell proliferation. Phenotypically, no changes in expression of CD25, CD45RB and $\alpha_4\beta_7$, were detected as a result of COX-2 inhibition (data not shown). As COX-2 and NS-398 have been described to directly affect T-cell apoptosis *in vitro* (27, 28), annexin-V expression was determined. After COX-2 inhibition, no differences in antigen-specific apoptotic cells could be observed (data not shown).

To assess whether COX-2 controls the cytokine profile of differentiating T cells, dividing CD4$^+$KJ1.26$^+$ cells in the MLN were monitored. Treatment with NS-398 significantly altered the cytokine profile of the differentiating T cells in the MLN. NS-398 enhanced the IL-4 secretion in T cells isolated from MLN at 48 h after OVA feed, as reflected by an increase in the percentage of IL-4-producing dividing OVA-specific T cells (Figure 2.2 C). Moreover, even though the percentage of interferon (IFN)-γ-secreting OVA-specific T cells did not change after COX-2 inhibition (Supplementary Figure 2.1 B), the overall release of this cytokine was

reduced in MLN cells from NS-398-treated mice (Figure 2.2 D). No differences in IL-5, IL-10, IL-13 or IL-17 were observed (data not shown). In agreement, NS-398 treatment of mice during OVA feed induced a two-fold increase in GATA-3 expression in differentiating OVA-specific T cells obtained from the gut-draining MLN, whereas T-bet expression was not significantly changed (Figure 2.2 E).
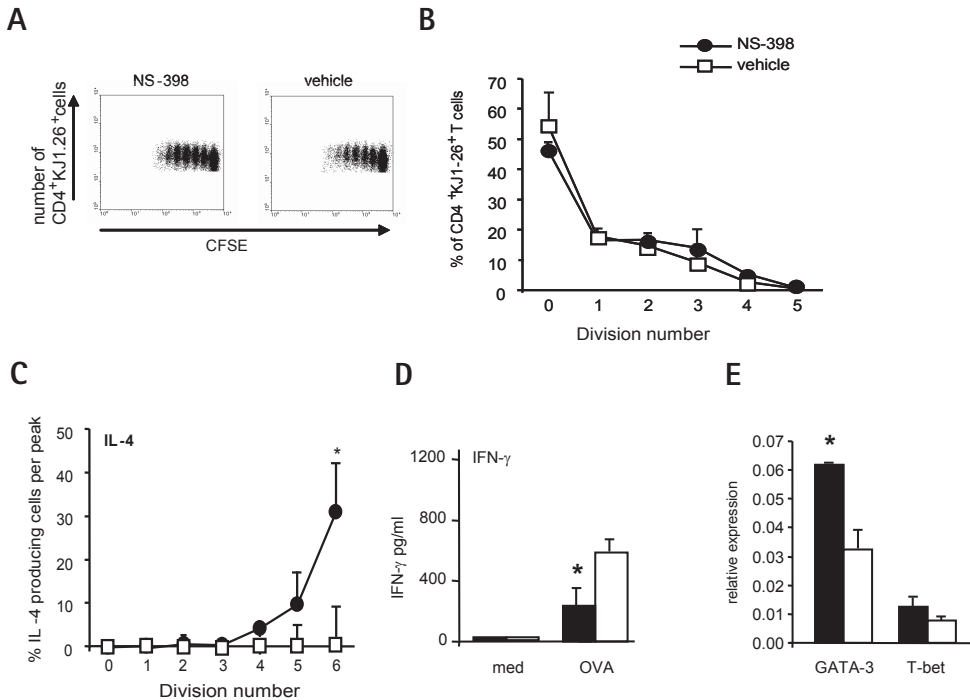
Figure 2.2 COX-2-dependent arachidonic acids inhibit differentiation of IL-4-producing T cells in MLN. Donor BALB/c mice were injected with 1×10^7 CD4$^+$ KJ1.26$^+$ cells i.v.. Starting the next day, mice were treated with NS-398 (black symbols) as described in Figure 2.1. Control groups were injected with vehicle consisting of a 1% DMSO solution in saline (white symbols). Tolerization was induced by a single i.g. dose of 70 mg OVA. At 48 h after OVA administration, MLN and PP (not shown) were isolated and single-cell suspensions were stained for the presence of CD4$^+$KJ1.26$^+$ cells. CFSE profiles of CD4$^+$KJ1.26$^+$ T cells were determined in MLN by flow cytometry. **(A)** Representative division plot at 48 h after OVA feed. **(B)** The percentages of CD4$^+$KJ1.26$^+$ T cells in each peak of division were calculated and are represented as the mean for at least 3 mice ± SD. **(C–E)** At 48 h after oral OVA administration, MLN cells were re-stimulated overnight with 0.5 mgml^{-1} OVA or medium at a concentration of 5×10^6 cells ml^{-1}. **(C)** Single-cell suspensions were stained for KJ1.26$^+$ cells and the percentage of IL-4-secreting cells per peak of division was determined with a cytokine secretion assay. Data are represented as mean percentage of OVA-induced cytokine-secreting antigen-specific T cells per peak of division of at least three separate experiments, with SD. **(D)** Cytokine concentrations in the supernatants of overnight cultures were determined by cytometric bead array. Data are represented as the mean cytokine concentration of at least three separate mice, with SD. **(E)** At 48 h after oral OVA administration, dividing OVA-specific T cells were isolated from MLN of three mice and expression of transcription factors GATA-3 and T-bet were performed by quantitative PCR. * Statistically significant (*P*<0.01). COX-2, cyclooxygenase-2; DMSO, dimethyl sulfoxide; DTH, delayed-type hypersensitivity; IFA, incomplete Freud's adjuvant; i.g., intragastric; IL, interleukin; i.v., intravenous; MLN, mesenteric lymph nodes; OVA, ovalbumin; PP, Peyer's patch.

COX-2 dependent arachidonic acid metabolites are required for development of functional Treg cells during oral tolerance induction

Conversion of naive T cells into suppressive Treg cells in the mucosa-draining lymph nodes is a pivotal event in mucosal tolerance induction (8, 9). Within 48-72 h post OVA feed, dividing Foxp3$^+$ T cells can be detected in MLN (Figure 2.3 A). Although it is unclear whether Foxp3 expression is essential for the functional suppressive capacity of adaptive mucosally induced Treg cells, it was questioned whether the enhanced IL-4-producing T-cell differentiation during
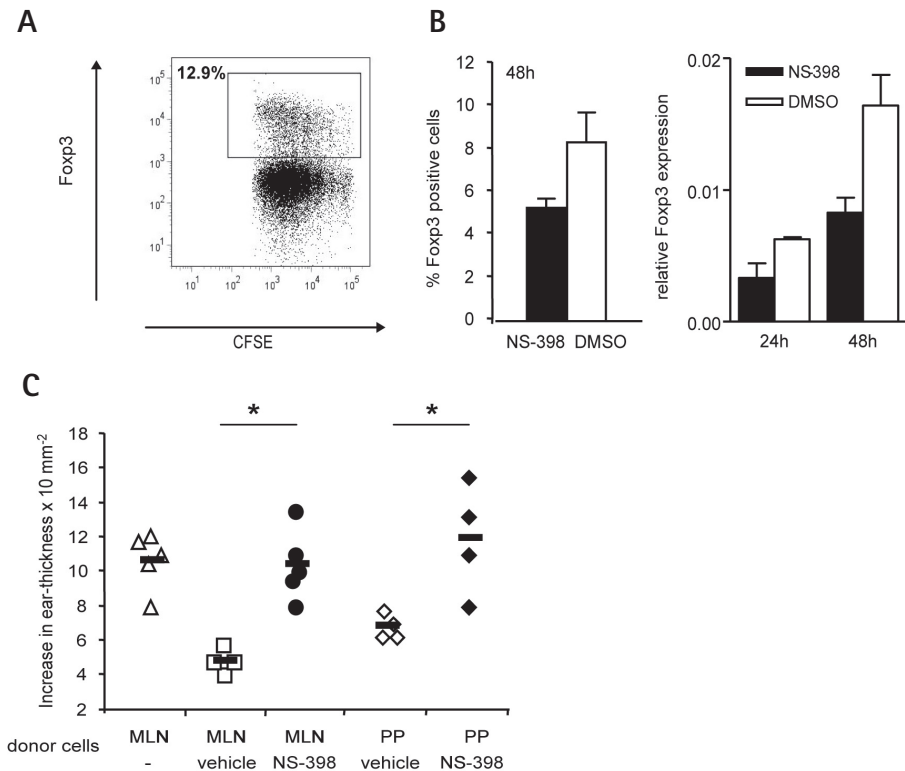

Figure 2.3 COX-2-dependent prostaglandins are required for functional Treg-cell induction in the gut-draining lymphoid tissue. **(A)** BALB/c mice enriched with CD4$^+$KJ1.26$^+$ x RAG$^{-/-}$ T cells were tolerized as described in Figure 2.1. Representative dot-plot of dividing Foxp3$^+$ T cells in the MLN at 72 h after OVA feed. **(B)** BALB/c mice enriched with CD4$^+$KJ1.26$^+$ cells were treated with NS-398 (black symbols) or vehicle control (white symbols). At 48 h after OVA administration, MLN were isolated and CD4$^+$KJ1.26$^+$ cells within single-cell suspensions were analyzed for the percentage of Foxp3$^+$ cells by flow cytometry and Foxp3 mRNA (24 and 48 h). **(C)** At 48 h after feeding, MLN and PP were isolated and single-cell suspensions were enriched for CD4$^+$ cells. 5 x 10^5 enriched CD4$^+$ cells from either NS-398-treated mice (black circles: MLN cells; black diamonds: PP cells) or vehicle-treated mice (white squares: MLN cells; white diamonds: PP cells) were transferred to naive BALB/c recipients. As a control, 5 x 10^5 enriched CD4$^+$ cells isolated from MLN of saline-treated mice as a DTH control (white triangles) were transferred to naive acceptor BALB/c mice. Acceptor mice were sensitized and challenged as described in Figure 2.1. * Statistically significant (P<0.05). COX-2, cyclooxygenase-2; DTH, delayed-type hypersensitivity; i.g., intragastric; MLN, mesenteric lymph nodes; OVA, ovalbumin; PP, Peyer's patch.

NS-398 treatment was associated with decreased Foxp3$^+$ T-cell differentiation. Thereto, dividing CD4$^+$KJ1.26$^+$ T cells in the MLN were analyzed for Foxp3 protein expression and mRNA. At 48 h after antigen application, a reduced percentage of dividing Foxp3$^+$KJ1.26$^+$ T cells in MLN was observed in NS-398-treated mice compared with vehicle control, which correlated with reduced Foxp3 mRNA levels at 24 h and 48 h post OVA (Figure 2.3 B).

Next, it was assessed whether the gain in IL-4-producing T-cell differentiation and concomitant loss of Foxp3$^+$ Treg-cell development resulted in loss of suppressive capacity of the mucosal T cells, explaining the absence of tolerance in COX-2 inhibitor-treated mice. Thereto, the dividing antigen-specific T cells from MLN of tolerized donors were adoptively transferred to naive recipients as described previously (8, 9). At 48 h after antigen application, CD4$^+$ cells were isolated from MLN and PP and transferred to naive BALB/c recipients that were sensitized and challenged for DTH to read out whether tolerance had developed. Although CD4$^+$ T cells from vehicle treated, tolerized donors could transfer tolerance, this suppressive activity was not seen after transfer of CD4$^+$ T cells from MLN and PP of NS-398-treated mice (Figure 2.3 C). This difference in tolerogenic capacity was not due to differences in frequency of OVA-specific KJ1.26$^+$ T cells within the CD4$^+$ fraction between NS-398-treated mice (3.3 % ± 1.2) and vehicle-treated mice (3.9 % ± 1.5). In conclusion, these data indicate that inhibition of COX-2 impedes the differentiation of functionally suppressive T cells in the MLN and is associated with enhanced IL-4 production and reduced Treg-cell induction.

Neutralization of IL-4 restores mucosal tolerance in NS-398-treated mice

To establish whether there is a causative relationship between IL-4 production, loss of Treg-cell differentiation and loss of functional suppression after NS-398 treatment, *in vivo* experiments were performed. NS-398-induced IL-4 secretion was neutralized before the inhibition of COX-2 and subsequent development of tolerance to OVA was assessed. Treatment of mice with anti-IL-4 antibodies before NS-398 and OVA administration restored oral tolerance when compared with mice treated with NS-398 and isotype control GL113 or saline (Figure 2.4). It should be noted that treatment with anti-IL-4 antibodies does not affect the DTH response in unfed controls (29). This implicates that enhanced IL-4 secretion after COX-2 inhibition causes abrogation of oral tolerance.

COX-2 expression in MLN-DC inhibits GATA-3 and IL-4 differentiation in favor of Treg-cell induction

Further understanding of how COX-2 regulates mucosal T-cell differentiation required the investigation *in vitro* modelling. Thereto, it was first established whether COX-2 expression occurred in mucosal antigen-presenting cells or whether expression is required in other cell types. At 24 h after oral treatment of mice with Alexa-Fluor 488-labeled OVA, CD11c$^+$OVA-488$^+$DCs were detected in MLN confirming that MLN-DC had taken up the antigen (Figure 2.5 A). Comparison of quantitative expression of COX-2 in purified CD11c$^+$MHCII$^+$ DC with expression
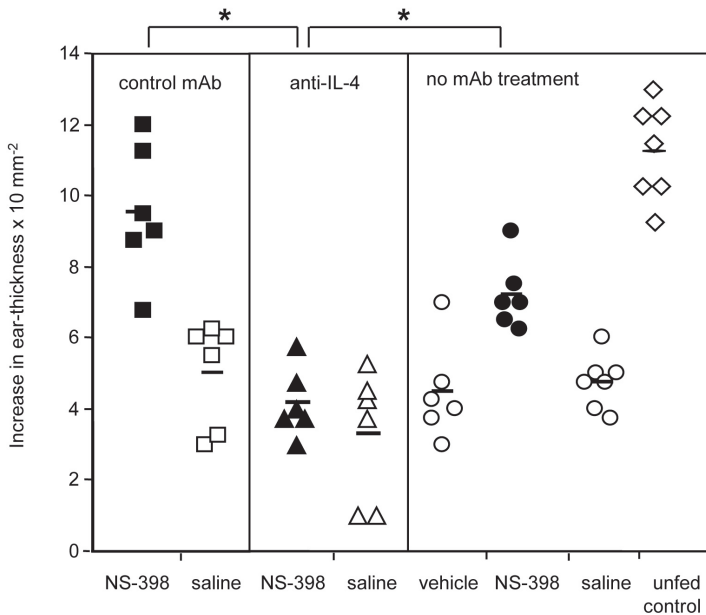
Figure 2.4 *In vivo* inhibition of IL-4 secretion during NS-398 treatment. Antibody-treated mice received 1 mg anti-IL-4 mAb (11B11) or control mAb (GL113) in 200 ml saline i.p.. Starting the next day, mice were injected with NS-398 during OVA treatment, sensitized and challenged as described in Figure 2.1. * Statistically significant (*P*<0.01). IL, interleukin; i.p., intraperitoneal; mAb, monoclonal antibody; OVA, ovalbumin.

in T cells, B cells and CD45$^-$ cells from MLN revealed that the enzyme is primarily expressed in MLN-DC (Figure 2.5 B). Moreover, COX-2 was expressed at relatively higher levels in MLN-DC compared with spleen-DC or peripheral lymph node (PLN)-DC (Figure 2.5 B) In particular, CD11b$^+$ myeloid DC from MLN constitutively expressed COX-2 mRNA, whereas this expression was very low in CD8α^+ lymphoid and GR1$^+$ plasmacytoid DC (Figure 2.5 C). The expression was not restricted to CD103$^+$ MLN-DC (COX-2 mRNA expression relative to cyclophillin in FACS-sorted cells: CD103-DC = 0.18 versus CD103$^+$ DC = 0.07) (11, 13).

To assess whether MLN-DC-derived COX-2 played a role in Treg-cell versus IL-4-producing T-cell differentiation, an *in vitro* mucosal Treg-cell differentiation assay was used. MLN-DC were pretreated with TGF-β and RA, loaded with OVA, and co-cultured with naive DO11.10 Tg x RAG$^{-/-}$ T cells in the presence of RA and TGF-β, which induces the differentiation of Foxp3$^+$ Treg cells *in vitro*. In agreement with other studies (13), stimulation of OVA-loaded MLN-DC with RA and TGF-β dramatically enhanced *de novo* expression of Foxp3 mRNA and protein expression in differentiating OVA-specific T cells (Figure 2.5 D). To establish that COX-2 expression in MLN-DC is involved in the conversion of naive T cells into Treg cells, we assessed whether incubation with NS-398 inhibited Treg-cell differentiation. Indeed, neutralization of COX-2 in the MLN-DC T-cell co-culture consistently inhibited Foxp3 mRNA expression and reduced
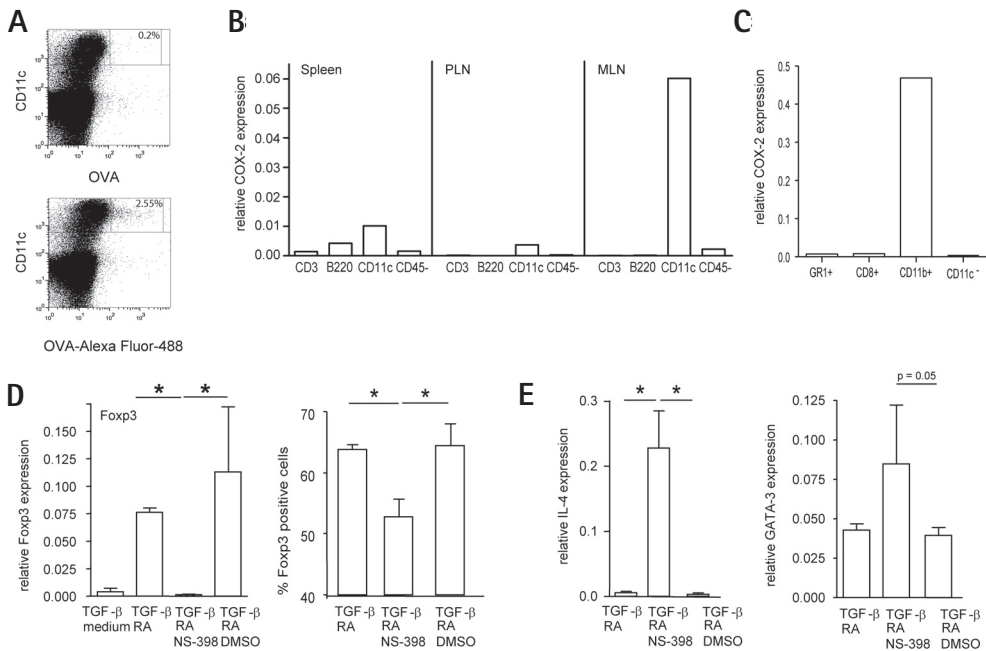
Figure 2.5 COX-2 expression in MLN-DC regulates mucosal Treg-cell conversion and suppresses IL-4-producing T-cell differentiation. **(A)** Mice were fed with 5 mg OVA labeled with Alexa Fluor-488 succinimydyl esther in 200 µl saline. At 24 h after antigen administration, MLN were isolated and single-cell suspensions were stained for CD11c. The percentage of DC that had ingested OVA were determined by gating on the CD11c and Alexa Fluor-488 double-positive population and compared with the overall CD11c+ population. **(B)** MLN, spleen and PLN of naive BALB/c mice were isolated, DC, T cells, B cells and CD45- cells were purified by flow-cytometric cell sorting and were compared for COX-2 expression relative to cyclophillin by quantitative PCR. **(C)** DC subsets were purified from MLN of naive BALB/c mice by flow-cytometric cell sorting and were compared for COX-2 expression relative to cyclophillin by quantitative PCR. **(D and E)** MLN-DC (2×10^4) were preincubated with 0.2 µg ml^{-1} OVA peptide with or without RA and with or without TGF-β for 6 h. After washing, the cells were incubated with 5×10^5 CFSE-labeled CD4+KJ1.26+ T cells in the presence of TGF-β and RA for 96 h. To assess the role of COX-2, NS-398 75 µM or DMSO control was added to the co-culture. At 96 h, CD4+ T cells were isolated from the culture by eliminating DC with mAbs and anti-rat magnetic beads and analyzed for **(D)** Foxp3 expression by Q-PCR and flow cytometry, **(E)** Th2 cytokine profile IL-4 and transcription factor GATA-3 mRNA expression by Q-PCR. * Statistically significant ($P<0.05$). COX-2, cyclooxygenase-2; DC, dendritic cell; DMSO, dimethyl sulfoxide; mAb, monoclonal antibody; MLN, mesenteric lymph nodes; OVA, ovalbumin; PLN, peripheral lymph node; RA, retinoic acid, TGF, transforming growth factor.

the frequency of Foxp3+ cells in the differentiating T cells (Figure 2.5 D). To assess whether the effect of COX-2 inhibition is the same in non-mucosal DC expressing low levels of COX-2, bone-marrow-derived DC, which express at least 2- to 10-fold lower levels of COX-2 mRNA, were used. As seen in Supplementary Figure 2.2 A-C, the inhibition of COX-2 in co-culture of bone-marrow-derived DC with TGF-β and RA reduced the percentage of differentiating Foxp3 positive cells (A), the degree of Foxp3 expression per cell (B) and the Foxp3 mRNA levels (C). Together, these data demonstrate that constitutive COX-2 expression in MLN-DC gives rise to

arachidonic acid metabolites that enhance Foxp3 induction in differentiating Treg cells.

To identify whether reduced Treg-cell differentiation during COX-2 inhibition is associated with concomitant IL-4-producing T-cell differentiation we monitored the cytokine profile of the T cells that differentiate in the presence and absence of NS-398 in MLN-DC T-cell co-culture. Thereto, MLN-DC were pretreated with TGF-β and RA, loaded with OVA, and co-cultured with CD4+KJ1.26+ T cells in the presence of NS-398 or DMSO. Differentiating T cells were analyzed for cytokine profile and transcription factor expression of the typical polarized effector cell subsets Th1 (IFN-γ), Th2 (IL-4) and Th17 (IL-17). Upon inhibition of COX-2, the differentiating T cells expressed increased amounts of IL-4 (Figure 2.5 E) but did not alter the expression of IFN-γ or IL-17 (data not shown). In agreement with the IL-4-producing profile, the differentiating T cells expressed increased amounts of the transcription factor GATA-3 (Figure 2.5 E).

As it has been reported that PGE_2 is the most predominant COX-2-dependent arachidonic acid metabolite in the intestine and as PGE_2 can induce *de novo* Foxp3 expression in CD25- T cells (30), we hypothesized that MLN-DC-derived PGE_2 may be one of the COX-2-dependent metabolites that modulates Foxp3 expression and Treg-cell conversion of OVA-specific T cells by MLN-DC. If so, then PGE_2 should be able to mimic COX-2 function in MLN-DC and enhance the differentiation of Foxp3+ T cells and suppress IL-4-producing T-cell differentiation. TGF-β-stimulated MLN-DC were loaded with OVA, incubated with OVA-specific T cells in the presence of PGE_2 or RA, and quantitative mRNA expression in dividing OVA-specific T cells was determined at 96 h of culture. Although incubation with PGE_2 slightly decreased the kinetics of T-cell division, it significantly enhanced differentiation of Foxp3+ Treg cells (Figure 2.6 A and B). Moreover, PGE_2 increased the mRNA expression of Foxp3 (Figure 2.6 B), demonstrating that PGE_2 can elicit *de novo* induction of Foxp3.

The finding that COX-2-derived prostaglandins could suppress IL-4-producing T-cell differentiation was corroborated by the observation that culture of OVA-loaded MLN-DC with PGE_2 and TGF-β suppressed IL-4 expression in dividing OVA-specific T cells at 96 h of culture as compared with TGF-β alone (Figure 2.6 C). This PGE_2-mediated IL-4 suppression was independent of MLN-DC activity as PGE_2 also inhibited IL-4 release of polyclonally-activated CD4+KJ1.26+ T cells (Figure 2.6 D).

In conclusion, MLN-DC-derived COX-2-dependent arachidonic acid metabolites suppress GATA-3 expression and IL-4 release in favor of mucosal Treg-cell differentiation.

NS-398-induced GATA-3 and IL-4 abrogate adaptive Treg-cell induction *in vitro*

To establish whether there is a direct mechanistic link between IL-4 production and loss of mucosal Treg-cell differentiation during NS-398 treatment, we assessed whether exogenous IL-4 could block MLN-DC-induced Treg-cell differentiation in the presence of TGF-β and RA *in vitro*. Thereto, MLN-DC were loaded with OVA and co-cultured with OVA-specific T cells in the presence of TGF-β, RA, and recombinant IL-4. When exogenous recombinant IL-4 was added
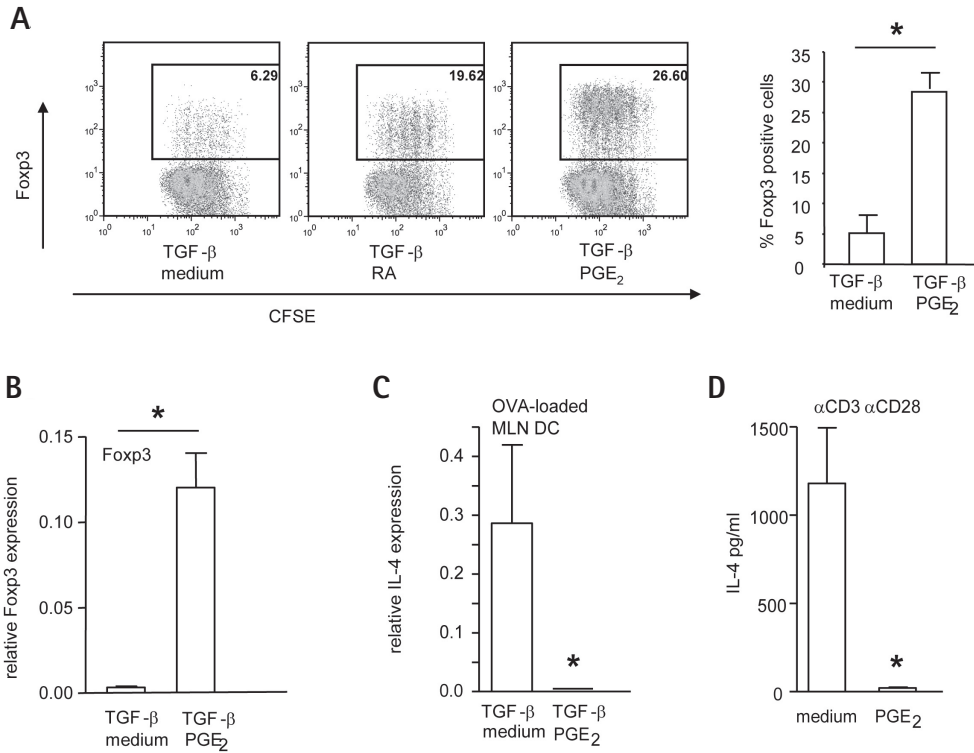
Figure 2.6 PGE$_2$ enhances Treg-cell differentiation and concomitantly inhibits Th2 differentiation. **(A–C)** MLN-DC (2×10^4) were preincubated with 0.2 µg ml^{-1} OVA peptide with or without RA and/or TGF-β for 6 h. After washing, the cells were incubated with 5×10^5 CFSE-labeled CD4$^+$KJ1.26$^+$ T cells in the presence of TGF-β with or without RA for 96 h. For analysis of PGE$_2$ effects, CD4$^+$KJ1.26$^+$ T cells were stimulated with 16,16-dimethyl PGE$_2$ (26 µM) 2 h before the start of the culture as well as during the 96 h culture. At 96 h, CD4$^+$KJ1.26$^+$ cells were analyzed for Foxp3 and IL-4. **(A)** Representative density plot of Foxp3 expression at 96 h of culture. **(B)** Foxp3 mRNA expression by Q-PCR (n=3). **(C)** IL-4 mRNA expression by Q-PCR (n=3). **(D)** 5×10^5 CFSE-labeled CD4$^+$KJ1.26$^+$ T cells were stimulated with αCD3 (10 µg ml^{-1}) and αCD28 (10 µg ml^{-1}) in the absence of TGF-β and presence of 16,16-dimethyl PGE$_2$ (26 µM) for 72 h. Release of IL-4 in cell-culture supernatants was determined by cytometric bead array. * Statistically significant ($P<0.05$). DC, dendritic cell; IL, interleukin; MLN, mesenteric lymph nodes; OVA, ovalbumin; PGE$_2$, prostaglandin E$_2$; RA, retinoic acid, TGF, transforming growth factor.

to the culture, differentiation of Foxp3$^+$ Treg cells was dramatically inhibited (Figure 2.7 A and B). Reciprocally, the addition of neutralizing anti-IL-4 antibodies during COX-2 inhibition with NS-398 partially restored the differentiation of Foxp3$^+$ Treg cells (Figure 2.7 C).

Likewise, transgenic overexpression of GATA-3 in the OVA-specific CD4$^+$KJ1.26$^+$ T cells during T-cell differentiation blocked the conversion of naive T cells into Foxp3$^+$ Treg cells (Supplementary Figure 2.2 D). The latter effect was only partially mediated by GATA-3-driven IL-4 secretion as neutralization of IL-4 partially restored the differentiation of Foxp3$^+$ cells (Supplementary Figure 2.2 E). Measurement of the concentration of IL-2 in the supernatant of the *in vitro* Treg-cell differentiation assay revealed that addition of exogenous IL-4 dramatically
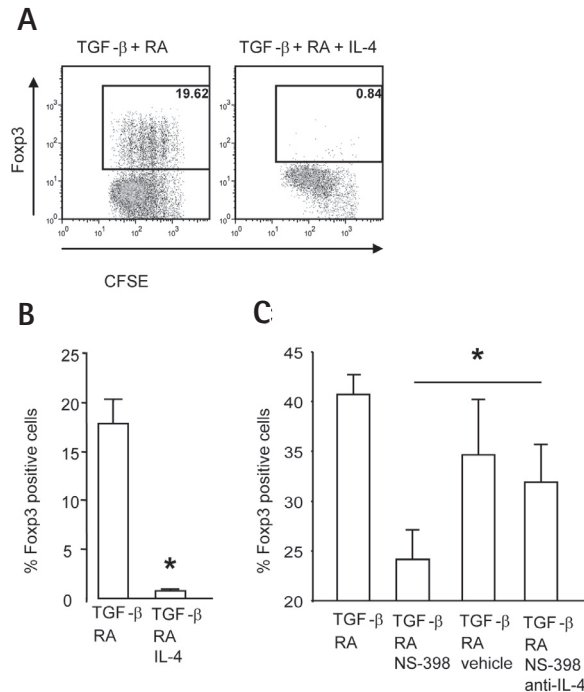
Figure 2.7   IL-4 inhibits TGF-β-induced adaptive Foxp3$^+$ Treg-cell conversion. MLN-DC were loaded with 0.2 μg ml^{-1} OVA peptide for 6 h. After washing, 2.5 x 10^4 MLN-DC were incubated with 5 x 10^5 CFSE-labeled CD4$^+$KJ1.26$^+$ T cells in the presence of TGF-β and RA for 96 h and analyzed for Foxp3 expression and CFSE content by flow cytometry. **(A)** Representative flow-cytometric analysis of Foxp3 expression in the presence or absence of exogenous recombinant IL-4 **(B)** combined data (n=3). **(C)** To determine whether the IL-4 that is produced during COX-2 inhibition directly interferes with Foxp3 expression, cells were cultured with NS-398 and anti-IL-4 (10 μg ml^{-1}; purified from 11B11 hybridoma). At 96 h, the cells were analysed for Foxp3 expression. * Statistically significant ($P<0.05$). DC, dendritic cell; IL, interleukin; MLN, mesenteric lymph nodes; OVA, ovalbumin; RA, retinoic acid; TGF, transforming growth factor.

reduced the concentration of IL-2 in the cultures (Supplementary Figure 2.2 F). This effect was also seen in the cultures with GATA-3 Tg x DO11.10 Tg T cells (Supplementary Figure 2.2 F). As it has become clear that presence of the cytokine IL-2 is crucial for the conversion of TGF-β-induced Treg cells, this could be one of the mechanisms by which IL-4 inhibits Foxp3$^+$ Treg-cell induction (10). These data establish that the activation of the GATA-3/IL-4 axis in differentiating T cells blocks the conversion to the TGF-β-elicited Foxp3$^+$ Treg-cell phenotype.

DISCUSSION

In this study, we show that COX-2 expression in MLN-DC is involved in the induction of Treg cells during oral tolerance. COX-2 acts by down regulating GATA-3 and IL-4 release in differentiating T cells. IL-4 was found to be a crucial factor that inhibits oral tolerance and impedes the

conversion of naive T cells into mucosal Treg cells during COX-2 inhibition. Our data shed new light on the earlier findings demonstrating a role of COX-2 in mucosal homeostasis (19-21), as they show that COX-2-dependent arachidonic acid metabolites determine the phenotype of the adaptive intestinal immune responses and regulate *de novo* Treg-cell induction in the mucosal immune system by suppression of IL-4-producing T cells.

It has been shown that orally induced antigen-specific Treg cells can develop in the absence of naturally-occurring Treg cells (31). Conversion of naive T cells into functional mucosal Treg cells occurs in the mucosa-draining lymphoid tissue within 48-72 h after antigen encounter (8, 9). In agreement, it was recently established that MLN-DC and lamina propria DC induce increased TGF-β-dependent Foxp3^{+} Treg-cell differentiation when compared with splenic DC (12, 13). One of the factors that control peripheral Treg-cell differentiation at this immune-privileged site is the vitamin A derivative RA (13). The current data show that COX-2 expression in MLN-DC contributes to this Treg-cell differentiation. Constitutive expression of COX-2 exclusively occurs in the intestine. Transient inhibition of this single enzyme during soluble protein feed suppressed Treg-cell differentiation in PP and MLN within 48 h. As COX-2 can be expressed by stromal cells in the lamina propria (18), as well as by DC in the MLN, its contribution to Treg-cell differentiation could be direct, by influencing DC-T-cell contact within the MLN, or indirect by "imprinting" lamina propria DC that migrate to the MLN. The present study reveals that MLN-DC-derived COX-2 is directly involved in the commitment of Foxp3^{+} Treg cells as inhibition of the enzyme in an MLN-DC-T-cell co-culture significantly inhibited the differentiation of TGF-β-induced Treg cells. One of the functions of COX-2 products is to negatively regulate GATA-3 expression and IL-4 synthesis. We and others have shown earlier that Th2 differentiation is competing with Treg-cell commitment (32-35). These novel data show that RA- and TGF-β-induced mucosal Treg-cell differentiation is also inhibited by IL-4. In part, this may be mediated by the direct suppression of the FOXP3 promotor by GATA-3 (32). The current *in vivo* experiments show that COX-2 inhibition drives GATA-3^{+} and IL-4-producing T cells in the MLN, which have lost their regulatory capacity. Neutralization of IL-4 production restored tolerance suggesting that IL-4 is a pivotal factor responsible for loss of tolerance *in vivo*. Thus, it can be hypothesized that there are two processes that may impede the differentiation of functionally suppressive T cells in the MLN during COX-2 inhibition. Firstly, COX-2 inhibition reduces the differentiation of Foxp3^{+} T cells. However, as this is not a full ablation the total prevention of transfer of tolerance by the MLN cells may additionally be caused by the reciprocal formation of inflammatory, IL-4-secreting, effector T cells. Whether COX-2 activity in stromal cells contributes to the adaptive T-cell response through "imprinting" of lamina propria DC remains to be established. Moreover, it is not expected that T-cell-derived COX-2 plays an important role as COX-2 expression was not observed in any of our experiments irrespective of whether T cells were differentiated *in vivo* or *in vitro*. Very recent data confirm that drug-mediated COX-2 inhibition is also effective to decrease T-Bet and IFN-γ in a model of autoimmune encephalomyelitis suggesting that COX-2 may regulate Th-cell differentiation depending on the inflammatory environment (36).

The finding that IL-4 inhibits Treg-cell differentiation and causes loss of mucosal tolerance is supported by studies demonstrating a detrimental role of IL-4 in intestinal homeostasis. In particular, it was shown that parasite-induced IL-4 can drive enteropathy, IL-4 causes crypt hyperplasia and recruitment of intraepithelial lymphocytes in murine jejunal graft versus host reaction, and IL-4 plays an important role in the development of colitis in TCRα$^{-/-}$ mice (22, 23, 37). Moreover, earlier reports have shown that IL-4-deficient mice are not hampered in tolerance induction (38). At first instant, the inhibitory effect of IL-4 on mucosal Treg-cell induction seems difficult to reconcile with the fact that mucosally induced Th3 cells generated by myelin basic protein feed are characterized by IL-4 production and can be expanded *in vitro* with this particular cytokine (1). However, the stage of differentiation at which the IL-4 is produced may crucially determine how it affects Treg-cell numbers. IL-4 potently inhibits IL-2 production (39). This is also seen in our *in vitro* Treg-cell differentiation experiments in which a strong reduction of IL-2 release was observed when OVA-specific T cells were cultured with IL-4 or when GATA3 Tg OVA-specific T cells were used. This IL-2 reduction will most likely have an effect on the initial stages of Treg-cell conversion as it was shown that IL-2 is essential for TGF-β to convert naive CD4$^+$CD25$^-$ T cells to CD25$^+$Foxp3$^+$ Treg cells (40). However, other common γ-chain cytokines, such as IL-4, IL-7, and IL-15, can sustain maintenance of committed Foxp3$^+$ Treg cells (40). Thus, it can be envisaged that IL-4 inhibits Treg cells at the stage of acquisition of the Treg-cell phenotype through the inhibition of IL-2 without affecting previously differentiated Foxp3$^+$ Treg cells. Besides facilitating Treg-cell differentiation through negatively regulating the GATA3/IL-4 pathway, COX-2-dependent metabolites may also positively induce Foxp3 expression. Earlier, it has been reported that the COX-2-dependent metabolite PGE$_2$ is able to induce Foxp3 gene expression in murine and human CD4$^+$CD25$^-$ T cells (30, 41). Although the molecular mechanism of this positive gene regulation is unclear we were able to show that PGE$_2$ can dramatically enhance Foxp3 expression during OVA-specific Treg-cell conversion induced by MLN-DC. Whether PGE$_2$ is the only main COX-2-dependent metabolite that controls Treg-cell conversion in MLN *in vivo* remains to be established. Similar properties may be shared by other PG metabolites as activation of the D prostanoid 1 receptor in a murine model for asthma was also associated with protection and induction of Treg cells and PGI$_2$ has been shown to down regulate DC functions (42, 43). However, PGE$_2$ clearly plays a crucial role in both small intestinal as well as colonic homeostasis. Quantitatively, in lamina propria stromal cells, PGE$_2$ is the most predominant COX-2-dependent arachidonic acid metabolite present, whereas COX-2 dependent production of PGF$_{2α}$, 6-keto PGF$_{1α}$, TXB$_2$ and PGD$_2$ is much lower (18, 19). Functionally, PGE$_2$ also seems to have dominant regulatory functions as deficiency in EP4, one of the G-coupled PGE$_2$ receptors, causes increased susceptibility to chemically-induced colitis, whereas deficiency in the PGD receptor (DP), the PGF receptor (FP), the PGI receptor (IP) or the TX receptor (TP) did not have this effect (44). Intriguingly, deficiency in the other three subtypes of PGE receptor (EP1, EP2, EP3) did not have the same effect (44).

The expression of COX-2 in DC in the gut-draining lymphoid tissue is very unique for this

microenvironment. Little is known about the factors that regulate the expression of the enzyme. Recently, it has been demonstrated that in the colon, COX-2 expression is dependent on TLR4 triggering by bacterial components (45). However, as TLR-4-independent COX-2 expression has been observed in lamina propria stromal cells (18), it is possible that local non-inflammatory mediators may also mediate COX-2 expression (46, 47). Although CD103^{+} MLN-DC were reported to selectively induce Foxp3^{-} and CCR9^{-} expression in differentiating T cells when compared with CD103^{-} DC (11, 16, 17), COX-2 expression was measured in both subsets but in contrast was particularly distinctive in CD11b^{+} DC.

Our data, generated in mouse models, show a crucial role for COX-2-dependent arachidonic acid metabolites in the induction of adaptive Treg cells and maintenance of mucosal tolerance in the intestine. Whether this exact mechanism of regulation also applies to the human situation will require extensive further research. Clearly, many reports support the notion that prostaglandin mediated regulation also occurs in the human intestine. In the past decades administration of inhibitors of COX enzymes, denoted as non-steroidal anti-inflammatory drugs, to patients with chronic inflammatory intestinal disease has become more controversial due to effects of exacerbation of the disease (48-51). Moreover, recently, a major new Crohn's disease susceptibility locus was identified close to the gene PTGER4 encoding for EP4 (52), further supporting the idea that modulating inflammation through long-term intake of COX-2 inhibitors may interfere with crucial immune regulatory processes and may therefore encompass threats to intestinal homeostasis.

In conclusion, besides unravelling the molecular mechanism of *de novo* Treg-cell induction in the peripheral mucosal immune system, the finding that COX-2-mediated prostaglandins regulate adaptive T-cell responses through inhibition of IL-4 will stimulate further research into the function of this enzyme in inhibiting chronic intestinal inflammatory disease.

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SUPPLEMENTARY FIGURES

A

B
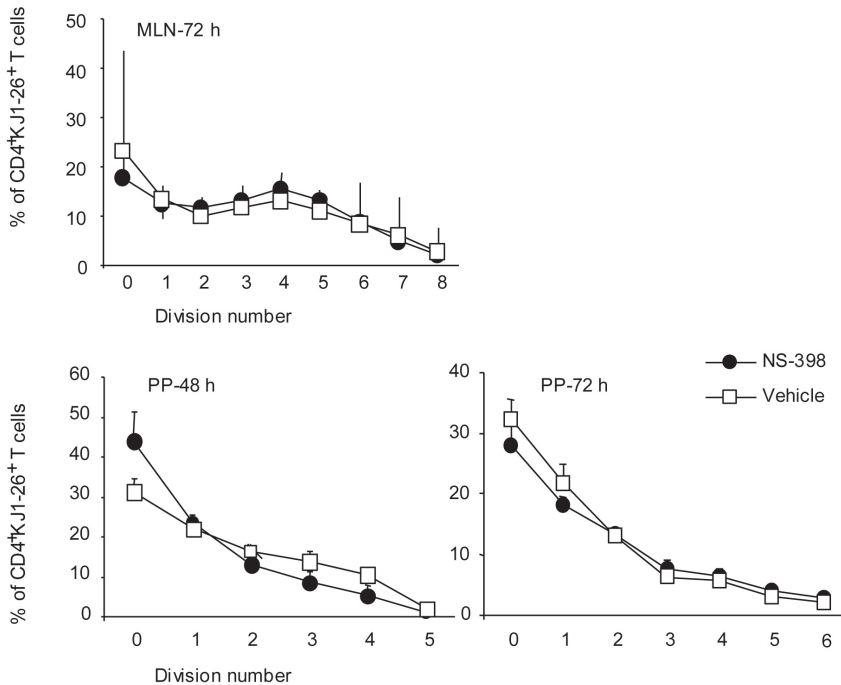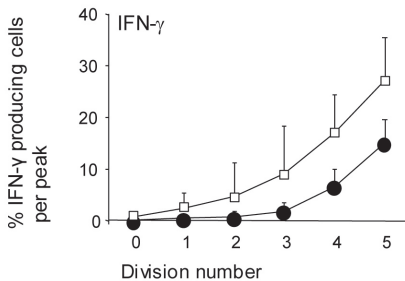
Supplementary Figure 2.1   Kinetics of T-cell division in MLN of NS-398-treated mice. Donor BALB/c mice were injected with 1×10^7 CD4+KJ1.26+ cells i.v.. Starting the next day, mice were treated with NS-398 as described in Figure 2.1. Control groups were injected with vehicle consisting of a 1% DMSO solution in saline. Tolerization was induced by a single i.g. dose of 70 mg OVA. **(A)** At 48 and 72 h after OVA administration, MLN and PP were isolated and single-cell suspensions were stained for the presence of CD4+KJ1.26+ cells. CFSE profiles of CD4+KJ1.26+ T cells were determined in MLN by flow cytometry. The percentages of CD4+KJ1.26+ T cells in each peak of division were calculated and are represented as the mean for at least three mice $\pm$ SD. **(B)** Single-cell suspensions were stained for KJ1.26+ cells and the percentage of IFN-γ secreting cells per peak of division was determined with a cytokine secretion assay. Data are represented as mean percentage of OVA-induced cytokine-secreting antigen-specific T cells per peak of division of at least three separate experiments, with SD. Differences were not statistically significant. DMSO, dimethyl sulfoxide; i.g., intragastric; IFN, interferon; i.v., intravenous; MLN, mesenteric lymph nodes; OVA, ovalbumin; PP, Peyer's patches.
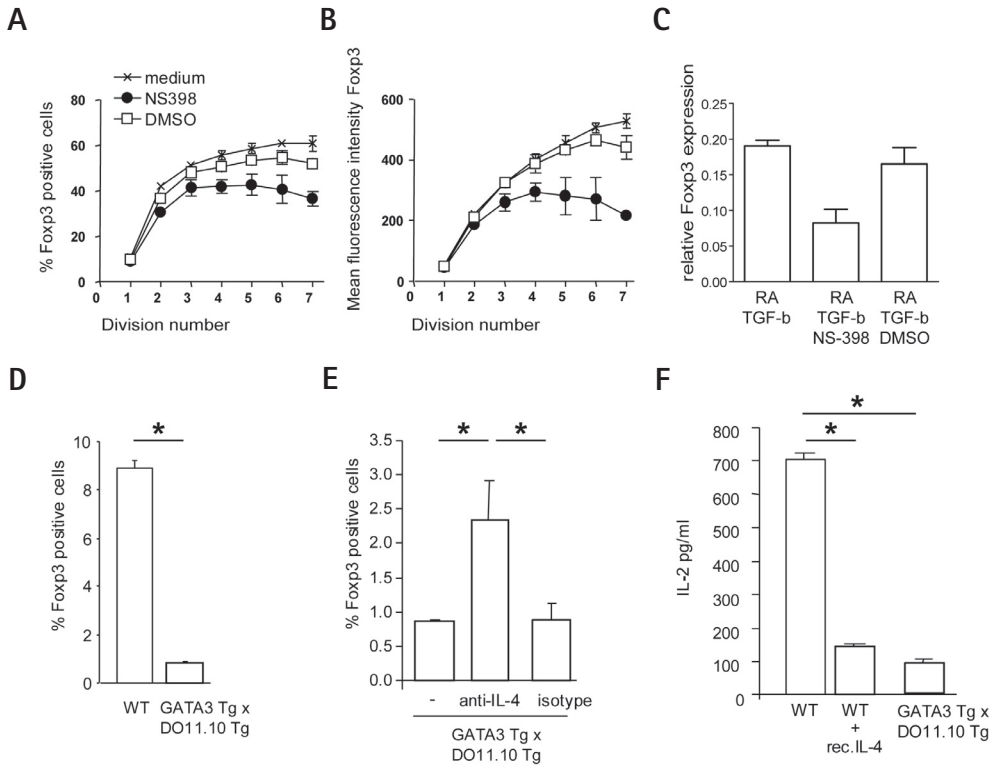
Supplementary Figure 2.2   IL-4 inhibits TGF-β-induced adaptive Treg-cell conversion. Bone-marrow-derived DC (2 x 10⁴) were loaded with 0.2 µg ml⁻¹ OVA peptide for 2 h. Thereafter, the cells were co-cultured with 5 x 10⁵ CFSE-labeled CD4⁺KJ1.26⁺ T cells in the presence of TGF-β and RA for 96 h. To assess the role of COX-2, NS-398 75 µM or DMSO control were added to the co-culture. At 96 h, CD4⁺ T cells were isolated from the culture by eliminating DC with mAbs and anti-rat magnetic beads and analyzed for Foxp3 protein by flow cytometry. **(A)** Foxp3-percentage positive cells, **(B)**: Foxp3-mean fluorescence intensity and **(C)** Foxp3 mRNA expression by Q-PCR. **(D)** Bone-marrow-derived DC were loaded with 0.5 mg ml⁻¹ OVA for 6 h. After washing, 2.5 x 10⁴ bone-marrow-derived DC were incubated with 5 x 10⁵ CFSE-labeled CD4⁺KJ1.26⁺ T cells from DO11.10 Tg x CD2-GATA3 Tg mice in the presence of TGF-β for 96h and analyzed for Foxp3 expression and CFSE content by flow cytometry. **(E and F)** To determine whether IL-4 directly interferes with Foxp3 expression, cells were cultured with anti-IL-4 (10 µg ml⁻¹; purified from 11B11 hybridoma), isotype control (10 µg ml⁻¹; purified from GL113 hybridoma) or recombinant IL-4. At 96 h, the cells were analysed for **(E)** Foxp3 expression and, **(F)** the concentration of IL-2 was determined in culture supernatant at 72 h. * Statistically significant ($P<0.05$). DC, dendritic cell; IL, interleukin; mAb, monoclonal antibody; OVA, ovalbumin; RA, retinoic acid; Tg, transgenic; TGF, transforming growth factor.

Differential regulation of mucosal tolerance in the small and large intestine

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ABSTRACT

In healthy individuals, exogenous soluble protein feed results in robust tolerance induction mediated by suppressive regulatory T (Treg) cells. This process is under strict regulation of resident mucosal dendritic cells (DC) that present antigen in the small-intestine draining lymphoid tissue. However, much less is known about the regulation of immune responses to protein antigens in the colon. Here we show that colonically-applied fluorescently-labeled protein antigen can be found associated with DC in the iliac lymph nodes (ILN) but not in the small-intestine draining mesenteric lymph nodes (MLN), while orally-adminstered antigen is exclusively found to be associated with DC in the MLN. In agreement, colonic OVA administration elicited T-cell proliferation in the ILN but not the MLN, while oral OVA induced a T-cell response in the MLN but not in the ILN. Despite the difference in the site of antigen presentation, both routes induced tolerance via the induction of functionally suppressive Treg cells. However, while OVA feed induced a substantial population of adaptive forkhead box P3 (Foxp3)-expressing T cells in the MLN, colonic application induced more limited numbers of these cells in the ILN. This regional difference in T-cell differentiation was associated with phenotypic differences of resident DC, as CD103$^+$ DC from ILN expressed much lower mRNA levels of the vitamin A-converting enzyme retinal dehydrogenase 2 (RALDH2) than MLN-derived CD103$^+$ DC. In contrast, ILN-derived DC expressed higher levels of the immunosuppressive cytokine interleukin (IL)-10. These data support the notion that there is regional compartmentalization within the intestinal immune system, and argue that the mechanisms driving small-intestinal and colonic tolerance to protein antigen are different.

INTRODUCTION

The intestines are continuously exposed to foreign antigens. Whereas it is critical that the intestinal immune system mounts protective immune responses against potential pathogens, it is equally important that tolerance develops to harmless proteins from food and the commensal flora. Loss of intestinal tolerance, as occurs in Crohn's disease, is associated with a chronic inflammatory T-cell response. Although disease in all patients is characterized by aberrant regulation of the mucosal immune response to bacterial proteins, the localization of inflammation can be strikingly different amongst individuals. Of adult patients, 85% has disease that is restricted to the colon, whereas 15% has small-intestinal lesions. In pediatric Crohn's disease, the frequency of individuals with isolated small-intestinal lesions is much lower (1), while a majority has extensive disease in small intestine and colon. The cause for this regional difference is largely unexplained. Unraveling the differences between the regulation of small-intestinal and colonic tolerance to protein antigen is required to better understand disease pathology and possibly identify new disease subtypes.

Tolerance to proteins from intestinal flora and tolerance to soluble food proteins both depend on the regulation of adaptive immune responses. Functional regulatory activity has been ascribed to several types of T cells that are commonly referred to as regulatory T (Treg) cells. In the intestine, both forkhead box P3 (Foxp3)$^+$ Treg cells and Foxp3$^-$ IL-10-secreting type 1 regulatory T (Tr1) cells are involved in maintaining mucosal tolerance (2-10). However,

regional differences in the phenotype of these subpopulations have been reported. For example, it has been demonstrated that the small intestine is rich in both Foxp3- IL-10-producing Tr1 cells and Foxp3$^+$ IL-10-producing Treg cells, whereas in the colon all IL-10-producing T cells are Foxp3$^+$ (11). Moreover, IL-10-receptor-deficient mice and IL-10-deficient mice develop spontaneous inflammation that is primarily restricted to the colon but not the small intestine (12, 13). These data suggest that the adaptive immune mechanisms driving small-intestinal and colonic tolerance are distinct.

We report that both oral and colonic application of soluble protein antigen induces mucosal tolerance, which is mediated by the induction of functionally-suppressive Treg cells. However, while oral antigen administration induced the differentiation of a substantial population of Foxp3$^+$ T cells in the mesenteric lymph nodes (MLN), T-cell differentiation in the iliac lymph nodes (ILN) induced by colonic antigen application was associated with fewer Foxp3$^+$ T cells. This localized difference in T-cell differentiation was associated with phenotypic differences of resident dendritic cells (DC). Taken together, our results demonstrate that within the intestine there are regional differences in the mechanisms that lead to adaptive immune tolerance.

METHODS

Mice

Specific pathogen free BALB/c mice (8-10 weeks) were purchased from Charles River, Maastricht, The Netherlands. DO11.10 transgenic (Tg), which have a Tg T-cell receptor (TCR) specific for the ovalbumin (OVA) 323-339 peptide, and DO11.10 Tg x RAG$^{-/-}$ mice were bred at our own facility. All mice were kept under routine animal housing conditions and experiments were approved by the animal experimental committee of the ErasmusMC.

Tracking of Alexa Fluor–488–labeled OVA

OVA (Calbiochem, San Diego, CA, USA) was labeled with Alexa Fluor-488 succinimidyl ester according to manufacturer's protocol (Molecular Probes, Leiden, The Netherlands). Mice received 3.5 mg OVA–Alexa-Fluor-488 by intragastric (i.g.) administration or 1.7 mg OVA–Alexa-Fluor-488 by intrarectal (i.r.) application. Control mice received the same amounts of unlabeled OVA. At 20 h after OVA administration, MLN and ILN were isolated and analyzed for Alexa-Fluor-488$^+$ cells by flow cytometry.

Adoptive transfer and CFSE labeling

Lymph nodes and spleens from DO11.10 Tg mice were isolated and single-cell suspensions were prepared, enriched for CD4$^+$ cells and labeled with 5,6-carboxy-succinimidyl-fluoresceine-ester (CFSE) (Molecular Probes) as described previously (5). For DO11.10 Tg x RAG$^{-/-}$ mice, whole splenocyte cell suspensions were labeled with CFSE. Purity of the population was assessed using

flowcytometry after staining with anti-CD4 (GK1.5) (BD-Pharmingen, Woerden, The Netherlands) and the DO11.10 Tg TCR specific antibody (KJ1.26, Invitrogen, Breda, The Netherlands). BALB/c acceptor mice were adoptively transferred with OVA-specific T cells by intravenous injection of 2 - 10 x 10^6 CFSE-labeled CD4$^+$KJ1.26$^+$ cells in 100 μl saline. One day after adoptive transfer, recipient mice were exposed to antigen by i.g. or i.r. administration (using a 2 cm long canula) of 70 mg 98% pure OVA, or by intramuscular (i.m.) immunization with 400 μg OVA (Calbiochem) in each hind limb. At 72 h after OVA administration draining lymph nodes and spleen were isolated and single-cell suspensions were stained for CD4, CD38 (90), CD62L (MEL-14, all BD), Foxp3 (JFK-16S, EMELCA Bioscience, Bergen op Zoom, The Netherlands) and DO11.10 Tg TCR (KJ1.26). Phenotype and cell division (based on fluorescence intensity of single CFSE peaks) were measured on a FACSCanto or FACSCalibur flow cytometer (BD).

Flow-cytometric cell sorting

To obtain purified CD103$^+$ and CD103$^-$ DC from MLN and ILN, lymph nodes were isolated and digested with Liberase Blenzyme 3 (Roche, Woerden, The Netherlands) in the presence of DNAse I (Roche) at 37 °C. DC were purified by negative enrichment using a mouse DC enrichment kit (Dynal, Oslo, Norway) and subsequent flow-cytometric cell sorting based on the expression of MHCII (M5/114), CD11c (HL3) and CD103 (M290, all BD) on a FACSAria flow-cytometric cell sorter (BD).

Q-PCR

RNA was purified from sorted cell subsets using the Nucleospin RNA-XS kit (Macherey-Nagel, Düren, Germany) and cDNA was synthesized. Real-time quantitative PCR was performed using SYBRgreen (Finnzymes, Espoo, Finland) on an AbiPrismR7900 Sequence Detection System (PE Applied Biosystems, CA, USA). Relative expression to cyclophillin was calculated as follows: relative expression = $2^{-\Delta Ct}$, whereby $\Delta Ct = Ct_{target} - Ct_{housekeeping}$. Specific primers were designed across different constant region exons resulting in these primers:

Cyclo: Fw: 5'-AACCCCACCGTGTTCT-3' Rv: 5'-CATTATGGCGTGTAAAGTCA-3'

FOXP3: Fw: 5'-ACCTGGGATCAATGTGG-3' Rv: 5'-TGGCAGTGCTTGAGAAA-3'

IL-10: Fw: 5'-CAAGCCTTATCGGAAATG-3' Rv: 5'-CATGGCCTTGTAGACACC-3'

RALDH2 (Aldh1a2): Fw: 5'-AGCCCATTGGAGTGTGT-3' Rv: 5'-CCAGCCTCCTTGATGAG-3'

Delayed-type hypersensitivity response

BALB/c mice received i.g. or i.r. administration of 25 mg OVA (Sigma Aldrich) in 200 or 150 μl saline respectively or saline alone. Five days after i.g. OVA administration, mice were sensitized subcutaneously in the tail base with 100 μg OVA in 50 μl of a 1:1 incomplete Freund's adjucant (IFA):saline solution (Difco, BD. Alphen a/d Rijn, The Netherlands). At day 11, mice were

challenged with 10 µg OVA in 10 µl saline in both ears, after 24 h increases in ear-thickness were determined and compared to values prior to challenge.

Transfer to assess regulatory function of T cells

BALB/c mice were adoptively transferred with CD4$^+$KJ1.26$^+$ T cells and received OVA i.g., i.r. or i.m. one day later. At 72 h after OVA administration, draining lymph nodes were isolated and enriched for CD4$^+$ T cells as described above. The enriched CD4$^+$ T-cell population contained 5–10% CD4$^+$KJ1.26$^+$ cells. 1 x 10^5 CD4$^+$KJ1.26$^+$ T cells were transferred to naive BALB/c mice. One day after the transfer, recipient mice were sensitized followed by a delayed–type hypersensitivity (DTH) challenge in the ears 5 days later.

RESULTS

Distinct lymphoid drainage of the small and large intestine

First, we determined which lymph nodes drain the intestines after oral and colonic OVA application. It has previously been established that protein feed results in the induction of oral tolerance that depends on DC-mediated antigen-transport to the gut-draining MLN, where the antigen is presented to naive T cells (2, 14).

To assess whether rectal OVA administration also results in DC-mediated transport to the MLN, mice were treated with Alexa-Fluor 488-labeled OVA (OVA-488) orally or rectally. As observed earlier (2), OVA-488$^+$CD11c$^+$ DC were detected in the MLN at 24 h after oral OVA-488 administration, indicating that these DC were associated with the antigen (Figure 3.1 A). In contrast, no OVA-488$^+$ DC could be detected in the MLN after rectal administration of the fluorescently-labeled protein (Figure 3.1 A). Analysis of CD11c$^+$ DC in the iliac and caudal lymph nodes (together denoted as ILN) revealed that DC in these lymph nodes were associated with the rectally-administered fluorescently-labeled antigen (Figure 3.1 A). No OVA-488$^+$ DC were observed in the ILN after oral OVA administration, indicating that these lymph nodes specifically drain the distal part of the large intestine (Figure 3.1 A).

To determine whether the drainage also resulted in T-cell proliferation in these lymph nodes, BALB/c mice were adoptively transferred with CFSE-labeled CD4$^+$KJ1.26$^+$ (OVA-specific) T cells and, one day later, received OVA either orally or rectally. At 72 h after OVA administration, MLN and ILN were analyzed for proliferation of responding T cells. As shown in Figure 3.1 B, oral OVA administration resulted in proliferation of OVA-specific T cells in the draining MLN, but not in ILN. Rectal OVA administration induced proliferation in the ILN and not in the MLN. Taken together, these data demonstrate that the distal large intestine is drained by the ILN and not by the MLN.

A

B
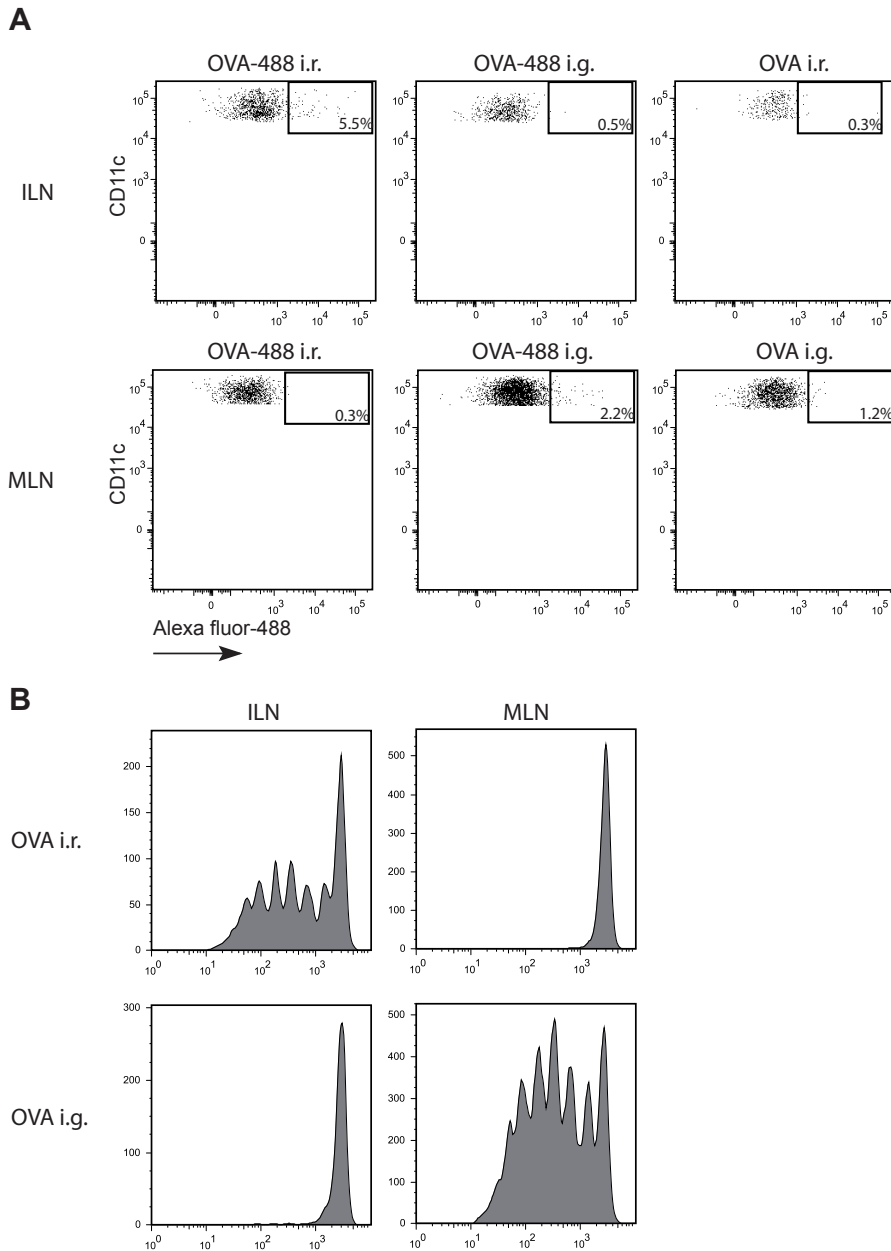
Figure 3.1 Distinct lymphoid drainage of the small and large intestine. **(A)** Mice received 3.5 mg OVA i.g., either labeled with Alexa Fluor-488 succinimidyl ester or unlabeled, or 1.7 mg OVA i.r.. At 20 h, single-cell suspensions of MLN and ILN were stained for CD11c and analyzed for Alexa-Fluor-488+ cells. **(B)** Mice were enriched with 6 x 10^6 CFSE-labeled CD4+KJ1.26+ OVA-specific T cells and given 70 mg OVA either i.g. or i.r.. At 72 h after OVA administration, MLN and ILN were isolated and single-cell suspensions were stained for the presence of CD4+KJ1.26+ T cells. Representative histogram plots of CFSE-profiles as determined by flow cytometry. I.g., intragastric; ILN, iliac lymph nodes; i.r., intrarectal; MLN, mesenteric lymph nodes; OVA, ovalbumin.

Preferential induction of Foxp3+ OVA-specific T cells in the MLN and to a lesser degree in ILN

It has previously been demonstrated that oral antigen application results in the differentiation of Foxp3+ T cells in the small intestine-draining MLN (2-4, 6, 7). This Foxp3+ Treg-cell differentiation is dependent on the local microenvironment in the gut-associated tissues. To establish whether antigen presentation in the ILN also leads to Foxp3+ T-cell differentiation *in vivo*, differentiation studies were performed as described previously (2). In short, OVA-specific CD4+ T cells were obtained from DO11.10 Tg x RAG$^{-/-}$ mice and adoptively transferred to naive BALB/c recipient mice, that were fed OVA one day after transfer. Oral OVA administration in this model resulted in the *de novo* expression of Foxp3 in responding CD4+KJ1.26+ T cells in the MLN (Figure 3.2 A). However, the percentage of differentiating OVA-specific Foxp3+ cells in the MLN depended on the number of transferred T cells. In particular, it was found that the percentage of Foxp3+ cells decreases with increasing numbers of intravenously injected CD4+KJ1.26+ T cells (Figure 3.2 A).

Next, we determined whether antigen-specific Foxp3+ Treg cells differentiate in the ILN after colonic antigen application. Thereto, BALB/c mice were adoptively transferred with CD4+KJ1.26+ T cells and received OVA i.r.. As shown in Figure 3.2 B, rectal OVA administration did induce the conversion of naive cells into Foxp3+ Treg cells in the ILN, although the percentage of converted Foxp3+ cells was slightly but significantly lower when compared with that seen
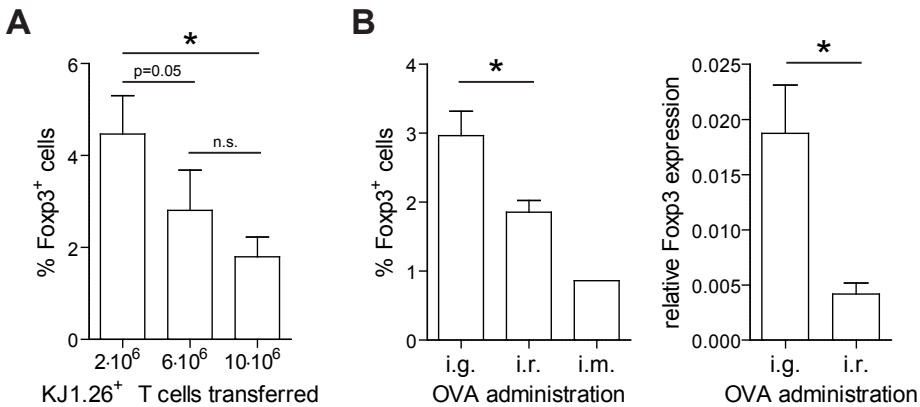
Figure 3.2 Preferential induction of Foxp3+ OVA-specific T cells in the MLN and to a lesser degree in ILN. **(A)** 2-10 x 10^6 CFSE-labeled CD4+KJ1.26+ T cells were transferred to naive mice by intravenous injection. The next day, mice received 70 mg OVA i.g.. 72 h after OVA administration, MLN were isolated and CD4+KJ1.26+ T cells within single-cell suspensions were analyzed for the percentage of Foxp3+ cells by flow cytometry (n=3). **(B)** 6 x 10^6 CFSE-labeled CD4+KJ1.26+ T cells were transferred to naive mice. The next day, mice received 70 mg OVA i.g. or i.r., or 400 µg i.m.. At 72 h after OVA administration, draining lymph nodes (MLN for i.g., ILN for i.r. and popliteal and inguinal lymph nodes for i.m.) were isolated and analyzed for expression of Foxp3 by flow cytometry (gated on CD4+KJ1.26+ T cells) and Q-PCR analysis (on FACS-sorted CD4+KJ1.26+ T cells). * Statistically significant (*P*<0.05). I.g., intragastric; ILN, iliac lymph nodes; i.m., intramuscular; i.r., intrarectal; MLN, mesenteric lymph nodes; OVA, ovalbumin.

after oral OVA administration. In agreement, Q-PCR analysis of Foxp3 mRNA levels in sorted CD4+KJ1.26+ T cells also revealed a decreased induction of Foxp3 in the ILN after rectal OVA administration (Figure 3.2 B).

Both oral and rectal antigen application induce mucosal tolerance

Next, we determined whether colonic antigen application results in the induction of mucosal tolerance in a similar way as oral antigen application. Thereto, naive BALB/c mice received OVA via the oral or rectal route and were subjected to a delayed type hypersensitivity (DTH) response consisting of sensitization with OVA in the tailbase and a subsequent challenge with OVA in the ears to assess whether tolerance was induced. As shown in Figure 3.3, colonic OVA application induced tolerance to a similar degree as oral OVA ingestion, as indicated by a decreased ear swelling when compared with mice that were treated with saline i.r.. To assess whether mucosal tolerance to orally and colonically applied antigen is both mediated through the function of mucosally-induced Treg cells, BALB/c mice were injected with CD4+KJ1.26+ OVA-specific T cells. One day after cell transfer, tolerance was induced by either oral or colonic application of OVA. As a control, mice were injected with OVA i.m. to induce an effector T-cell response. At 72 h, draining lymph nodes were isolated and enriched for CD4+ T cells. 1 x 10^5 CD4+KJ1.26+ T cells were transferred to naive BALB/c acceptor mice, that were subsequently sensitized and challenged with OVA in the ears according to the DTH protocol. As depicted in Figure 3.4, transfer of CD4+KJ1.26+ T cells that were activated in peripheral lymph nodes by an i.m. injection of OVA did not induce tolerance in the naive BALB/c acceptor mice, as indicated by a high increase in ear thickness. As shown previously (5), CD4+KJ1.26+ T cells that differentiated in the MLN after OVA feed suppressed the OVA-specific DTH response in the ears, indicating that these cells had a regulatory function (Figure 3.4). Here we show that colonic
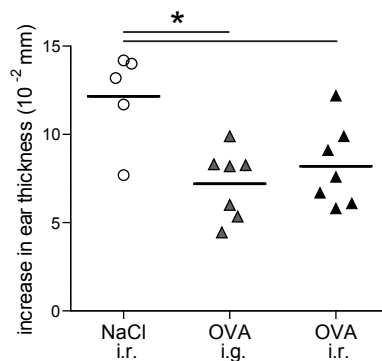
Figure 3.3   Both oral and rectal antigen application induce mucosal tolerance. Mice were treated with saline i.r., 25 mg OVA i.g. or 25 mg OVA i.r.. Five days after OVA administration, mice were sensitized subcutaneously in the tailbase with 100 µg OVA in IFA. At day 11, mice were challenged with 10 µg OVA in 10 µl saline in the auricle of both ears. After 24 h, increases in ear thickness were determined and compared with values before challenge. * Statistically significant (*P*<0.05). I.g., intragastric; i.r., intrarectal; OVA, ovalbumin.
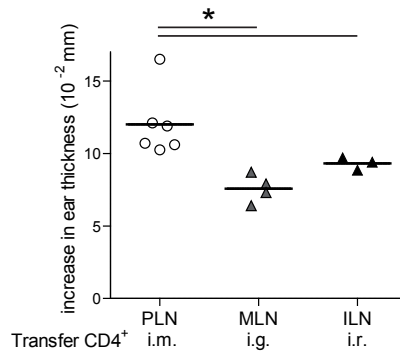
Figure 3.4 CD4^{+} T cells in MLN and ILN transfer tolerance. Mice adoptively transferred with CD4^{+}KJ1.26^{+} T cells received OVA i.g. or i.r. as described in Figure 3.1 B, or 400 µg OVA i.m.. At 72 h after OVA administration, draining lymph nodes were isolated and enriched for CD4^{+} T cells. 1 x 10^{5} CD4^{+}KJ1.26^{+} T cells were transferred to naive mice. One day after the transfer, recipient mice were sensitized with OVA in IFA, followed by a DTH challenge in the ears 5 days later as described in Figure 3.3. * Statistically significant ($P<0.05$). DTH, delayed type hypersensitivity; IFA, incomplete Freund's adjuvant; i.g., intragastric; ILN, iliac lymph nodes; i.m., intramuscular; i.r., intrarectal; MLN, mesenteric lymph nodes; OVA, ovalbumin; PLN, peripheral lymph nodes (inguinal and popliteal lymph nodes).

OVA application induces mucosal Treg cells in a similar way, as OVA–specific CD4^{+}KJ1.26^{+} T cells isolated from ILN after colonic OVA application also suppressed the DTH response upon adoptive transfer (Figure 3.4).

Taken together, these data demonstrate that both oral and colonic antigen application induces mucosal tolerance and that this is mediated through the differentiation of T cells with regulatory capacity.

Distinct dendritic–cell subsets in MLN and ILN

Since both colonic and oral OVA application induced Treg cells that were able to suppress a DTH response, but since the two routes differ in the degree of Foxp3 expression induced in these cells, we hypothesized that induction of mucosal tolerance through these two routes may be differentially regulated. CD103^{+} DC from MLN are efficient inducers of Foxp3^{+} Treg-cell differentiation during oral tolerance. The ability of CD103^{+} DC to induce *de novo* Foxp3 expression was shown to rely on their expression of RALDH2, a retinal dehydrogenase that mediates the conversion of dietary vitamin A into retinoic acid (3). Hence, we here determined whether the lower degree of Foxp3 induction during rectal tolerance induction could be explained by differences in the CD103^{+} DC subset. ILN contained a detectable population of CD103^{+} DC which comprised approximately 60% of CD11c^{hi} cells, which is similar to the CD103^{+} DC population in the MLN (data not shown). However, relative expression levels of RALDH2 mRNA in CD103^{+} DC from ILN were much lower in comparison with CD103^{+} DC from MLN and did not exceed those of CD103^{-} DC (Figure 3.5). Expression of RALDH1 was not detectable in
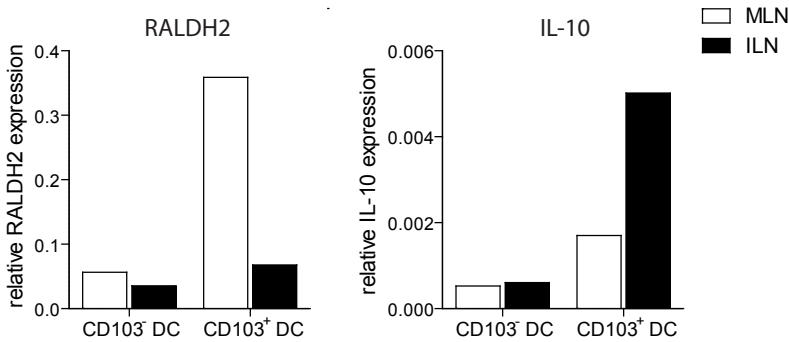
Figure 3.5　Analysis of CD103$^+$ DC subsets in MLN and ILN. Purified CD103$^+$ and CD103$^-$ DC subsets were obtained from MLN and ILN by flow-cytometric cell sorting expression of RALDH1 (aldh1a1) (not shown), RALDH2 (aldh1a2) and IL-10 relative to cyclophillin was determined by Q-PCR analysis. DC, dendritic cell; IL, interleukin; ILN, iliac lymph nodes; MLN, mesenteric lymph nodes; RALDH, retinal dehydrogenase.

any of the DC subsets analyzed (data not shown). In contrast, CD103$^+$ DC from ILN were found to express high levels of the immunosuppressive cytokine IL-10, while this was not observed in CD103$^+$ DC obtained from MLN (Figure 3.5).

DISCUSSION

In this study we show that small-intestinal and colonic tolerance to protein antigen are differentially regulated. In contrast to responses after protein feed, colonic antigen application results in T-cell differentiation in the ILN, but not to the MLN. This compartmentalization can be explained by the selective lymphatic drainage as in mice the MLN drain the small intestine, cecum and the ascending colon, whereas the descending colon and the rectum are drained by the caudal or ILN (15). This regional compartmentalization not only determined the anatomical localization of the immune response, but also had consequences for the phenotype of the T-cell response. Although both oral and colonic antigen application resulted in differentiation of functionally suppressive T cells, the degree of Foxp3 induction was lower in T cells that divided in the ILN after colonically applied OVA. *De novo* induction of Foxp3$^+$ Treg cells in the mucosa-draining lymphoid tissue after protein feed was shown to be essential for establishing mucosal tolerance to orally encountered protein antigens (3, 4, 6, 16). Whether this also holds true for colonic tolerance is unclear and will be investigated. However, it is not excluded that other populations of Treg cells participate in these responses. Functional Treg-cell differentiation within the ILN occurred within 72 h after colonic protein application, a timeframe which is highly similar to that seen in the MLN after protein feed (4). DC are pivotal antigen presenting cells for this process. In particular small-intestinal CD103$^+$ DC, which have the capacity to drive Foxp3$^+$ Treg-cell conversion through their ability to convert dietary vitamin A into retinoic acid,

which acts in synergy with TGF-β (3, 6, 16). Here we show that a CD103$^+$ DC population can also be detected in the ILN as was reported previously (17). The proportion of CD103$^+$ DC within the total DC population was comparable to that seen in the MLN. This is of relevance as *in vivo*, the frequency dividing T cells that convert into Foxp3$^+$ T cells depended on the number of naive OVA-specific T cells that were transferred (Figure 3.2 A). Transfer of lower numbers of T cells increased the frequency of Foxp3 expression suggesting that ratio of DC – T-cell interactions may limit the induction as has also been observed for *in vitro* Foxp3$^+$ Treg-cell differentiation (3). Therefore, to further substantiate that there are intrinsic differences in Foxp3$^+$ induction between MLN and ILN, the CD103$^+$ DC subsets were studied in more detail. Unlike CD103$^+$ DC from MLN, ILN-derived CD103$^+$ DC expressed low levels of RALDH 1 and 2, the enzymes required for vitamin A conversion. As a result, we hypothesize that a disability of these DC to produce retinoic acid may explain the reduced Foxp3$^+$ Treg-cell differentiation during the induction of colonic tolerance. Importantly, retinoic acid production by mucosal DC also stimulates expression of the chemokine receptor CCR9 and the adhesion molecule $\alpha_4\beta_7$ on T cells which is required for migration into the small intestinal tissue (18, 19) (20-23). In particular, CCR9 expression selectively allows migration into the small intestine and is absent on T cells in the colonic lamina propria (24-27). In contrast, expression of $\alpha_4\beta_7$ is required for T-cell entry into both the small and the large intestine (28). Therefore, it will be of interest to determine whether a difference in RALDH2 expression between CD103$^+$ DC from MLN and ILN has consequences for the gut-homing capacity of T cells and primarily on the expression of $\alpha_4\beta_7$.

Intriguingly, CD103$^+$ DC from ILN expressed high amounts of IL-10 when compared with CD103$^+$ DC from MLN. These data agree with the well established role for IL-10 in specifically maintaining homeostasis in the large intestine. It has previously been demonstrated that mice deficient for IL-10 spontaneously develop colitis (12). Although the mechanisms through which IL-10 protects from colitis remain poorly understood, it has become clear that this is at least in part mediated by IL-10-expressing antigen presenting cells (APC) (29). Murai et al. have demonstrated that IL-10 derived from CD11b$^+$ myeloid cells can support the function of Foxp3$^+$ Treg cells in the inflamed large intestine (29). In addition, tolerogenic IL-10-producing APC have been shown to induce IL-10-producing Tr1 cells (30-33). Notably, T-cell derived IL-10 does not play an essential role in maintaining tolerance to fed antigens (34). The latter seems to establish that tolerance to dietary antigens is regulated differently than tolerance to microbial proteins. However, it has not been resolved whether the independence of dietary-antigen tolerance from T cell-derived IL-10 is due to the fact that soluble antigens are primarily encountered in the small intestine or whether tolerance to soluble antigen is regulated differently than that to bacterial antigen. Therefore, future research is required to determine whether IL-10 has a role in the development of mucosal tolerance to protein antigens in the distal part of the large intestine.

It is unclear why the expression of retinal dehydrogenases mainly occurs in lymphoid DC from tissues associated with the small intestine and not in the ILN, while the opposite effect

is seen for IL-10. It is likely that local microenvironmental factors steer this process. As such, epithelial-cell derived TGF-β and retinoic acid have been shown to condition DC by imprinting their tolerogenic capacity (35, 36). However, also stromal cells in the intestine-draining lymph nodes may contribute to this process, as it has been demonstrated that these cells express high levels of retinoic acid-metabolizing enzymes and support the induction of gut-homing receptors on T cells (37, 38). In conclusion, the current study provides new insights into regional differences in regulation of mucosal tolerance in the small and large intestine. More knowledge regarding regional differences in Foxp3 and IL-10-mediated tolerance may have consequences for understanding of loss of tolerance as occurs in inflammatory bowel diseases and advance research aimed at identifying new subtypes of disease.

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Tolerance to ingested deamidated gliadin in mice is maintained by splenic Tr1–like cells

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ABSTRACT

Permanent intolerance to gluten as seen in coeliac disease (CD) occurs in approximately 1:100 individuals. This high frequency raises the question whether oral tolerance to gluten differs from that to other food proteins. Using mice transgenically expressing HLA-DQ2 and a gliadin-specific humanized T-cell receptor (TCR), we compared gluten-specific T-cell responses with the tolerogenic mucosal T-cell responses to the model food protein ovalbumin (OVA). Consistent with earlier data, the OVA-specific response occurred in the mesenteric lymph nodes (MLN) and induced forkhead box P3 (Foxp3)[+] regulatory T (Treg) cells. In contrast, deamidated gliadin feed induced dominant T-cell division in the spleen and very little in MLN. The gliadin-reactive T cells had an effector-like phenotype and secreted large amounts of interferon (IFN)-γ but also interleukin (IL)-10. Despite their effector-like phenotype, gliadin-reactive T cells exhibited regulatory capacity as transfer of the cells suppressed a gliadin-induced delayed type hypersensitivity (DTH) response. Thus, in the healthy intestine deamidated gliadin ingestion induces the differentiation of tolerogenic type 1 regulatory T (Tr1)-like cells in spleens of HLA-DQ2 transgenic (Tg) mice. These data infer that the default T-cell response to deamidated gliadin feed is tolerance which is not conditioned by the mucosal immune system but instead relies on IL-10 induction imposed by antigen presentation in the spleen.

INTRODUCTION

Coeliac disease (CD) is one of the most common food intolerances, affecting approximately 1% of the western world (1). CD is induced by the ingestion of gluten from wheat, rye and barley and can be treated by avoiding gluten in the diet. A central role for CD4^{+} T cells in the pathogenesis of CD is well established. Inflammatory gluten-specific CD4^{+} T cells can be isolated from the small-intestinal mucosa of CD patients but not of healthy individuals (2). These interferon (IFN)-γ-secreting gluten-specific T cells (3-5) recognize a diverse array of gluten peptides that are presented exclusively in the context of HLA-DQ2 or HLA-DQ8 (6). In agreement, the majority of CD patients carry HLA-DQ2, whereas HLA-DQ2-negative patients usually express HLA-DQ8 (7). A crucial event in the binding of gluten peptides to these HLA molecules is peptide deamidation by the enzyme transglutaminase 2 (TG2). Generally, gluten peptides have a low affinity for HLA-DQ2 and HLA-DQ8 but TG2 strongly facilitates this interaction by the introduction of negative charges in gluten peptides (8, 9).

Despite our understanding of the role of HLA-DQ2 (and HLA-DQ8) in development of CD, it is still unclear why oral tolerance to complex gluten is permanently broken in a significant proportion of the HLA-DQ2-carrying population whereas other HLA-DQ2 positive individuals remain tolerant. Oral tolerance to soluble proteins is strictly regulated by the microenvironment in mucosa-draining lymphoid tissue. Within mesenteric lymph nodes (MLN) and Peyer's patches (PP) food protein-specific Treg cells differentiate that acquire forkhead box P3 (Foxp3) under the control of the mucosal environment (10-15). As oral tolerance to soluble protein is such a controlled mechanism it can be questioned why permanent intolerance to gluten is relatively frequent. We hypothesize that tolerance to gluten may be induced by different mechanisms compared to other food proteins.

To test this hypothesis, we have generated mice that transgenically express HLA-DQ2 and an HLA-DQ2-restricted gliadin-specific T-cell receptor (TCR) derived from a T cell of the coeliac lesion and determined the response to orally-administered deamidated gliadin in these mice. We report that T-cell division after deamidated gliadin feed predominantly occurs in the spleen but not in the gut-draining MLN. The proliferating T cells in the spleen had an effector-like phenotype and secreted large amounts of interferon (IFN)-γ but also secreted interleukin (IL)-10. Despite their inflammatory phenotype, these gluten-reactive T cells exhibited regulatory capacity thus resembling type 1 regulatory T (Tr1) cells. Taken together, these results demonstrate that the default response to deamidated gluten feed is tolerance mediated by Tr1-like cells that differentiate in the spleen. These data provide a new perspective on the mechanisms that lead to oral tolerance and may provide new strategies for unravelling the pathogenesis of CD.

METHODS

Mice

HLA-DQ2 (DQA1*0501, DQB1*0201=HLADQ2.5).MHCII$^{\Delta/\Delta}$ transgenic (Tg) mice, gliadin-TCR. MHCII$^{\Delta/\Delta}$ Tg mice, which have a Tg T-cell receptor (TCR) specific for the DQ2-γ-I epitope (16, 17), and DO11.10 Tg mice, which have a Tg TCR specific for the ovalbumin (OVA) 323–339 peptide, were bred at the Erasmus MC. HLA-DQ2 and gliadin-TCR Tg mice were maintained on a C57BL/6 – DBA/2 mixed background. HLA-DQ2.gliadin-TCR.MHCII$^{\Delta/\Delta}$ double Tg mice were obtained by crossing DQ2.MHCII$^{\Delta/\Delta}$ Tg with gliadin-TCR.MHCII$^{\Delta/\Delta}$ Tg mice. BALB/c mice (8-10 weeks) were purchased from Charles River, Maastricht, The Netherlands. All mice were kept under specific pathogen-free housing conditions and experiments were approved by the animal experimental committee of the Erasmus MC. HLA-DQ2.MHCII$^{\Delta/\Delta}$ Tg, gliadin-TCR.MHCII$^{\Delta/\Delta}$ Tg and HLA-DQ2.gliadin-TCR.MHCII$^{\Delta/\Delta}$ double Tg mice were bred and maintained on a gluten-free chow (Arie Blok BV, Woerden, The Netherlands).

Generation of HLA–DQ2–transgenic and gliadin–TCR–transgenic mice

A 68 kb fragment containing the DQA1*0501 and DQB1*0201 (DQ2.5) genes including promoters and regulatory elements was purified from bacteriophage clone p797a11 and used for microinjection. For generation of TCR chimeric constructs genomic DNA was purified from the T-cell clone 4.32, that has been generated from a small-intestinal biopsy of a patient with CD challenged *in vitro* with pepsin and trypsin-digested gliadin (17). V(D)J regions of rearranged TCRα and TCRβ chains were PCR amplified from genomic DNA and cloned into a pair of cassette expression vectors containing the murine TCR constant sequences as well as natural mouse TCR promoter/enhancer elements (18). Transgenic mice were generated by microinjecting the constructs without vector sequences into fertilized eggs from (DBA/2xC57BL/6)F1 matings. Viable embryos were transferred to the oviducts of pseudo-pregnant females for development to term. Tg offspring were backcrossed twice to the MHCII$^{\Delta/\Delta}$ mice (19).

Proteins and peptides

Gliadin. Crude gliadin from wheat (Sigma Aldrich, Zwijndrecht, The Netherlands) was dissolved in a 0.1 M NH_4HCO_3 2 M urea buffer (100 mg ml^{-1}) and digested with 50 µg ml^{-1} α–chymotrypsin (CT) (Sigma) at room temperature for 24 h. Digestion was stopped by heating to 98 °C for 10 min. The chymotrypsin–treated gliadin (CT–gliadin) was centrifuged (5000 g, 45 min), filter-sterilized (0.45 µm) and dialysed against sterile PBS. Protein concentration was determined using a bicinchoninic acid assay (BCA assay, Perbio Science, Etten-Leur, The Netherlands). To obtain deamidated gliadin, CT–gliadin was treated with guinea pig liver transglutaminase (TG2) (0.08 U mg^{-1}) (Zedira GmbH, Darmstadt, Germany) for 16 h at 37 °C. For *in vivo* experiments, mice received 75 mg to 100 mg CT–gliadin (here termed non–deamidated gliadin) or TG2–treated CT–gliadin (here termed deamidated gliadin) by oral gavage (i.g.) or 500 µg intramuscularly (i.m.) by injection into the thigh muscle of each hind limb. For *in vitro* experiments, the synthetic native gliadin–γ1–Q peptide (QPQQPQQSFPQQQRPF) and the synthetic deamidated gliadin–γ1–E peptide (QPEQPQQSFPEQERPF) were used (5 µg ml^{-1}).

   Ovalbumin. In all *in vivo* experiments intact 98% pure OVA (either from Sigma Aldrich or from Calbiochem, San Diego, CA, USA) was used. In *in vitro* experiments either intact OVA (0.5 mg ml^{-1}, Calbiochem) or OVA$_{323-339}$ peptide (0.2 µg ml^{-1}) was used.

In vitro proliferation assays

^{3}H–Thymidine incorporation. Splenocytes from DQ2.gliadinTCR mice and MHCII$^{\Delta/\Delta}$ control mice were cultured in the presence or absence of 0.5 mg ml^{-1} deamidated gliadin for 72 h. Proliferation was assessed by incorporation of ^{3}H–thymidine.

   ***In vitro* T–cell differentiation.** Dendritic cells (DC) were isolated from the spleens of DQ2 mice by digesting the tissue with Liberase Blenzyme 3 (Roche, Woerden, The Netherlands) in the presence of DNAse I (Roche) for 30-35 min in a 37 °C incubator. After erythrocyte lysis, CD11c$^+$ cells were isolated using anti-CD11c MACS beads (Miltenyi Biotec, Bergisch Gladbach, Germany). To obtain CD4$^+$ gluten-specific T cells, lymph nodes and spleens were obtained from DQ2.gliadinTCR mice and strained through a 100 µm gauze. After erythrocyte lysis, the single-cell suspension was enriched for CD4$^+$ T cells by depletion of B cells, macrophages, monocytes and CD8$^+$ cells with rat antibodies against B220 (clone 6B2), F4/80, CD11b (MAC-1), MAC-2, MHCII (M5/114) and CD8 (53.6.72) and anti-rat magnetic Dynabeads (Invitrogen). The enriched CD4$^+$ T cells were fluorescently labeled with 5,6-carboxy-succinimidyl-fluoresceine-ester (CFSE) (Molecular Probes, Leiden, The Netherlands). 2 x 10^4 DQ2$^+$ DC were incubated with 5 x 10^5 CFSE-labeled CD4$^+$Vβ1$^+$ gliadin-specific T cells for 96 h in the presence of non–deamidated or deamidated gliadin (0.5 mg ml^{-1}), OVA (0.5 mg ml^{-1}), gliadin–γ1 peptide (0.5 µg ml^{-1}) or OVA323-339 peptide (0.2 µg ml^{-1}). Anti-IL-10-receptor (IL-10R) purified from 1B1.2 hybridoma, 10 µg ml^{-1} gift from Schering-Plough Biopharma) and appropriate isotype control (GL113) were used to neutralize IL-10R signalling.

Adoptive transfer

Lymph nodes and spleens from DO11.10 mice or DQ2.gliadinTCR mice were isolated and single-cell suspensions were prepared, enriched for CD4$^+$ cells and labeled with CFSE as described above. BALB/c acceptor mice received 1 x 10^7 CD4$^+$KJ1.26$^+$ cells in 100 µl saline by intravenous injection, DQ2 acceptor mice received 7 x 10^6 CD4$^+$Vβ1$^+$ cells. One day after transfer, DQ2 mice received 75-100 mg of non-deamidated or deamidated gliadin by 3-4 oral gavages with intervals of at least 4 h. In BALB/c acceptor mice, tolerance was induced by giving a single dose of 70 mg OVA i.g. or immunity by giving 400 µg OVA i.m.. Spleens and draining lymph nodes (MLN for i.g., PLN for i.m.) were isolated at 72 h after antigen administration and analyzed by flow cytometry for division of transferred cells or used for *in vitro* restimulation experiments.

In vitro restimulation

For *in vitro* restimulation experiments, CFSE$^+$CD4$^+$Vβ1$^+$ cells were purified by flow-cytometric cell sorting from spleens of DQ2 acceptor mice that were enriched with CD4$^+$Vβ1$^+$ cells and had received non-deamidated or deamidated gliadin i.g.. Bone-marrow-derived DC were generated from DQ2 mice as described earlier (20). 1 x 10^5 sorted CD4$^+$Vβ1$^+$ cells were restimulated *in vitro* with 1 x 10^4 DQ2$^+$ bone-marrow-derived DC and 0.5 mg ml^{-1} deamidated gliadin for 48 h. For intracellular detection of cytokines, the protein transport inhibitor monensin (Golgistop; BD-Pharmingen, Woerden, The Netherlands) was added during the last 4 h of culture.

For Q-PCR analysis, sorted CFSE$^+$CD4$^+$Vβ1$^+$ cells were restimulated with PMA / CaI (50 ng ml^{-1} and 0.1 µg ml^{-1}, respectively) for 14 h.

Flow cytometry

The following antibodies were used for flow cytometry: Anti-CD3 (145-2C11), anti-CD4 (GK1.5), anti-CD8 (53-6.7), anti-CD62L (MEL-14), anti-CD25 (PC61), anti-MHCII (M5/114; all BD), DO11.10 Tg TCR (KJ1.26; Invitrogen, Breda, The Netherlands), anti-Foxp3 (JFK-16S), anti-IL-10 (JES5-16E3), anti-IFN-γ (XMG1.2; all EMELCA Bioscience, Bergen op Zoom, The Netherlands), anti-TCR Vβ1 (BL37.2) (Beckman Coulter, Woerden, The Netherlands). Anti-HLA-DQ2 (XIII.358.4) was a kind gift of Dr. M.C. Mazilli, Rome, Italy. Anti-HLA-DQ (SPV-L3) was kindly provided by Dr. H. Spits, Amsterdam, The Netherlands. Phenotype and cell division (based on fluorescence intensity of single CFSE peaks) were measured on a FACSCanto or FACSCalibur flow cytometer (BD).

Detection of cytokines

Concentrations of IL-10, IFN-γ, IL-12, IL-6, TNF-α and MCP-1 in supernatants were determined by BD Cytometric Bead Array according to manufacturers' instructions (BD). Quantitative ELISAs for IL-2, IFN-γ, IL-4, IL-17 and IL-10 were performed using the following antibody pairs and recombinant cytokines: recIL2, anti-IL-2, anti-IL-2-bio (all BD), recIFN-γ, anti-IFN-γ

(Biolegend, Uithoorn, The Netherlands) anti-IFN-γ-bio (cultured from R46A2 hybridoma), recIL-4 (BD), anti-IL-4 (cultured from 11B11 hybridoma), anti-IL-4-bio (BVD6-24G2), recIL-17, anti-IL-17 (TC11-18H10.1), anti-IL-17-bio (TC11-8H4), recIL-10, anti-IL-10 (SXC1.1), anti-IL-10-bio (JES5.2A5; all BD).

Real-time PCR

Total RNA was purified from purified CFSE$^+$CD4$^+$Vβ1$^+$ cells using the Nucleospin RNA XS kit (Macherey-Nagel, Düren, Germany). RNA was reverse transcribed to single-stranded cDNA using a mix of random hexamers (2.5 μM) and oligodT primers (20 nM). The RT reaction was performed in a total volume of 25 μL containing 0.2 mM of each dNTP (Amersham Pharmacia BioTech, Piscataway, NJ), 200 U Moloney murine leukemia virus reverse transcriptase (M-MLV RT; Promega, Madison, WI), and 25 U RNAsin (Promega). Conditions for the RT reaction were 37 °C for 30 minutes, 42 °C for 15 minutes, and 94 °C for 5 minutes. Real-time quantitative PCR was performed using an AbiPrismR 7900 Sequence Detection System (PE Applied Biosystems, CA, USA) based on specific primers and general fluorescence detection with SYBR green. Cyclophillin was used to control for sample loading and to allow normalization between samples. The expression levels relative to cyclophillin were calculated following the equation: relative expression level = $2^{-\Delta Ct}$, whereby $\Delta Ct = Ct_{target} - Ct_{housekeeping}$. Specific primers were designed across different constant region exons resulting in these primers:

Cyclo: Fw: 5'-AACCCCACCGTGTTCT-3' Rv: 5'-CATTATGGCGTGTAAAGTCA-3'

IL-10: Fw: 5'-CAAGCCTTATCGGAAATG-3' Rv: 5'-CATGGCCTTGTAGACACC-3'

Delayed type hypersensitivity response

Dividing CD4$^+$ gliadin-TCRtg T cells were FACS sorted from spleens of DQ2 mice that were enriched with CFSE$^+$ CD4$^+$ gliadin-TCRtg T cells and were fed 75 mg TG2-gliadin one day after cell transfer. 2.5 x 10^5 dividing CD4$^+$ Vβ1$^+$ cells were adoptively transferred to DQ2.gliadinTCR by intravenous injection in 100 μl saline. Gliadin-specific Foxp3$^+$ Treg cells were generated *in vitro* by culturing 5 x 10^5 CD4$^+$Vβ1$^+$ cells with 2 x 10^4 splenic DQ2$^+$CD11c$^+$ DC and 0.5 mg ml^{-1} non-deamidated gliadin. After 96 h, dividing CD4$^+$CD25$^+$Vβ1$^+$ cells were sorted and 2.5 x 10^5 cells were transferred to DQ2.gliadinTCR acceptor mice. Expression of Foxp3 in sorted *in vitro* generated Treg cells was 83%, as determined by flow cytometry. One day after adoptive transfer, recipient mice were sensitized subcutaneously in the tail base with 100 μg of TG2-gliadin in 50 μl of a 1:1 incomplete Freund's adjuvant (IFA): saline solution (Difco, BD. Alphen a/d Rijn, The Netherlands). 5 days later, mice were challenged with 10 μg TG2-gliadin in 10 μl saline in both ears, after 24 h increases in ear thickness were determined and compared to values prior to challenge. As a control, a delayed type hypersensitivity (DTH) response was induced in DQ2. gliadinTCR that did not receive transferred cells.

Histology

Segments of approximately one cm were collected from the small intestine of DQ2.gliadinTCR mice, fixed in 4% formalin solution and embedded in paraffin. Sections of 4 μm thickness were stained with hematoxylin (Vector Laboratories) and eosin (Sigma) and analyzed by microscopy. The length of five representative villi and adjacent crypts was measured to calculate villous/crypt ratios. For immunohistochemical detection of CD3 (rabbit anti-CD3, Dako Heverlee, Belgium), sections were deparaffinized and endogenous peroxidases were quenched with 3% H_2O_2 in methanol for 20 min. Antigen retrieval was achieved by microwave treatment in citrate buffer (10 mM pH 6.0). Sections were blocked for 1 h in 10 mM Tris, 5mM EDTA, 0.15 M NaCl, 0.25% gelatine, 0.05% Tween-20, 10% normal mouse serum, pH 8.0. Antibody incubation was performed overnight at 4 °C. Immunoreactions were detected using biotinylated secondary goat-anti-rabbit serum with the Vectastain ABC Elite Kit (Vector Laboratories) and 3,3'-diaminobenzidine tetrahydrochloride (Sigma-Aldrich). Sections were counterstained with hematoxylin.

Statistics

Data are expressed as the mean $\pm$ SD and analyzed either using Student's t-test or ANOVA. $P<0.05$ was considered significant.

RESULTS

Generation of HLA–DQ2–transgenic and gliadin–TCR–transgenic mice

HLA-DQ2 Tg mice were generated on the basis of a genomic fragment containing the DQA1*0501, DQB1*0201 (DQ2.5) genes as well as promotor and regulatory elements. One founder animal was selected of which all offspring exhibited a similar expression of the transgene product. Gliadin-TCR Tg mice were generated with a chimeric TCR construct in which the variable domains were derived from a human gliadin-specific T-cell clone whereas the constant regions and regulatory elements were of mouse origin. HLA-DQ2 Tg and gliadin-TCR Tg mice were backcrossed twice to MHCII$^{\Delta/\Delta}$ mice (19). HLA-DQ2.MHCII$^{\Delta/\Delta}$ Tg mice were crossed with gliadin-TCR.MHCII$^{\Delta/\Delta}$ Tg mice to obtain HLA-DQ2.gliadin-TCR.MHCII$^{\Delta/\Delta}$ double Tg mice (DQ2.gliadinTCR mice). Expression of the transgene products was assessed by flow cytometry on splenocytes of DQ2.gliadinTCR mice. HLA-DQ2 was expressed on all B cells (Figure 4.1 A) and DC (Figure 4.1 B) whereas the humanized gliadin-specific TCR was present on CD3$^+$ T cells (Figure 4.1 C). Analysis of the peripheral CD4:CD8 T-cell ratio confirmed proper thymic selection of gliadin-TCRtg CD4$^+$ T cells in DQ2.gliadinTCR mice (Figure 4.1 D). In contrast, >95% of CD3$^+$ T cells in DQ2 negative gliadin-TCR.MHCII$^{\Delta/\Delta}$ Tg mice are CD8$^+$CD4$^-$ T cells, and only very small numbers of CD4$^+$CD8$^-$, double positive and double negative CD3$^+$ T cells are detected in the periphery (Figure 4.1 E). These data indicate that in DQ2.gliadinTCR mice, HLA-DQ2 is required for the selection of CD4$^+$ T cells. All mice are healthy and have normal intestinal morphology (data not shown).
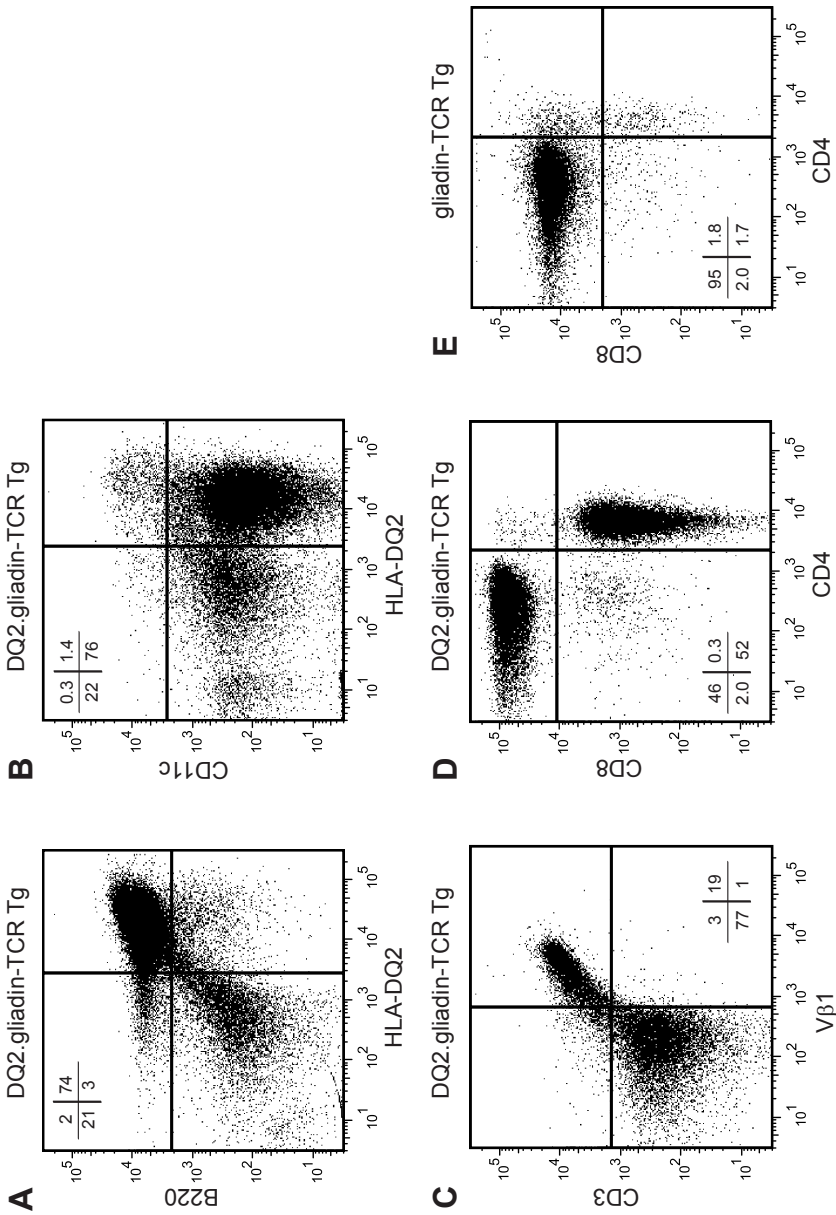
Figure 4.1 Transgenic mice express HLA-DQ2 on APC and gliadin-TCR on CD4+ T cells. **(A, B)** Splenocytes of DQ2.gliadinTCR mice were stained for HLA-DQ2 (SPV-L3) and B220 **(A)** or CD11c **(B)**. **(C)** Dot plot of FACS staining for murine CD3 and the human TCR Vβ1 on splenocytes from DQ2.gliadinTCR mice. **(D)** Dot plot of FACS staining for CD4 and CD8, gated on CD3+ splenocytes from DQ2.gliadinTCR mice. **(E)** Dot plot of FACS staining for CD4 and CD8, gated on CD8, gated on CD3+ splenocytes from gliadinTCR mice. APC, antigen presenting cell; TCR, T-cell receptor; Tg, transgenic.

Gliadin–TCRtg T cells only respond to deamidated gliadin

To determine whether the HLA-DQ2 and gliadin-TCR transgenes support a functional T-cell response, splenocytes from DQ2.gliadinTCR mice and non-transgenic MHCII$^{\Delta/\Delta}$ control mice were cultured in the presence or absence of deamidated gliadin (TG2-treated CT-digested gliadin, denoted as TG2-gliadin). At 72 h, proliferation was assessed by incorporation of 3^H-thymidine. Proliferation in response to deamidated gliadin was only detected with splenocytes from DQ2. gliadinTCR mice, but not from MHCII$^{\Delta/\Delta}$ mice, indicating that there is functional presentation of the antigen in cells from DQ2.gliadinTCR mice (Figure 4.2 A). The specificity of the response was further assessed by stimulation of CD4$^+$ gliadin-TCRtg T cells with splenic DQ2$^+$ DC in the presence of non-deamidated or deamidated gliadin or the irrelevant protein OVA. Deamidated gliadin induced high levels of IL-2 (Figure 4.2 B). In contrast, the IL-2 production in response to non-deamidated gliadin, OVA or medium control was very low (Figure 4.2 B). In agreement, flow-cytometric analysis of CFSE-labeled CD4$^+$ gliadin-TCRtg T cells in the same experimental setup revealed up to 7 cycles of division for the majority of CD4$^+$ gliadin-TCRtg T cells whereas a very small fraction of T cells proliferated to non-deamidated gliadin (Supplementary Figure 4.1 A). Stimulation with deamidated gliadin predominantly induced secretion of IFN-γ and moderate amounts of IL-4, IL-17 and IL-21 (Figure 4.2 C).

As complex gliadin protein has been shown to have adjuvant activity (21-24), the phenotypes of T-cell responses induced by non-deamidated CT-gliadin were compared with those induced by a synthetic gliadin-γ1 peptide, a peptide harboring the DQ2-γ-I epitope recognized by the transgenic TCR. Stimulation of gliadin-TCRtg T cells with a synthetic native gliadin-γ1 peptide (QPQQPQQSFPQQQRPF, denoted as "γ1-Q") induced a very low but detectable amount of proliferation (Supplementary Figure 4.1 A). In contrast, a synthetic variant of the deamidated gliadin-γ1 peptide (QPEQPQQSFPEQERPF, denoted as "γ1-E") resulted in T-cell proliferation (Supplementary Figure 4.1 A) and IL-2 release (Supplementary Figure 4.1 B) which were slightly higher than what observed for deamidated gliadin. Stimulation with deamidated gliadin-γ1 peptide resulted in a somewhat lower release of IFN-γ but an increased production of IL-4 as compared to stimulation with deamidated gliadin (Supplementary Figure 4.1 B). In sum, these data demonstrate that T cells of the DQ2.gliadinTCR double Tg mice mount a response to the deamidated DQ2-γ-I epitope in the context of HLA-DQ2.

Oral deamidated gliadin induces dominant T–cell proliferation in the spleen but not in the mesenteric lymph nodes

Using a DO11.10 transfer model, we and others have previously reported that OVA feed exclusively induces T-cell differentiation in the intestinal immune system, in particular in the gut-draining MLN and PP (12, 25). To elucidate whether deamidated gliadin feed induces a comparable response a DQ2.gliadinTCR T-cell transfer model was setup. Thereto, CD4$^+$ gliadin-TCRtg T cells were purified from DQ2.gliadinTCR mice, labeled with CFSE and intravenously injected in HLA-DQ2.MHCII$^{\Delta/\Delta}$ Tg acceptor mice (DQ2 mice). Starting the day after T-cell
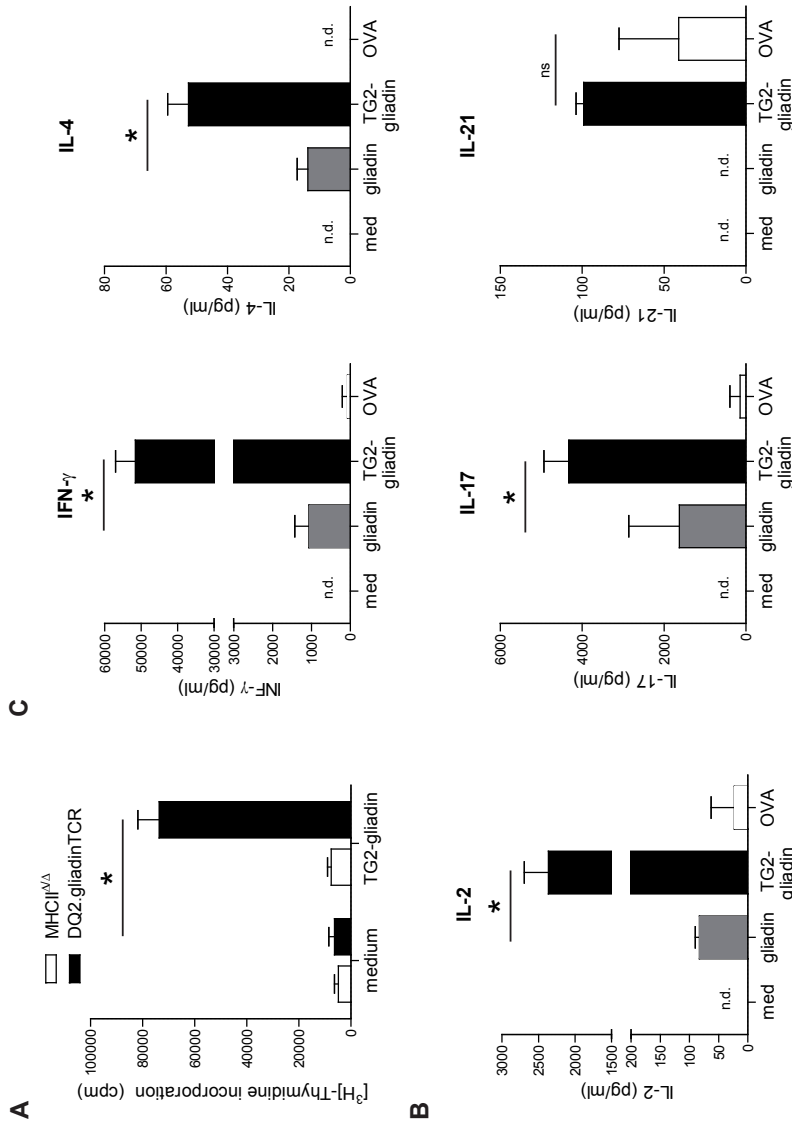
Figure 4.2 Gliadin-specific TCRtg CD4^{+} T cells respond to deamidated but not to non-deamidated gliadin. **(A)** 5 x10^{5} splenocytes from DQ2.gliadinTCR mice and MHCII[Δ/Δ] control mice were cultured in the presence or absence of 0.5 mg ml^{-1} deamidated gliadin (TG2-gliadin) for 72 h. Proliferation was assessed by incorporation of 3[H]-Thymidine. **(B, C)** Gliadin-specific CD4+Vβ1+ T cells (5 x 10^{5}) were stimulated with splenic DQ2+ DC (SPL-DC) (2 x 10^{4}) in the presence or absence of gliadin, TG2-gliadin or OVA. **(B)** Release of IL–2 at 48 h of culture as measured by ELISA. **(C)** Release of IFN–γ, IL–4, IL–17 and IL–21 at 96 h of culture as measured by ELISA. * Statistically significant (*P<0.05*). IFN, interferon; IL, interleukin; n.d, non-detectable; n.s., non-significant; OVA, ovalbumin; TCR, T-cell receptor; Tg, transgenic; TG2, transglutaminase 2.

transfer, DQ2 mice received 75-100 mg deamidated gliadin through multiple gavages. In parallel experiments, normal BALB/c mice received OVA-specific CD4$^+$ DO11.10tg T cells and 24 h later were either fed 70 mg OVA to induce a tolerogenic T-cell response or received 400 μg OVA intramuscularly to induce an inflammatory T-cell response (26). At 72 h after feed, lymphoid organs and spleens were analyzed for the division of transferred protein-specific T cells. Confirming earlier data ((12) and Chapter 5 of this thesis), oral OVA administration resulted in proliferation of OVA-specific T cells in the gut-draining MLN at 72 h after ingestion (Figure 4.3 A, middle panel). In addition, intramuscular injection of OVA induced proliferation of OVA-specific T cells in the draining inguinal and popliteal lymph nodes (peripheral lymph nodes (PLN)) and the spleen; (Figure 4.3 A, right panel and (26)). However, after deamidated gliadin feed virtually no proliferation was detected in the MLN of DQ2 acceptor mice whereas proliferation predominantly occurred in the spleen (Figure 4.3 A, left panel). To exclude that division of gliadin-specific T-cells in MLN may have occurred earlier, different time points after protein feed were investigated. Also at 48 h very few dividing CD4$^+$ gliadin-TCRtg T cells were observed in MLN (Supplementary Figure 4.2) whereas dividing gliadin-specific T cells in the spleen had already undergone three divisions (Supplementary Figure 4.2). It should be noted that 72 h after OVA feed proliferating OVA-specific T cells can also be detected in the spleen (Figure 4.3 A, middle panel), but are still absent at 48 h (Supplementary Figure 4.2). This agrees with earlier reports that the cells observed at 72 h have migrated to spleen after initial activation and division in the MLN (25). It was excluded that the unexpected localization of the gliadin-specific T-cell response in the spleen was due to an effect of the digestion/deamidation process, as neither mock-deamidation of OVA, nor cofeeding of OVA + deamidated gliadin altered the localization of the OVA-specific T cells in the MLN (data not shown).

To finally establish that the gliadin-specific T-cell response was specific for deamidated gliadin only, DQ2 mice received multiple oral gavages of non-deamidated or deamidated gliadin. At 72h after gliadin feed, spleens were analyzed for the division of transferred gliadin-specific T cells. As depicted in Figure 4.3 B, the proliferation of CD4$^+$ gliadin-TCRtg T cells to non-deamidated gliadin was insignificant whereas gliadin-specific T cells specifically proliferated in response to oral deamidated gliadin (Figure 4.3 B). These results indicate that under homeostatic conditions gliadin is not deamidated *in vivo* by murine TG2. It has been demonstrated that TG2 in the murine intestine is inactive during intestinal homeostasis and is transiently activated upon tissue damage (27). Indeed, we observed that inducing tissue damage by intramuscular injection of non-deamidated gliadin was sufficient for the proliferation of gliadin-TCRtg T cells in the draining PLN (Figure 4.3 B).Taken together, these data demonstrate that, in the DQ2.gliadinTCR mouse model, the CD4$^+$ T-cell response to deamidated gliadin occurs predominantly in the spleen.
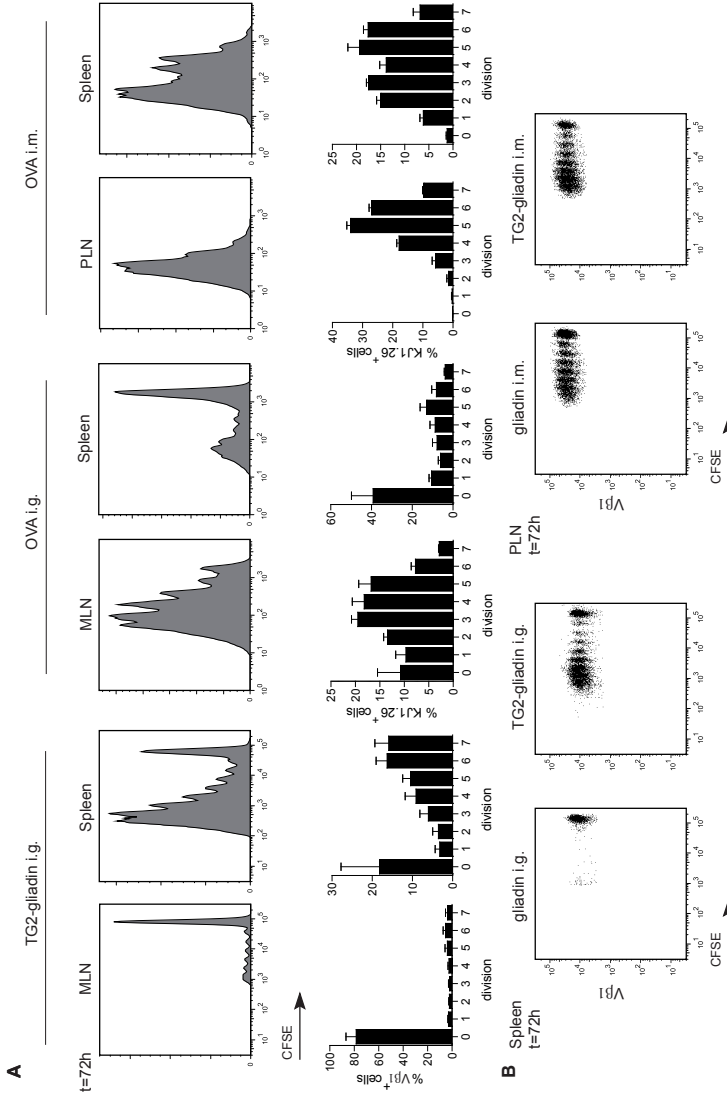
Figure 4.3 Oral deamidated gliadin induces a dominant T-cell proliferation in the spleen but not in the mesenteric lymph nodes. **(A)** DQ2 mice were intravenously injected with 7 x 10⁶ CFSE-labeled CD4⁺ Vβ1⁺ cells following by the oral administration of 100 mg TG2-gliadin the next day. BALB/c mice were enriched with 1 x 10⁷ CFSE-labeled CD4⁺KJ1-26⁺ cells. One day later, either tolerance was induced by giving 70 mg OVA i.g. or immunity by giving 400 µg OVA i.m.. Spleens and draining lymph nodes (MLN for i.g., PLN for i.m.) were isolated at 72 h after antigen administration and analyzed by flow cytometry for division of transferred cells. For each group, a representative histogram plot and dot plot showing CFSE dilution (gated on transgenic CD4⁺ Vβ1⁺ or CD4⁺KJ1-26⁺ cells) is depicted. The percentages of CD4⁺ Vβ1⁺ or CD4⁺KJ1-26⁺ cells in each peak of division were calculated and are represented as the mean ± SD for 5 mice (TG2-gliadin i.g.) and 3 mice (OVA i.g./i.m.) **(B)** DQ2 mice enriched with CFSE-labeled CD4⁺Vβ1⁺ T cells were given 100 mg of non-deamidated gliadin or deamidated TG2-gliadin by oral gavage, or 500 µg i.m.. At 72 h after gliadin administration, spleens (for gliadin i.g.) or draining PLN (for gliadin i.m.) were isolated and CD4⁺Vβ1⁺ T cells were analyzed by flow cytometry for CFSE dilution. i.g., intragastric; i.m., intramuscular; MLN, mesenteric lymph nodes; OVA, ovalbumin; PLN, peripheral lymph nodes; SD, standard deviation; TG2, transglutaminase 2.

Dividing gliadin–TCRtg T cells have an inflammatory T–cell phenotype

We have demonstrated that the mucosal administration of a harmless food protein such as OVA results in the differentiation of functionally-suppressive mucosal Treg cells in the MLN that are characterized by a rapid downregulation of CD62L and the acquisition of Foxp3 expression (Chapter 5). To determine whether the difference in localization between the T-cell responses induced by oral OVA and deamidated gliadin is of consequence to the type of immune response that is initiated, we determined the phenotype of the gliadin-specific T cells that differentiate in the spleen and compared it to the phenotype of OVA-specific T cells that are induced after oral or intramuscular OVA administration. The majority of gliadin-specific T cells expressed high levels of CD62L, the homing receptor that allows migration into lymph nodes (Figure 4.4 A (upper left plot) and Figure 4.4 B). In addition, deamidated gliadin feed did not induce expression of Foxp3 (Figure 4.4 A, lower left plot, and Figure 4.4 B). This CD62L^{hi}Foxp3$^-$ phenotype was opposite to the tolerogenic T-cell response that was seen in the MLN after OVA feed (Chapter 5 & Figure 4.4 A, middle plots, Figure 4.4 B). By comparison, the response was similar to that after encounter of OVA via a non-mucosal route, i.e. through injection in the thigh muscle, which resulted in a non-tolerogenic T-cell response that was characterized by minor Foxp3 expression and a CD62L^{hi} phenotype (Chapter 5 & Figure 4.4 A, right plots, Figure 4.4 B). It should be noted that even in the cases when T-cell division was seen in the PP, the phenotype of the dividing cells was identical to that of the cells dividing the spleen (data not shown). In sum, the phenotype of gliadin-specific T cells that differentiated in the spleen in response to oral deamidated gliadin administration closely resembled the effector T-cell response that occurs in the PLN upon intramuscular OVA injection.

To further characterize the T-cell response that is induced by feeding deamidated gliadin, we determined the cytokine profile of responding gliadin-specific T cells. Thereto, DQ2 mice were enriched with CD4$^+$ gliadin-TCRtg T cells and were fed either deamidated gliadin or non-deamidated gliadin. After 72 h, both responding gliadin-TCRtg T cells (deamidated gliadin feed) and non-responding (non-deamidated gliadin feed) were isolated from the spleen and restimulated *in vitro* with DQ2$^+$ bone-marrow-derived DC loaded with deamidated gliadin. At 48 h of restimulation, the secreted cytokines were measured. As shown in Figure 4.4 C, the reactive T cells from mice that were fed deamidated gliadin had a T helper 1 (Th1)-like effector phenotype, as indicated by prominent secretion of IFN-γ and the presence of IL-6. This response was specific for restimulated cells as the non-responding cells isolated from mice that received non-deamidated gliadin feed produced lower amounts of these cytokines. The cells in the culture also secreted TNF-α, MCP-1 and IL-12p70 (Figure 4.4 C). However, the latter cytokines were not specifically associated with restimulation as the cells isolated from mice that received non-deamidated gliadin feed produced equal amounts. These findings demonstrate that deamidated gliadin feed induces the differentiation of gliadin-specific T cells with a Th1-like phenotype.
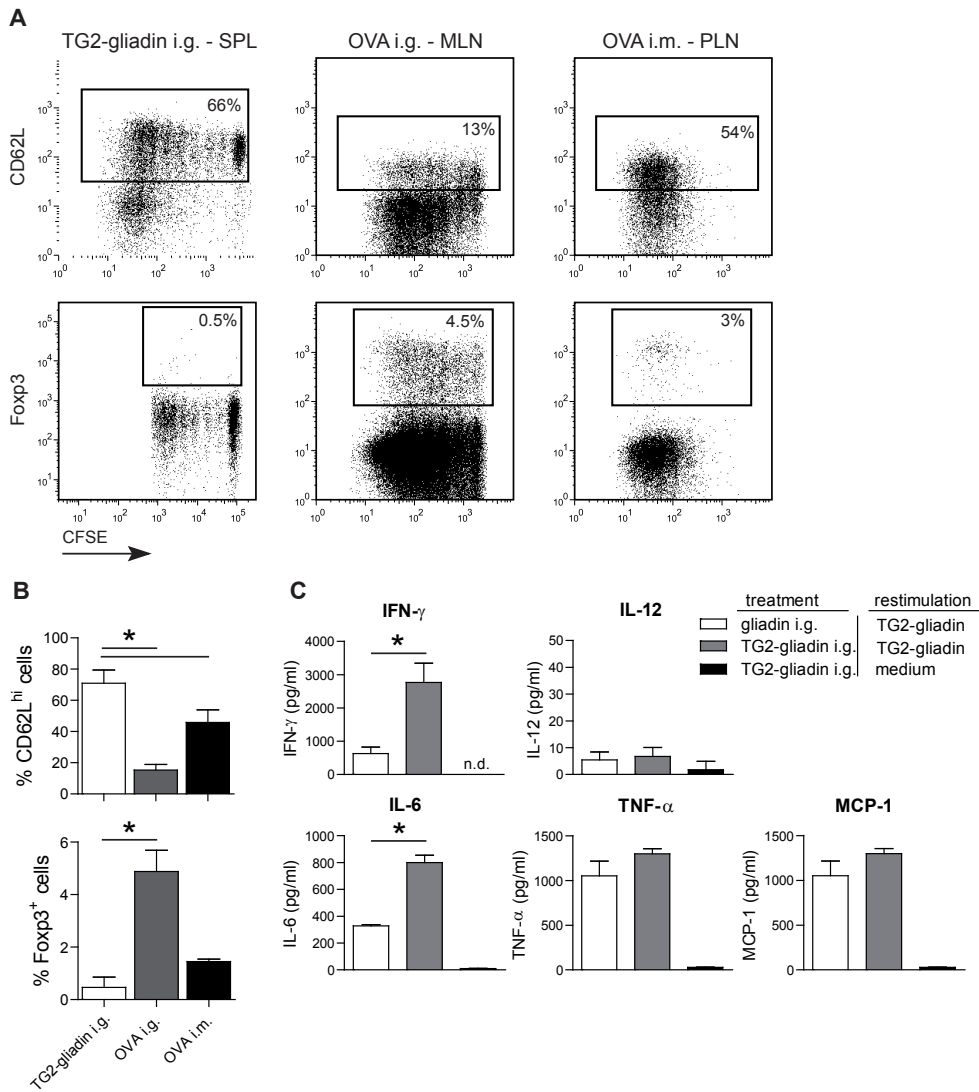
A

B **C**

Figure 4.4 Dividing gliadin-TCRtg T cells have an inflammatory phenotype. **(A, B)** DQ2 mice and BALB/c mice were enriched with CFSE-labeled gliadin-specific or OVA-specific T cells, respectively, and the next day, TG2-gliadin or OVA were administered as described in Figure 4.3. At 72 h after antigen administration, the phenotype of dividing transferred T cells was analyzed by staining single-cell suspensions of the draining lymphoid tissue (i.e. the spleen for TG2-gliadin i.g., MLN for OVA i.g. and PLN for OVA i.m.). **(A)** Representative dot plot showing staining for CD62L and Foxp3. **(B)** The percentage of CD62L^{hi} and Foxp3^{+} cells were calculated and are represented as the mean $\pm$ SD for at least 3 mice. **(C)** DQ2 mice were enriched with CFSE-labeled gliadin-TCRtg T cells and, the next day, received gliadin or TG2-gliadin i.g.. At 72 h, gliadin-TCRtg T cells were purified from the spleens and restimulated *in vitro* with TG2-gliadin-loaded bone-marrow-derived DQ2^{+} DC. After 48 h of restimulation, supernatants were analyzed for secreted cytokines. * Statistically significant (*P*<0.05). IFN, interferon; i.g., intragastric; IL, interleukin; i.m., intramuscular; MCP, monocyte chemoattractant protein; MLN, mesenteric lymph nodes; n.d, non-detectable; OVA, ovalbumin; PLN, peripheral lymph nodes; SPL, spleen; TCR, T-cell receptor; Tg, transgenic; TG2, transglutaminase 2; TNF, tumor necrosis factor.

Gliadin–TCRtg T cells also secrete IL–10

Next, we determined whether differentiating splenic gliadin-specific T cells also secreted anti-inflammatory cytokines, as it has previously been reported that suppressive gliadin-specific IL-10-producing Tr1 cells can be detected in patients with established CD (28). Similar to the experiment described in Figure 4.4 C, CD4+ gliadin-specific T cells were transferred to DQ2 acceptor mice that were fed non-deamidated or deamidated gliadin. At 72 h after gliadin feed, gliadin-specific T cells were purified from the spleens of DQ2 mice by flow-cytometric cell sorting and restimulated *in vitro* with deamidated–gliadin loaded DQ2+ bone-marrow-derived DC to determine whether these cells secrete the immunoregulatory cytokine IL-10. Gliadin-specific T cells that had not encountered deamidated gliadin *in vivo* (i.e. that were obtained from mice that were fed non-deamidated gliadin) secreted very low amounts of IL-10-protein upon stimulation *in vitro* with deamidated gliadin (Figure 4.5 A). However, T cells that had been activated *in vivo* by oral administration of deamidated gliadin and that received a second stimulation *in vitro* were found to secrete very high levels of the immunosuppressive cytokine (Figure 4.5 A). Moreover, these gliadin-specific T cells produced significantly lower amounts of IL-2 when stimulated for the second time (Figure 4.5 A). Q-PCR analysis revealed that IL-10 mRNA levels were elevated as well in proliferating gliadin-specific T cells from mice that were fed deamidated gliadin (Figure 4.5 B). Many subsets of CD4 T cells can secrete IL-10. Both IL-10-secreting Th1 cells as well as Tr1 cells that secrete variable amounts of IFN-γ have been reported to exist (29). As gliadin-specific T cells in our model secreted high amounts of IFN-γ concurrent to IL-10 (as showed above in Figure 4.4 C), we determined whether both cytokines were produced by the same cell subset. After *in vitro* restimulation and intracellular cytokine staining, splenic gliadin-specific T cells that were induced by deamidated gliadin feed could be subdivided into three different subsets: IFN-γ-producing cells (4.85% ± SD 0.63, n=3), IL-10-producing cells (13.18% ± SD 0.55, n=3) and cells that produced both cytokines (4.57% ± SD 0.59, n=3) (Figure 4.5 C). These data infer that multiple subsets of gliadin-specific T cells contribute to the high amounts of IFN-γ and IL-10.

To assess whether the IL-10 release had functional effects on the T-cell response, gliadin-TCRtg T cells were stimulated *in vitro* with splenic DQ2+ DC and deamidated gliadin. At day 3, T cells were harvested and restimulated with fresh DQ2+ bone-marrow-derived DC and deamidated gliadin in the presence of neutralizing αIL-10R antibodies. As shown in Figure 4.5 D, the interference with IL-10R signaling resulted in an augmented IFN-γ release. Together these data demonstrate that deamidated gliadin feed induces differentiation of a splenic suppressive Tr1-like T-cell population.

Transfer of dividing gliadin–TCRtg T cells induces tolerance

Dividing OVA-specific T cells from the MLN of BALB/c mice that are orally tolerized with OVA can induce tolerance upon adoptive transfer to naive acceptor mice, as demonstrated by a suppression of an OVA-specific DTH response in the ears (12). To assess the function of the
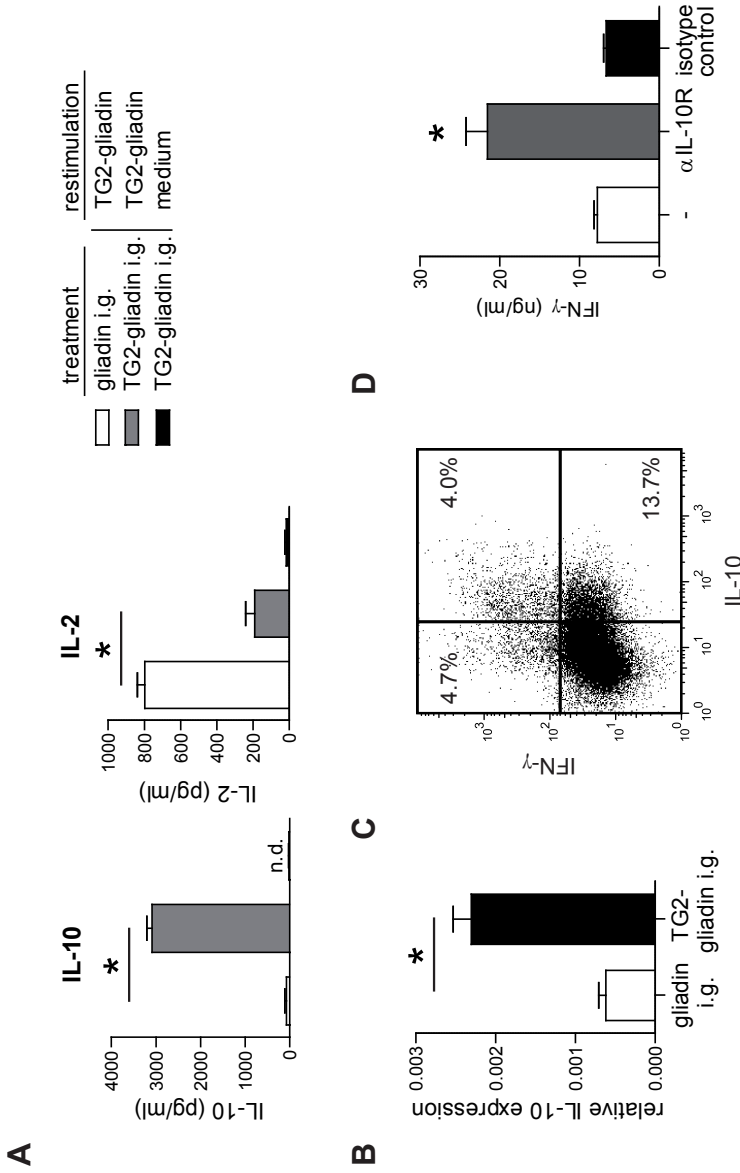
Figure 4.5 Gliadin–TCRtg T cells that responded to oral gliadin or TG2-gliadin secrete IL-10. **(A)** Gliadin–TCRtg T cells that were enriched with CD4+Vβ1+ cells and had received gliadin or TG2-gliadin i.g. and restimulated as described in Figure 4.4 C. **(B)** Restimulation of sorted splenic gliadin–TCRtg T cells with PMA/Cal for 14 h. Relative expression of IL-10 mRNA was determined by real-time PCR. **(C)** Splenic gliadin–TCRtg T cells were obtained and restimulated with TG2-gliadin as described in Figure 4.4 C. After 48 h of restimulation, cells were harvested and stained intracellulary for Vβ1, IFN-γ and IL-10. One representative dotplot is shown (gated on Vβ1+ cells, n=3). **(D)** Naive gliadin–TCRtg T cells (5 x 10⁵) were stimulated with deamidated gliadin–loaded splenic DQ2+ DC (2 x 10⁴) for 72 h, harvested and restimulated with fresh deamidated gliadin–loaded DQ2+ bone-marrow–derived DC in the presence or absence of a neutralizing αIL-10R antibody (1B1.2) or isotype control (GL113). Release of IFN-γ at 48 h of culture was measured by ELISA. * Statistically significant (*P*<0.05). DC, dendritic cell; IFN, interferon; i.g., intragastric; IL, interleukin; n.d, non-detectable; OVA, ovalbumin; SPL, spleen; TCR, T-cell receptor; Tg, transgenic; TG2, transglutaminase 2.

differentiating gliadin-TCRtg Tr1-like cells *in vivo* a similar approach was taken. Dividing gliadin-specific T cells from the spleen were isolated by flow-cytometric sorting and transferred to naive DQ2.gliadinTCR mice. As a control, two DQ2.gliadinTCR mice received Foxp3+ gliadin-specific Treg cells (83% Foxp3+), which were generated *in vitro* by culture of naive gliadin-TCRtg T cells with gliadin-loaded splenic DQ2+ DC. One day after cell transfer, the DQ2.gliadinTCR acceptor mice were subjected to a DTH response consisting of sensitization with deamidated gliadin in the tail base and a subsequent challenge with deamidated gliadin in the ears. As seen in Figure 4.6, DQ2.gliadinTCR mice that had received no cell transfer developed a gliadin-specific DTH response after injection of deamidated gliadin in the ears following a sensitization in the tail base. However, transfer of the dividing gliadin-specific CD4+ splenic T cells to naive DQ2. gliadinTCR mice suppressed the gliadin-specific DTH response as demonstrated by a significantly lower increase in ear thickness which was comparable to that of mice that received *in vitro* generated Foxp3+ gliadin-specific T cells (Figure 4.6). These data demonstrate that, despite their inflammatory phenotype and their ample secretion of effector cytokines, the gliadin-specific T cells that differentiate in the spleen after gliadin feed are tolerogenic.
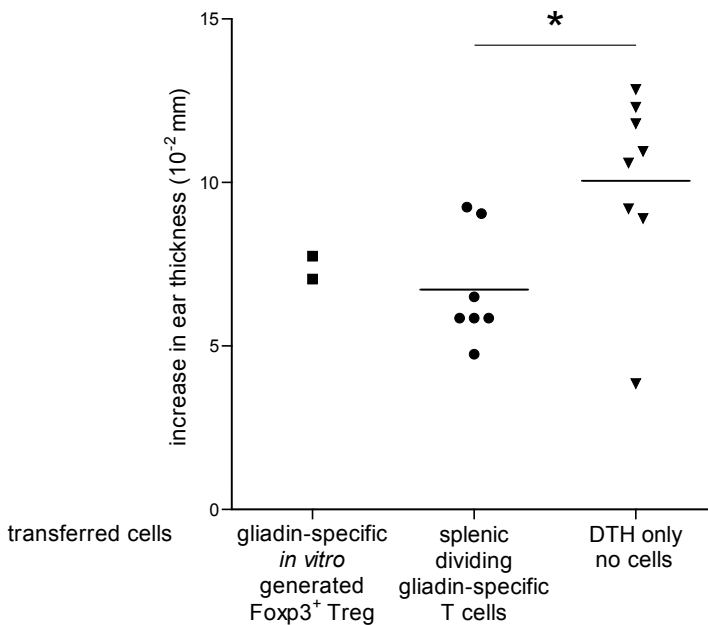
Figure 4.6 Transfer of gliadin-TCRtg T cells induces tolerance. Dividing CD4+ gliadin-TCRtg T cells were FACS sorted from spleens of DQ2 mice that were enriched with CFSE+ CD4+ gliadin-TCRtg T cells and were fed 75 mg TG2-gliadin. 2.5×10^5 cells were adoptively transferred to DQ2.gliadinTCR mice. Two mice received 2.5×10^5 purified *in vitro* generated gliadin-specific Foxp3+ Treg cells. Control mice did not receive any transferred cells. One day after adoptive transfer, both groups were sensitized subcutaneously with 100 µg TG2-gliadin. 5 days later, mice were challenged with 10 µg TG2-gliadin in both ears and, after 24 h, the increase in ear-thickness was determined and compared to values prior to challenge. * Statistically significant ($P<0.05$). DTH, delayed type hypersensitivity; TCR, T-cell receptor; Tg, transgenic; TG2, transglutaminase 2; Treg, regulatory T cell.

DQ2.gliadinTCRtg mice do not develop gluten-induced enteropathy

As these data established that gliadin feed elicited oral tolerance while systemic sensitization with deamidated gliadin dissolved in adjuvant induced an inflammatory DTH response, we wished to determine whether the presence of such an inflammatory gliadin-specific T-cell response was sufficient to induce intestinal pathology. DQ2.gliadinTCRtg mice were sensitized by injecting deamidated gliadin in the presence of IFA in the tailbase. The generation of gliadin-specific inflammatory T-cell response was confirmed by a positive DTH upon challenge in the ears (data not shown). After the DTH response, mice were transferred to a gluten-containing chow or kept on the standard gluten-free diet. Ten weeks after the start of the gluten-containing diet, all mice were killed and intestines were obtained for histological analysis. As shown in Figure 4.7 A, gluten-fed animals showed a normal villous architecture. Moreover, measurement of the length of villi and crypts of these mice revealed no differences in villous/crypt ratio between mice that were maintained on gluten-containing or gluten-free diet (Figure 4.7 B). Lastly, immunohistochemical staining for CD3 showed no increase in intra epithelial lymphocytes (IEL), one of the hallmarks of CD (Figure 4.7 C, D). In sum, these data demonstrate that the generation of a systemic inflammatory T-cell response is not sufficient to induce intestinal pathology.

DISCUSSION

Here we show that tolerance to deamidated gliadin ingestion is regulated by systemic Tr1-like cells without evidence for a role for Foxp3+ cells. Even though mechanistically different from other harmless food proteins the net outcome of deamidated gliadin ingestion is tolerance. However, this tolerance is predominantly mounted outside of the intestinal immune system, namely in the spleen where IL-10-secreting Tr1-like cells are formed but virtually no adaptive Foxp3+ T cells.

In our mouse model of double transgenic mice that express HLA-DQ2 and a chimeric TCR of a coeliac disease patient specific for deamidated gliadin peptide, feeding non-deamidated gliadin induced no signs of CD pathology nor T-cell division. Conversely, intramuscular injection of non-deamidated gliadin resulted in robust T-cell proliferation in the draining lymph nodes. This may suggest that in mice fed non-deamidated gliadin there is no TG2 activity in the intestine that will create the deamidated epitope, and that this can be one reason for the lack of T-cell response in these mice. In the normal mouse intestine Siegel et al. found no signs of constitutive TG2 activity (27) which would be in keeping with our results. Induction of TG2 activity in the intestine could however be induced by treating the mice systemically with poly(I:C). Whether treatment of DQ2.gliadinTCR mice with poly(I:C) in conjunction with non-deamidated gliadin feed results in T-cell division remains to be elucidated.

Notably, the feeding of deamidated gliadin resulted in a gliadin-specific T-cell response in the spleen, but not in the MLN, in the DQ2.gliadinTCR mice. In addition to the MLN, oral antigens induce proliferation in the PP (12). However, in our model the response to deamidated gliadin in PP was highly variable. In 38% of the mice that received deamidated gliadin orally,
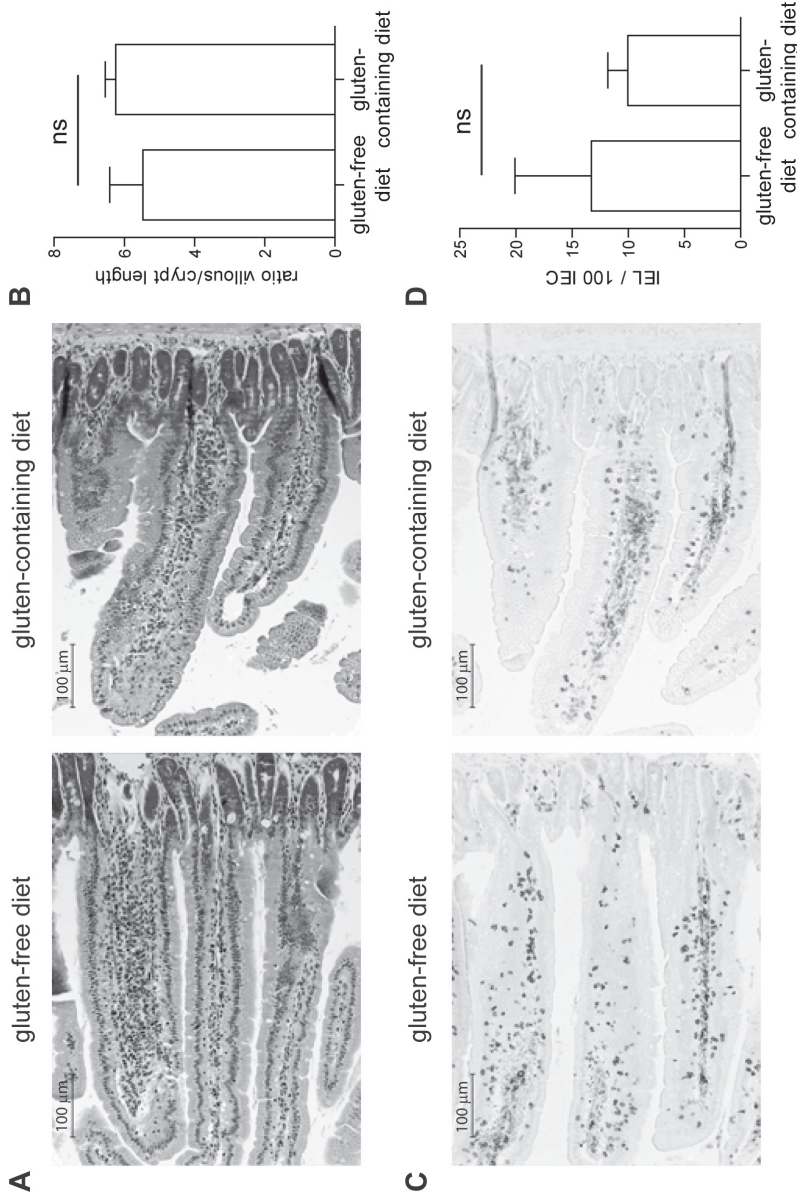
Figure 4.7 DQ2.gliadinTCR mice do not develop gluten-induced enteropathy. DQ2.gliadinTCR mice were sensitized subcutaneously with TG2-gliadin and subjected to a TG2-gliadin-specific DTH response in the ears. After the DTH, mice were either transferred to a gluten-containing diet or kept on the standard gluten-free diet. After 10 weeks, mice were killed for histological analysis. **(A)** Hematoxylin and eosin staining of a segment in the proximal small intestine of one representative animal. **(B)** Quantitative analysis of villous/crypt ratio. **(C)** Immunohistochemical CD3 staining of proximal small intestine of one representative animal. **(D)** Quantitative analysis of the number of IEL/100 enterocytes. DTH, delayed type hypersensitivity; IEL, intraepithelial lymphocytes; IEC, intestinal epithelial cells; IEL, intraepithelial lymphocytes; n.s., non-significant; TG2, transglutaminase 2.

virtually no dividing cells could be detected (In the remaining 62%, the percentage of cells that had proliferated varied from 34% to 80%; data not shown). The variation in the PP responses was confined to the PP as for these same mice no such variation could be observed in the splenic response.

This new model allowed us to demonstrate that deamidated gliadin, when given by the oral route, induces tolerance. The mechanisms that underlie oral tolerance to deamidated gliadin however appear strikingly different from that seen for other food proteins thus far. One is the topical localization in the spleen. Another is that within 48 h after antigen intake most cells have an activated phenotype and lack Foxp3 expression. This is exceptional considering that feeding harmless soluble proteins to naive mice normally induces oral tolerance that is associated with the induction of antigen-specific Foxp3$^+$ mucosal Treg cells in mucosa draining lymphoid tissue (10, 12, 15, 25, 30). Thus, because oral tolerance to deamidated gliadin occurs in the spleen, it does not occur under the strict control of mucosal resident DC and the mucosal microenvironment. In consequence, impaired tolerance in CD patients may not be due to defects in the mucosal immune response but rather be related to altered splenic tolerance. Splenic presentation of the antigen may be related to its molecular structure. For example feeding whole digested deamidated gliadin could differ from feeding deamidated peptide which may be more readily taken up by microfold (M) cells. In a DQ2-α-II epitope-dependent mouse model, De Kauwe et al. (31) have shown that a three-day feeding regime with deamidated gluten peptide in peanut oil resulted in a limited but detectable division in the PP and MLN. However, as the deamidated gluten peptide was emulsified in peanut oil this may have affected its uptake. Whether gliadin presentation in MLN or PP would lead to mucosal control and impose the typical Foxp3$^+$ CD62L^{neg} Treg-differentiation is questionable, as at the times that proliferation was detected in PP in our study we also observed the development of Foxp3$^-$CD62L^{hi} cells. The latter agrees with De Kauwe et al., who also report no increased Foxp3 induction in mucosally differentiating cells (31). To fully resolve which properties of deamidated gliadin drive splenic presentation detailed future analysis is required. It is difficult to establish whether gliadin-specific responses also predominantly occur in the spleen in humans. However, it is clear that inflammatory immune responses in CD are not always restricted to the intestine. For example patients can suffer from infertility, arthritis, metabolic bone disease and neuropsychiatric disorders (32, 33). Moreover, CD is frequently associated with dermatitis herpitiformis (32, 33) and, although the pathogenesis is not known, several reports have described hyposplenism in association with CD (34-36).

Functional analysis of the dividing gliadin-specific T cells in the spleen revealed these cells have a Tr1 phenotype characterized by suppressive activity in a DTH model, low IL-2 release and IL-10-dependent inhibition of IFN-γ production. However, simultaneously to the production of IL-10, the cells do secrete relatively large amounts of IFN-γ, which would argue denoting them as having a Th1 phenotype. Intracellular cytokine staining revealed that despite originating from the same HLA-DQ2-TCR interaction the differentiating T cells consist of three separate subpopulations. One only IFN-γ producing, one IFN-γ$^+$IL-10$^+$ and one only

IL-10[+]. This heterogeneity could either imply that the dividing T cells differentiate into distinct subpopulations or denote that these cells are Th1 cells that develop into IL-10-secreting cells in a specific phase of differentiation (29, 37-43). For this reason we have chosen to denote the cells as "Tr1-like". Although future experiments need to reveal which pathways determine gliadin-specific Tr1-like-cell differentiation, our *in vitro* T-cell proliferation assays revealed that IL-10-producing T cells can be induced by both splenic CD11c^{+} DC and B cells (data not shown), suggesting that the induction of these cells can be mediated by different subsets of antigen presenting cells. It should be noted that the cytokine profile of differentiating gliadin-specific T cells was dependent on the nature of the protein. *In vitro* comparison of whole protein stimulation with peptide stimulation resulted in a reduced secretion of IFN-γ but an increased production of the Th2-cytokine IL-4. This may be related to possible adjuvant effects of complex gliadin that are lost when using the gliadin-γ1 peptide.

The finding that oral tolerance to deamidated gliadin is mediated by Tr-1-like cells suggests that impaired tolerance in CD patients may be related to dysregulation in the Tr1-like T-cell response. Indeed IL-10 release has been observed in isolated intestinal T-cell subsets from pediatric CD patients that had been challenged with a gluten-containing diet (44). Moreover, it has previously been demonstrated that IL-10-producing gliadin-specific T-cell clones can be isolated from the mucosa of treated CD patients. Importantly, these Tr1-cell clones contain suppressive capacity as they could inhibit the proliferation of gliadin-specific effector T-cell clones (28). Future research will reveal whether particular defects in the induction of Tr1-like cells can be observed in CD patients.

Our finding that deamidated gliadin feed leads to induction of tolerance associated with Tr1-like regulatory cells may partially explain previous observations in other HLA-DQ2 or HLA-DQ8 transgenic mice models (31, 45) which showed that gluten feed did not result in small intestinal enteropathy. However, also in our model the presence of systemic inflammatory T-cells after sensitization and DTH failed to induce intestinal disease. In view of accumulating evidence that the innate immune system plays an important role in the pathogenesis of CD (21-24) these data may imply that chronic activation of the intestinal innate immune system is required for intestinal pathology.

In sum, this study provides a new mouse model that allows to further dissect the mechanisms that underlie the induction of tolerogenic gluten-specific Tr1-like cells and may provide new strategies to unravel how these mechanisms are disturbed leading to the inflammatory T cells that are seen in active CD.

ACKNOWLEDGEMENTS

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SUPPLEMENTARY FIGURES

A

B
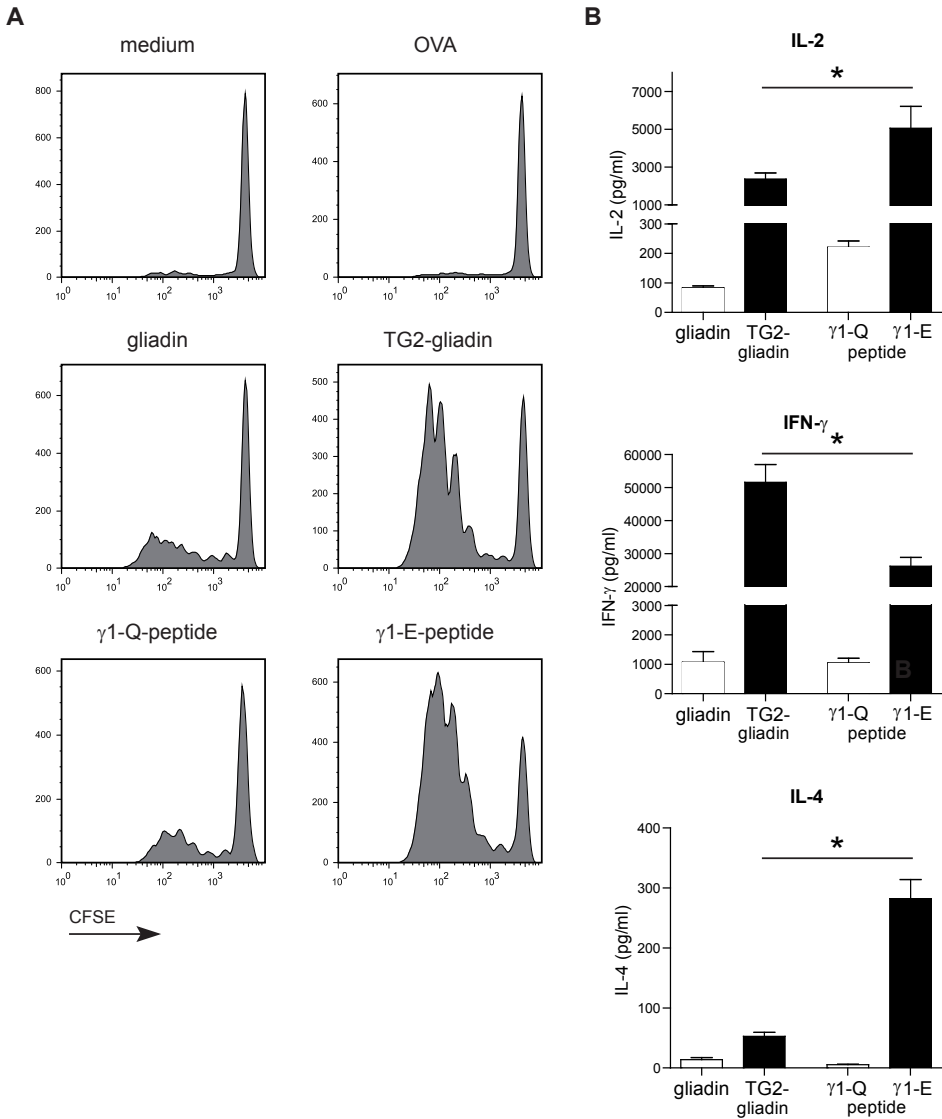
Supplementary Figure 4.1 *In vitro* proliferation of Gliadin-TCRtg CD4^{+} T cells to gliadin and gliadin γ1-peptide. **(A, B)** Gliadin-specific CD4^{+}Vβ1^{+} T cells (5 x 10^{5}) were stimulated with splenic DQ2^{+} DC (2 x 10^{4}) with OVA, gliadin, TG2-gliadin, and synthetic peptides of gliadin-γ1 in its native (γ1-Q) and deamidated (γ1-E) form for 96 h. **(A)** Representative histogram plots showing CFSE dilution. **(B)** Release of IL-2 (at 48 h), IFN-γ and IL-4 (both at 96 h) as measured by ELISA. * Statistically significant (*P*<0.05). IFN, interferon; IL, interleukin; OVA, ovalbumin; TCR, T-cell receptor; Tg, transgenic; TG2, transglutaminase 2.
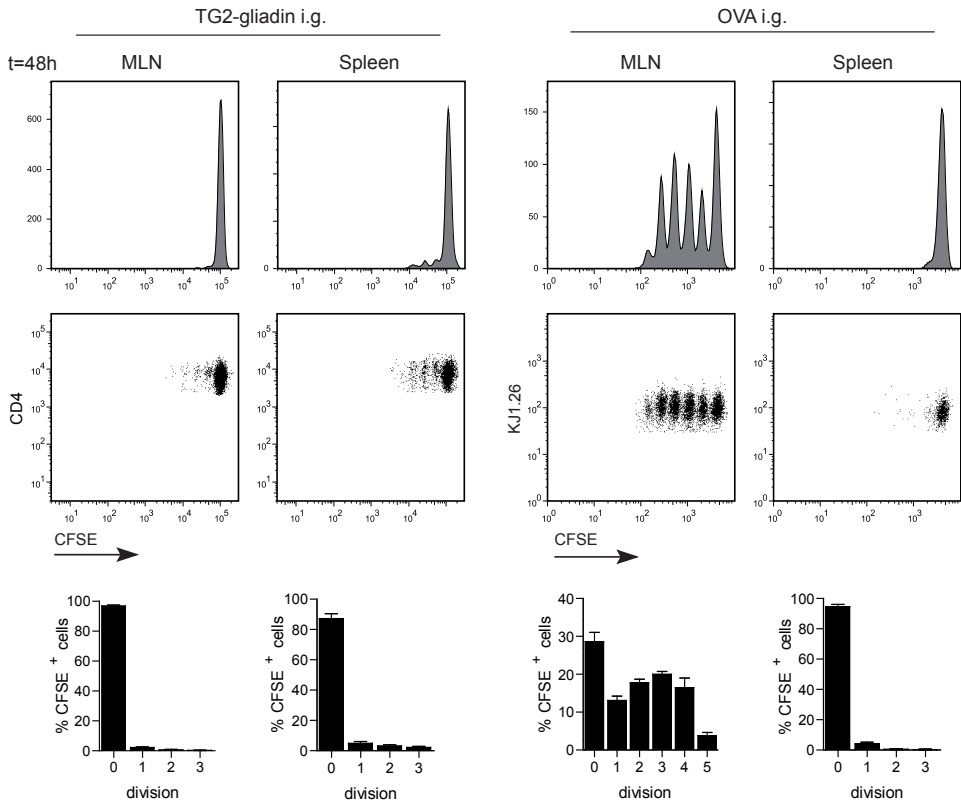
Supplementary Figure 4.2 Oral deamidated gliadin induces a dominant T-cell proliferation in the spleen. DQ2 mice were intravenously injected with 7 x 10⁶ CFSE-labeled CD4⁺ Vβ1⁺ cells following by the oral administration of 100 mg TG2-gliadin the next day. BALB/c mice were enriched with 1 x 10⁷ CFSE-labeled CD4⁺KJ1-26⁺ cells and given 70 mg OVA i.g.. At 48 h after antigen administration, spleens and MLN were isolated and analyzed by flow cytometry for division of transferred cells. One representative histogram plot and dot plot of the CFSE dilution profile is shown for each group, quantitative data demonstrate the percentages of CD4⁺ Vβ1⁺ cells in MLN (n=2), spleen (n=3) and CD4⁺KJ1-26⁺ cells in MLN and spleen (both n=3). TG2, transglutaminase 2; MLN, mesenteric lymph nodes; OVA, ovalbumin.

CD62L^{neg}CD38$^+$ expression on circulating CD4$^+$ T cells identifies mucosally–differentiated cells in protein–fed mice and in human coeliac disease patients and controls

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ABSTRACT

Objective The aim of this study was to identify new markers of mucosal T cells in order to monitor ongoing intestinal immune responses in peripheral blood. **Design** Expression of cell-surface markers was studied in mice on ovalbumin (OVA)-specific T cells in the gut-draining mesenteric lymph nodes (MLN) after OVA feed. The effect of the local mucosal mediators retinoic acid (RA) and transforming growth factor (TGF)-β on the induction of a mucosal phenotype was determined in *in vitro* T-cell differentiation assays with murine and human T cells. Tetramer stainings were performed to study gluten-specific T cells in the circulation of patients with coeliac disease (CD), a chronic small-intestinal inflammation. **Results** In mice, proliferating T cells in MLN were CD62L^{neg}CD38+ during both tolerance induction and abrogation of intestinal homeostasis. This mucosal CD62L^{neg}CD38+ T-cell phenotype was efficiently induced by RA and TGF-β in mice, whereas for human CD4+ T cells RA alone was sufficient. The CD4+CD62L^{neg}CD38+ T-cell phenotype could be used to identify T cells with mucosal origin in human peripheral blood, as expression of the gut-homing chemokine receptor CCR9 and β$_7$-integrin were highly enriched in this subset, whereas expression of cutaneous leukocyte-associated antigen was almost absent. Tetramer staining revealed that gluten-specific T cells appearing in blood of treated CD patients after oral gluten challenge were predominantly CD4+CD62L^{neg}CD38+. The total percentage of circulating CD62L^{neg}CD38+ cells within the CD4+ T-cell populatoin was not an indicator of intestinal inflammation as percentages did not differ between pediatric CD patients, inflammatory bowel disease (IBD) patients and respective controls. However, the phenotypic selection of mucosal T cells allowed cytokine profiling as upon restimulation of CD62L^{neg}CD38+ cells IL-10 and IFN-γ transcripts were readily detected in circulating mucosal T cells. **Conclusions** By selecting for CD62L^{neg}CD38+ expression which comprises 5–10% of the cells within the total CD4+ T-cell pool we are able to highly enrich for effector T cells with specificity for mucosal antigens. This is of pivotal importance for functional studies as this purification enhances the sensitivity of cytokine detection and cellular activation.

STUDY HIGHLIGHTS

What is the current knowledge?

- It is difficult to characterize defects in mucosal T-cell responses that contribute to chronic intestinal diseases as no ideal markers are available to monitor ongoing intestinal immune responses in peripheral blood.

- Mucosal dendritic cell-derived factors TGF-β and vitamin-A steer the mucosal T-cell response.

What is new here?

- Intestinal factors TGF-β and the vitamin-A derivative RA induce CD62L^{neg}CD38+ expression on CD4+ T cells in mouse and man.

- CD62L^{neg}CD38+ expression reflects mucosal imprinting irrespective of regulatory or inflammatory function.

- After a gluten challenge in CD patients, the majority of the gluten-specific T cells were detected in the small $CD4^+CD62L^{neg}CD38^+$ peripheral blood T-cell subset, establishing that the latter phenotype better defined a mucosally-imprinted population than the known markers CCR9 and $\alpha_4\beta_7$.

How might it impact on clinical practice in the foreseeable future?

- The value of defining this new subset lies in lowering the threshold for detection of luminal antigen specific T cells within the circulating $CD4^+$ T-cell pool. By selecting for $CD62L^{neg}CD38^+$ expression, which comprises 5–10% of the cells within the total $CD4^+$ T-cell pool, it is now possible to highly enrich for effector T cells with specificity for mucosal antigens. This is of pivotal importance for functional studies as this method removes 90–95% of $CD4^+$ non-gut-imprinted effector T cells and naive T cells from the analysis.

INTRODUCTION

The mucosal surfaces of our gastrointestinal tract are continuously exposed to foreign antigens. While inflammatory immune responses are needed to eliminate invasive bacteria and protect the body from infection, harmless soluble proteins and commensal flora induce mucosal tolerance (1). Inappropriate regulation of these responses can lead to chronic inflammatory disorders of the intestine, such as coeliac disease (CD), caused by intolerance to the dietary protein gluten, and inflammatory bowel diseases (IBD) caused by an aberrant inflammatory response to intestinal microbiota. Despite significant advances in the understanding of CD and IBD ongoing T-cell responses in patients are difficult to study as T cells from mucosal origin are not easily identified in peripheral blood.

Both effector T cells and regulatory T (Treg) cells differentiate from naive T cells upon antigen presentation by mucosal dendritic cells (DC) in the Peyer's Patches (PP) and gut-draining mesenteric lymph nodes (MLN) following antigen feed (2-4). Upon activation in the PP or MLN, T cells acquire high expression levels of the integrin $\alpha_4\beta_7$ and the chemokine receptor CCR9, that allow their migration to the small intestine (5-7). Imprinting of this gut-homing phenotype is efficiently induced in response to protein feed regardless of the functional outcome of the T-cell response, as CCR9 is efficiently induced both in the absence and presence of the mucosal adjuvant cholera toxin (CT) (8).

Although the role for CCR9 in migration to the small intestine is well established, the expression of CCR9 is not absolutely required for the localization of $CD4^+$ T cells in the small intestine (9). $CCR9^{-/-}$ mice have normal numbers of $CD4^+$ T cells in the small intestine, whereas the $CD8^+$ population is reduced (10, 11). In agreement with these findings $CD4^+$ T cells in the lamina propria express a more heterogeneous chemokine receptor profile including CCR9, CXCR3, CXCR6, as well as CCR5 and CCR6 (12). Moreover, expression of CCR9 on human peripheral blood T cells does not seem to be restricted to gut-migrating T cells, as CCR9-expressing naive

T cells can be found that lack $\alpha_4\beta_7$ and are presumed to be recent thymic emigrants (13). In addition, a large proportion of $\alpha_4\beta_7^+$ T cells in blood do not express CCR9 (13). These findings indicate that CCR9 may not exclusively identify all gut-imprinted T cells in peripheral blood.

Hence, we investigated whether mucosal imprinting results in a distinctive phenotype that could be used to identify mucosally-educated T cells in the periphery.

MATERIALS AND METHODS

Mice

Specific pathogen-free BALB/c mice (Charles River, Maastricht, The Netherlands), DO11.10 transgenic (Tg) and DO11.10 Tg x RAG$^{-/-}$ mice (breeding at ErasmusMC), which have a Tg T-cell receptor (TCR) for the ovalbumin (OVA)$_{323-339}$–peptide were kept under routine animal housing conditions. Experiments were approved by the animal experimental committee of the ErasmusMC.

Adoptive transfer and CFSE labeling

BALB/c mice received an intravenous injection of 6-10 x 10^6 5,6-carboxy-succinimidyl-fluoresceine-ester (CFSE)-labeled DO11.10 cells as described previously (2, 14). The next day, mice received 70 mg 98% pure OVA (Sigma Aldrich, Zwijndrecht, The Netherlands) intragastically (i.g.) or 400 µg OVA (Calbiochem, San Diego, CA) intramuscularly (i.m.). To break tolerance, mice received 70 mg OVA with 20 µg CT (Sigma) i.g.. At 72 h after OVA administration, cells from draining lymph nodes and spleen were stained for CD4 (GK1.5), CD38 (90), CD62L (MEL-14, all BD-Pharmingen, Woerden, The Netherlands), Foxp3 (JFK-16S, EMELCA Bioscience, Bergen op Zoom, The Netherlands) and DO11.10 Tg TCR (KJ1.26, Invitrogen, Breda, The Netherlands). Phenotype and cell division (based on fluorescence intensity of single CFSE peaks) were measured on a FACSCanto or FACSCalibur flow cytometer (BD). Gating of CD38 on CD4$^+$KJ1.26$^+$ cells was set according to expression on CD38$^+$ lymphocytes (mainly B cells) in the same lymph node. CD38^{int} was defined as a level of expression with fluorescence intensity in between CD38^{neg} and CD38^{hi} lymphocytes (Supplementary Figure 5.1).

In vitro T-cell differentiation assays (mouse)

For polyclonal T-cell differentiation experiments, CFSE$^+$CD4$^+$KJ1.26$^+$ cells were obtained as described above. CD25$^+$ cells were depleted with PC61 antibody (kindly provided by Dr. J. den Haan, Amsterdam, The Netherlands). 5 x10^5 CFSE$^+$CD4$^+$KJ1.26$^+$ T cells were stimulated with plate-bound anti-CD3e (145-2C11, 10 µg ml^{-1}, BD) and soluble anti-CD28 (37.51, 10 µg ml^{-1}, BD) for 72 h.

DC from MLN, peripheral lymph nodes (PLN) and spleens for OVA-specific T-cell differentiation assays were obtained as described previously (15). Naive CD4$^+$KJ1.26$^+$ T cells

were obtained from lymph nodes and spleens of DO11.10 Tg x RAG$^{-/-}$ mice by positive selection using biotinylated KJ1.26 and the CELLection biotin-binder kit (Invitrogen). 2 x 10^4 DC were cultured with 5 x 10^5 CFSE-labeled CD4$^+$KJ1.26$^+$ cells, OVA$_{323-339}$ peptide for 96 h and/or 20 ng ml^{-1} rhTGF-β (Preprotech, Rocky Hill, NJ) and 10 nM RA (Sigma). Concentrations of rhTGF-β and RA were determined in a dose response experiment and chosen based on optimal induction of Foxp3 (not shown). In some experiments recIL-4 (300 ng ml^{-1}, R&D systems, Abingdon, UK), lipopolysaccharide (LPS) (E. coli 0111:B4, 100 ng ml^{-1}, Difco), RA receptor antagonists LE540 (10 μM, Wako Chemical, Osaka, Japan) or LE135 (10 μM, Tocris Bioscience, Bristol, UK) were added.

Peripheral blood

Healthy controls: Peripheral blood cells from healthy volunteers were stained for CD4 (RPA-T4), CD38 (HIT2), CD62L (DREG-56, all BD), CD45RA (MEM-56, Invitrogen), Foxp3 (PCH101, EMELCA), CCR9 (FAB1791A, R&D Systems, Abingdon, UK), CLA (HECA-452), β$_7$-integrin (FIB504, both BD) and appropriate isotype controls. Peripheral blood mononuclear cells (PBMC) were isolated and stimulated with CD3/CD28 T-cell expander beads (Dynal) in the presence of 20 ng ml^{-1} rhTGF-β and/or 10 nM RA. After 96 h the cells were analyzed by flow cytometry. All participants gave written informed consent for the study.

Pediatric coeliac disease and inflammatory bowel disease patients: Peripheral blood cells were obtained from pediatric patients who underwent an esophago-gastro-duodenoscopy with suspicion of CD in the Erasmus MC – Sophia Children's Hospital, Rotterdam, The Netherlands. Children with biopsy-proven CD were included in the patient group (34 patients), whereas children with a normal intestinal histology were included in the control group (21 controls). In addition, peripheral blood from IBD patients was analyzed. All IBD patients (29 patients) were diagnosed by endoscopy, histopathological and clinical characteristics according to the Porto criteria (16). Age matched orthopedic patients (14 patients) with no underlying inflammatory or intestinal diseases served as controls. Written informed consent from the parents was obtained for each patient.

Adult coeliac disease patients: For detection of gluten-specific T cells in peripheral blood, six CD patients on a gluten-free diet were recruited (study approved by the Regional Ethics Committee for Southern Norway). The patients ate four slices of standard white bread daily for three days (approximately 160 g, Bakers AS), thereafter continued their gluten-free diet. On day 6, blood was drawn and gluten-reactive T cells were stained with tetramers as described below and reported previously (17).

Biopsies

Duodenal biopsy specimens were obtained from patients who underwent gastroendoscopy for their diagnostic work-up for abdominal pain and diarrhoea (2 patients) or Barret esophagus (one patient). Histology revealed normal mucosal architecture. Epithelium was removed by incubation

in 2 mM EDTA (2 x 30 min.), followed by digestion of the tissue in 1 mg ml^{-1} collagenase H (Sigma) (2 x 30 min.). Released cells were stained with specific antibodies against CD45 (HI30, eBioscience), CD62L (DREG-56), CD4 (SK3), CD38 (HB7) and TCRαβ (T10B9.1A-31, all BD) and treated with 0.2 μg ml^{-1} propidium iodide to exclude dead cells. Collection of clinical samples was approved by the regional ethics committee for Southern Norway and the patients gave written informed consent.

Gliadin-specific T-cell staining using α-gliadin tetramers

Water-soluble DQ2 (DQA1*0501/DQB1*0201) molecules with three different peptides were produced as previously described (17); two gliadin-tetramers with deamidated T-cell epitopes of α-gliadin (i.e., DQ2-αI tetramer and DQ2-αII tetramer) and one control tetramer containing the invariant chain peptide CLIP2 (18). Tetramers were made by conjugating DQ2–peptide complexes with streptavidin-PE (Molecular Probes, Eugene, OR). Specificity of the tetramers was assessed after conjugation by staining two T-cell clones TCC 380.E.2 (specific for the DQ2-α-I epitope) and TCC 412.5.28 (specific for the DQ2-α-II epitope) with all three DQ2 tetramers.

CD4⁺ lymphocytes were isolated from PBMC by positive selection (Invitrogen) and stained as described previously (17) with slight modifications. Briefly, 1 to 4 million CD4⁺ T cells were stained in 100 μl with 10 μg ml^{-1} tetramer at 37 °C for 30 min cooled and stained with specific antibodies. At least 0.8 million cells were analyzed on an LSRII instrument (BD). T cells were identified as CD4⁺CD11c⁻ cells. The following antibodies were used: CD62L (DREG-56), CD38 (HIT2), CD11c (3.9), CD45RA (HI100, all eBioscience), CD4 (SK3, BD) and CCR9 (FAB1791A, R&D).

Q-PCR

RNA was purified from purified murine CD4⁺ T cells or T-cell subsets using the Nucleospin RNA-XS kit (Macherey-Nagel, Düren, Germany) and cDNA was synthesized. Real-time quantitative PCR was performed using SYBRgreen (Finnzymes, Espoo, Finland) on an AbiPrismR7900 Sequence Detection System (PE Applied Biosystems, CA, USA). Relative expression to cyclophillin was calculated as follows: relative expression = $2^{-\Delta Ct}$, whereby $\Delta Ct = Ct_{target} - Ct_{housekeeping}$. Specific primers were designed across different constant region exons resulting in these primers:

CYCLO: Fw: 5'-AACCCCACCGTGTTCT-3' Rv: 5'-CATTATGGCGTGTAAAGTCA-3'

FOXP3: Fw: 5'-ACCTGGGATCAATGTGG-3' Rv: 5'-TGGCAGTGCTTGAGAAA-3'

CD38: Fw: 5'-GCTGCCTCATCTACACTCA-3' Rv: 5'-TTTGCTCCAAAAGAGAGTCT-3'

Statistics

Data are expressed as the mean ± SD and analyzed either using Student's t-test or ANOVA. $P<0.05$ was considered significant.

RESULTS

Under homeostatic conditions mucosal T cells have a CD62L^{neg}CD38^{int} phenotype

Previously, we have shown that within 48 h after OVA feed, proliferating antigen-specific T cells in the MLN are CD62L^{neg} and express CD38 (2). To assess whether CD62L^{neg}CD38$^+$ expression is characteristic for gut-imprinted cells *in vivo*, we compared the T-cell response in the gut draining MLN after OVA feed with the T-cell response in popliteal and inguinal lymph nodes referred to as peripheral lymph nodes (PLN) after i.m. OVA injection. Expression of CD62L rapidly decreased upon differentiation of tolerogenic OVA-specific T cells in the MLN after OVA feed, whereas expression levels remained high on differentiating T cells in the PLN after OVA i.m. (Figure 5.1 A). The differentiating CD62L$^+$ T cells in the PLN co-expressed high levels of CD25 (data not shown). Moreover, the CD62L$^+$ phenotype remained upon injection of OVA in incomplete Freud's adjuvant, indicating that absence of CD62L downregulation is not due to suboptimal T-cell activation (data not shown). As a result, the percentage of cells that had become CD62L^{neg} in the MLN was significantly higher in comparison with the PLN (Figure 5.1 B). Downregulation of CD62L on differentiating T cells in the MLN was not influenced by the APC – T-cell ratio, as adoptive transfer of different numbers of CD4$^+$KJ1.26$^+$ T cells resulted in an equal percentage of CD62L^{neg} cells (Supplementary Figure 5.2).

The mean fluorescence intensity (MFI) of CD38 on dividing T cells in the MLN gradually increased upon division towards a CD38^{int} phenotype, whereas in PLN most dividing cells were CD38^{neg} (Figure 5.1 A, B). A small proportion of the OVA-specific T cells expressed CD38 at high levels. As these cells were CD62L$^+$ this phenotype may reflect precursors for central memory cells, as CD62L$^+$CD38^{hi} cells are also observed during a secondary flu infection in the lung (observations Frances Lund). Taken together, the CD62L^{neg}CD38^{int} phenotype is characteristic for T cells that differentiate in the MLN after protein feed (Figure 5.1 C). Differentiating MLN T cells elicited by OVA feed are Treg cells that suppress an inflammatory delayed-type hypersensitivity response upon adoptive transfer (2). A proportion of these Treg cells *de novo* expresses Foxp3 ((15) and Figure 5.1 D). To assess whether the CD62L^{neg}CD38^{int} phenotype correlated with the differentiation of Foxp3$^+$ Treg cells, we analyzed dividing Foxp3$^+$ and Foxp3 negative T cells in the MLN. Both Foxp3$^+$ and Foxp3$^-$ T cells in the MLN were predominantly CD62L^{neg} (Figure 5.1 E). In contrast, the majority of Foxp3$^+$ T cells that differentiated in PLN were CD62L$^+$ (Supplementary Figure 5.3). The percentage of CD38$^+$ cells was significantly higher in the Foxp3+ T-cell population when compared with Foxp3 negative cells in both MLN (Figure 5.1 E) and PLN (Supplementary Figure 5.3), indicating that CD38 expression correlates with the *de novo* induction of Foxp3. In sum, during mucosal tolerance induction, antigen-specific T cells preferentially have a CD62L^{neg}CD38^{int} phenotype.
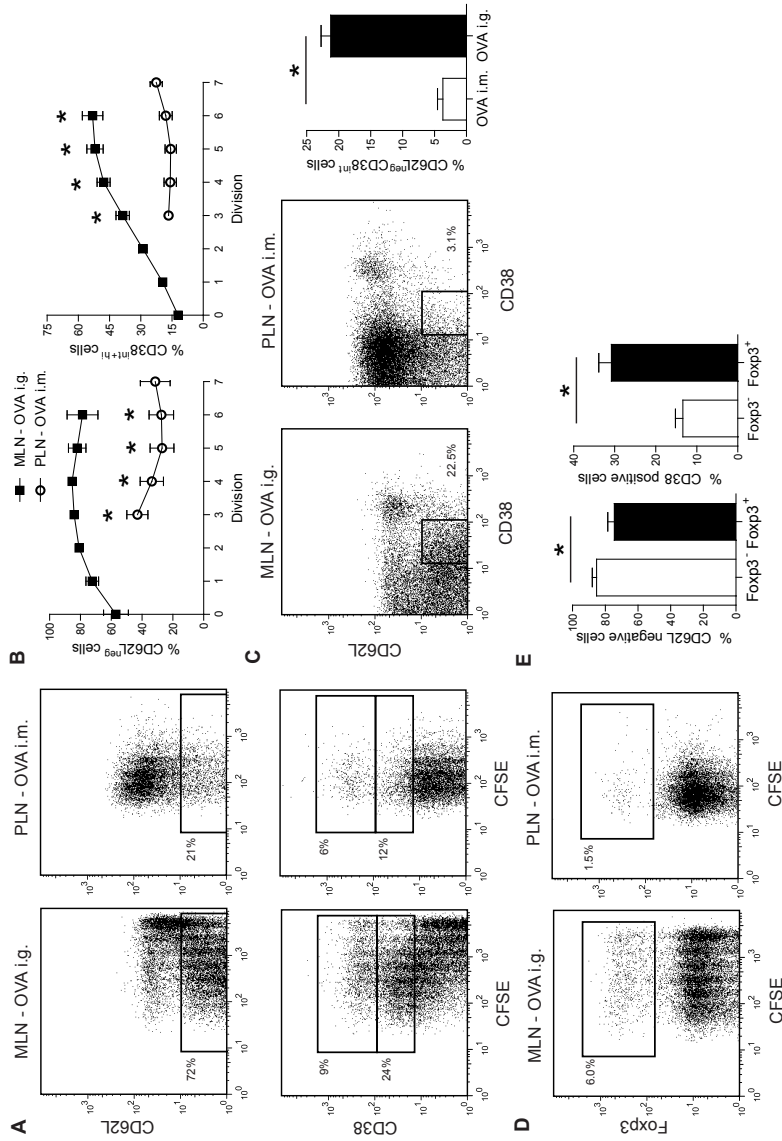
Figure 5.1 Mucosal T cells express CD62LⁿᵉᵍCD38ⁱⁿᵗ under homeostatic conditions. BALB/c mice received CFSE-labeled CD4⁺KJ1.26⁺ cells and OVA i.g. or OVA i.m.. At 72 h after OVA, cells from gut-draining MLN- (OVA i.g.) and thigh muscle draining popliteal and inguinal lymph nodes (denoted as PLN; OVA i.m.) were analyzed by flow cytometry. **(A)** CD62L or CD38 expression gated on CFSE⁺KJ1.26⁺ cells. **(B)** Percentage CD62Lⁿᵉᵍ and CD38ⁱⁿᵗ⁺ʰⁱ cells of CD4⁺KJ1.26⁺ T cells in each division peak (n=3). **(C)** Combined expression of CD38 and CD62L and CD62LⁿᵉᵍCD38ⁱⁿᵗ T cells as percentage of CD4⁺KJ1.26⁺ T cells (n=3). **(D)** Representative dotplots showing Foxp3 staining of CFSE⁺CD4⁺KJ1.26⁺ T cells in draining lymph nodes 72 h after OVA i.g. and OVA i.m. **(E)** Quantitative analysis of CD62Lⁿᵉᵍ and CD38⁺ cells within Foxp3⁺CD4⁺KJ1.26⁺ and Foxp3ⁿᵉᵍCD4⁺KJ1.26⁺ gated cells (n=3). * Statistically significant (P<0.05). Plots are representative of n=3. I.g., intragastric; i.m., intramuscular; MLN, mesenteric lymph nodes; OVA, ovalbumin; PLN, peripheral lymph nodes.

## TGF-β and retinoic acid induce differentiation of CD62L^{neg}CD38^{+}T cells

To determine whether the CD62L^{neg}CD38^{int} phenotype is driven by factors in the gastrointestinal microenvironment, we stimulated naive CD4^{+} T cells with αCD3αCD28, TGF-β and RA. Without TGF-β and RA, T-cell stimulation resulted in CD62L^{hi} expression for most activated T cells. Upon the addition of rhTGF-β the expression of CD62L was rapidly lost (Figure 5.2 A). As rhTGF-β inhibited T-cell activation, these results demonstrate that loss of CD62L does not exclusively reflect T-cell activation. Addition of RA to TGF-β-stimulated T cells only slightly further increased the percentage of CD62L^{neg} cells (Figure 5.2 A, B), while culturing with RA alone also induced a loss of CD62L (data not shown).

T-cell stimulation without TGF-β and RA did not result in induction of CD38 expression (Figure 5.2 A). In contrast, TGF-β induced a moderate increase in CD38 expression at both protein (Figure 5.2 A, B) and mRNA levels (Figure 5.2 C). This TGF-β-mediated induction of CD38 occurred simultaneously to Foxp3^{+} Treg-cell differentiation (Figure 5.2 A). Culture with RA and TGF-β together further increased the induction of CD38 and Foxp3. In particular, culture with RA and TGF-β induced a three-fold increase in the number of CD38^{+} cells (Figure 5.2 B) and a four-fold increase in CD38 mRNA levels compared with polyclonal stimulation alone (Figure 5.2 C). No effect on CD38 expression was observed when RA was used alone (data not shown). Thus, RA and TGF-β drive the reduction of CD62L and increase CD38 expression on differentiating mucosal Treg cells.

Next, we assessed whether mucosal DC, that are able to convert vitamin-A into RA (19), are more efficient in inducing a CD62L^{neg}CD38^{+} phenotype *in vitro* when compared with non-mucosal DC. Thereto, OVA-specific T cells were stimulated with OVA-loaded MLN-DC, PLN-DC or splenic DC. As previously reported, CD62L expression rapidly decreased upon stimulation with DC, irrespective of their origin (5), and the downregulation of CD62L was further enhanced by TGF-β (data not shown). No differences in CD38 expression could be detected between T cells cultured with MLN-DC, PLN-DC or splenic DC (data not shown). Addition of TGF-β significantly increased CD38 expression on T cells stimulated by MLN-DC but not by PLN-DC or splenic DC (Figure 5.3 A, Supplementary Figure 5.4 A). To establish that the imprinting of CD38 by MLN-DC could be explained by their synthesis of RA, we added selective RA-receptor antagonists LE135 and LE540. Both antagonists inhibited the induction CD38 by MLN-DC (Figure 5.3 B), demonstrating that synergy between endogenous RA and TGF-β was responsible for imprinting of CD38 expression. Moreover, interference with RA signaling resulted in a reduction of CD62L^{neg} cells (Figure 5.3 B).

Addition of exogenous RA together with TGF-β to T cells cultured with OVA-loaded MLN-DC further increased the expression of CD38 (Figure 5.3 C, Supplementary Figure 5.4 B). Furthermore, exogenous RA and TGF-β abolished the differences between mucosal and non-mucosal DC in the imprinting of CD38 expression on T cells (Figure 5.3 D). In sum, in the presence of TGF-β, mucosal DC efficiently induce expression of CD62L^{neg}CD38^{+} on differentiating T cells in an RA-dependent manner.

A
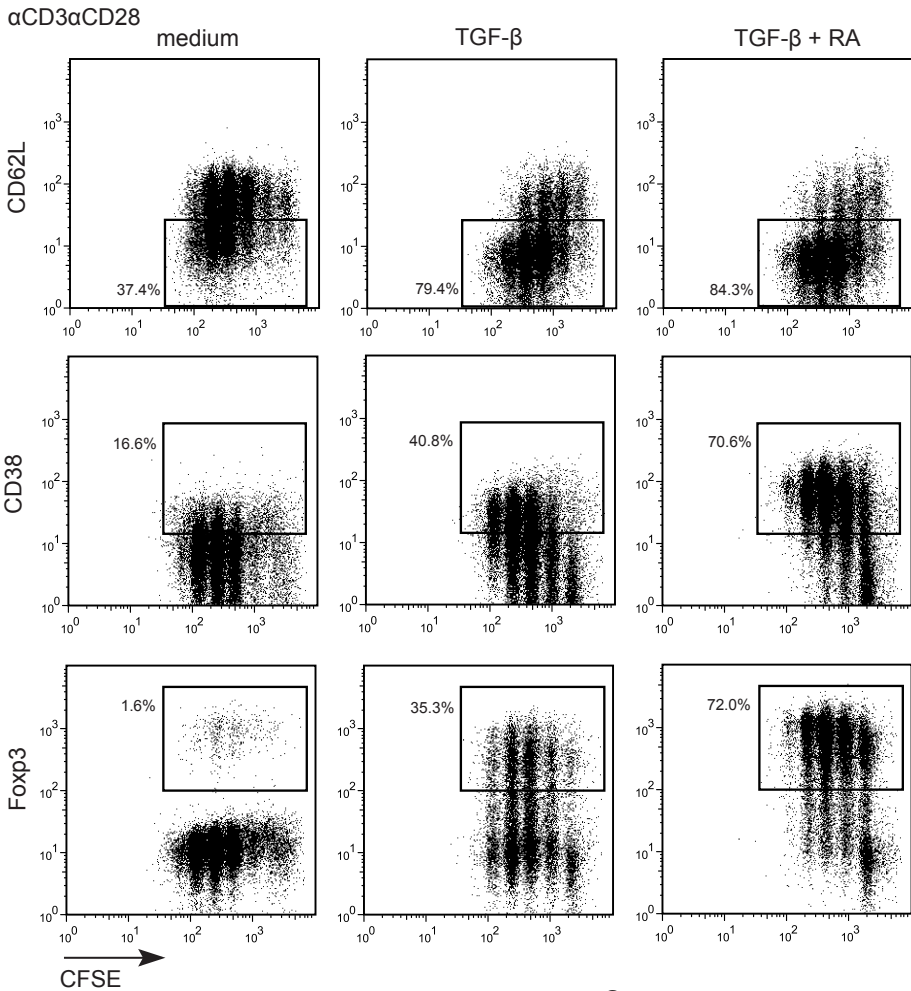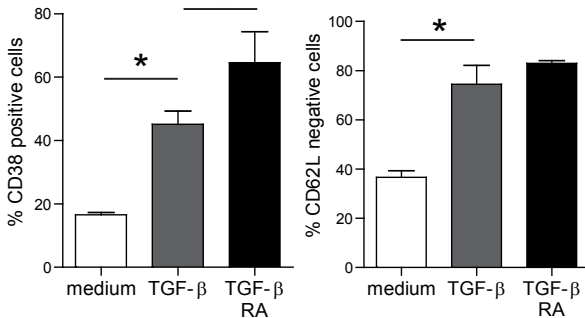
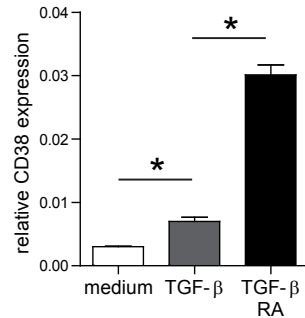**Figure 5.2** TGF-β and RA induce a CD62L^neg CD38+ phenotype on activated T cells. CFSE-labeled murine CD4+ T cells were stimulated with αCD3αCD28 +/- rhTGF-β and/or RA. **(A)** Flow cytometry of CD62L, CD38 and Foxp3. **(B)** Quantitative data of flow cytometry (n=3). **(C)** CD38 mRNA expression (n=3). * Statistically significant (*P*<0.05). RA, retinoic acid; TGF, transforming growth factor.
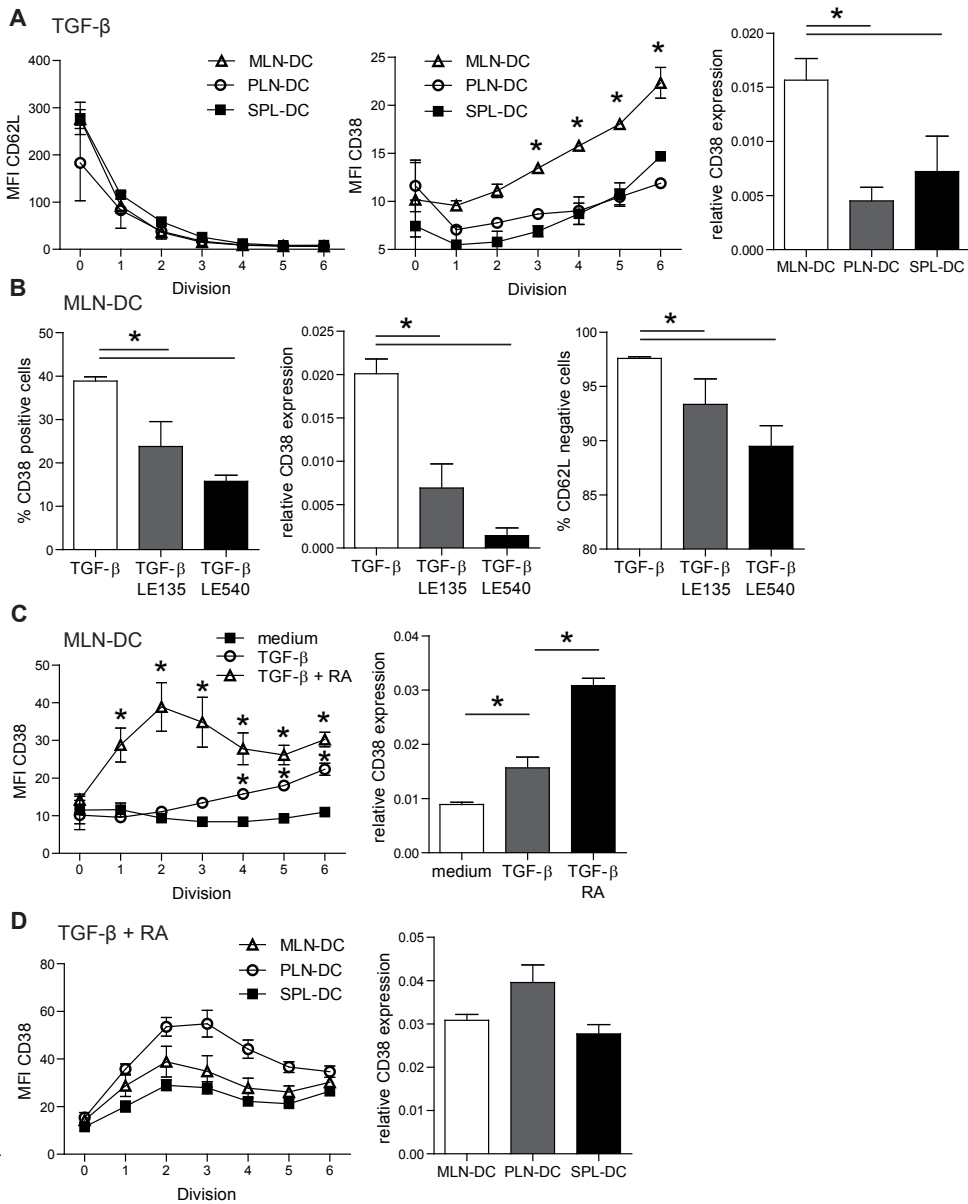
Figure 5.3 Expression of CD38 is preferentially induced by mucosal DC in a TGF-β and RA-dependent manner. OVA-loaded murine DC and CFSE⁺CD4⁺KJ1.26⁺ cells were cultured in different conditions. At 96 h relative CD38 mRNA expression and flow cytometry analysis was performed on the T cells. **(A)** culture with TGF-β. CD62L and CD38 MFI expression shown per peak of T-cell division and mRNA expression (n=3). **(B)** MLN-DC and T cells cultured with TGF-β and RA-receptor antagonists LE135 and LE540. Percentage CD38⁺ and CD62L^neg cells and CD38 mRNA levels (n=3). **(C)** MLN-DC and T cells cultured with TGF-β and TGF-β+RA. MFI and mRNA expression of CD38 on T cells (n=3). **(D)** DC from MLN, PLN and spleen cultured with TGF-β and RA. MFI of CD38 and mRNA expression (n=3). * Statistically significant (*P*<0.05). DC, dendritic cell; MFI, mean fluorescence intensity; MLN, mesenteric lymph nodes; OVA, ovalbumin; PLN, peripheral lymph nodes; RA, retinoic acid; SPL, spleen; TGF, transforming growth factor.

CD38 is expressed irrespective of regulatory or effector T-cell function

To test whether the imprinting of the CD62L^neg CD38+ phenotype by MLN-DC is maintained upon inhibition of adaptive Treg-cell induction, we cultured naive CD4+ T cells with MLN-DC in the presence of RA, TGF-β and rIL-4, which abolishes the differentiation of mucosal Foxp3+ Treg cells (15, 20, 21), or lipopolysaccharide (LPS). Both rIL-4 and LPS decreased Foxp3 mRNA levels. However, the suppression of Foxp3+ Treg-cell differentiation did not affect the imprinting of the CD62L^neg CD38+ phenotype (Figure 5.4). No differences in the downregulation of CD62L were observed (data not shown). The CD38+ phenotype was maintained in the absence of Foxp3 expression, however the percentage of CD38+ cells was slightly decreased by both rIL-4 and LPS (Figure 5.4 B, C). Strikingly, rIL-4 regulated the expression of CD38 as the MFI of CD38+ cells and CD38 mRNA levels were increased (Figure 5.4 D). In agreement with previous studies (22), rIL-4 also induced the expression of CCR9 on T cells cultured with MLN-DC in the presence of RA and TGF-β (data not shown). *In vivo*, the CD62L^neg CD38+ phenotype was also maintained upon abrogation of tolerance with CT (Supplementary Figure 5.5 A–C). In conclusion, these data suggest that the CD62L^neg CD38+ phenotype is maintained upon inhibition of Foxp3+ T-cell differentiation.

CD62L^neg CD38+ phenotype can be used to identify mucosal T cells in human peripheral blood of healthy donors

We hypothesized that the CD62L^neg CD38+ phenotype can be used to identify T cells from mucosal origin in humans. First, we determined the expression of CD62L and CD38 on normal small-intestinal biopsy specimens. Almost all CD4+ T cells in human duodenum are CD62L^neg CD38+ (Figure 5.5 A). Next, we assessed whether RA and TGF-β could induce the CD62L^neg CD38+ phenotype on human CD4+ T cells. Thereto, PBMC were stimulated with anti-CD3, anti-CD28 in the presence of RA, TGF-β or medium. Stimulation in the presence of RA induced an on average three-fold increase of the CD62L^neg CD38+ T-cell subset (fold increase 3.22 ± 0.60 relative to anti-CD3, anti-CD28-treated medium control, Figure 5.5 B), resulting in CD62L^neg CD38+ expression on approximately 50% of CD4+ T cells. The addition of TGF-β to the RA cultures did not further enhance the percentage of CD62L^neg CD38+ T cells (fold increase compared with anti-CD28-treated medium control 2.68 ± 0.60, Figure 5.5 B). Stimulation in the presence of TGF-β did not enhance the expression of CD38 on human CD4+ T cells (Figure 5.5 B). Thus, upon polyclonal stimulation in the presence of the mucosal factor RA, human CD4+ T cells acquire a CD62L^neg CD38+ phenotype.

Next, we determined whether the RA-induced CD62L^neg CD38+ T-cell population can be identified in fresh peripheral blood. A clear CD62L^neg CD38+ cell subset was observed consisting of 5.69 ± 2.17 (n=9) percent of total CD4+ lymphocytes (Figure 5.5 C). Distribution of the other CD62L/CD38 subsets was as follows: CD62L^neg CD38^neg: 10.66 ± 4.23, CD62L+CD38^neg: 26.44 ± 7.91 and CD62L+CD38+: 57.65 ± 10.90 (n=9). To determine the proportion of naive and memory cells within these subsets we co-stained for CD45RA. Naive CD45RA+ T cells
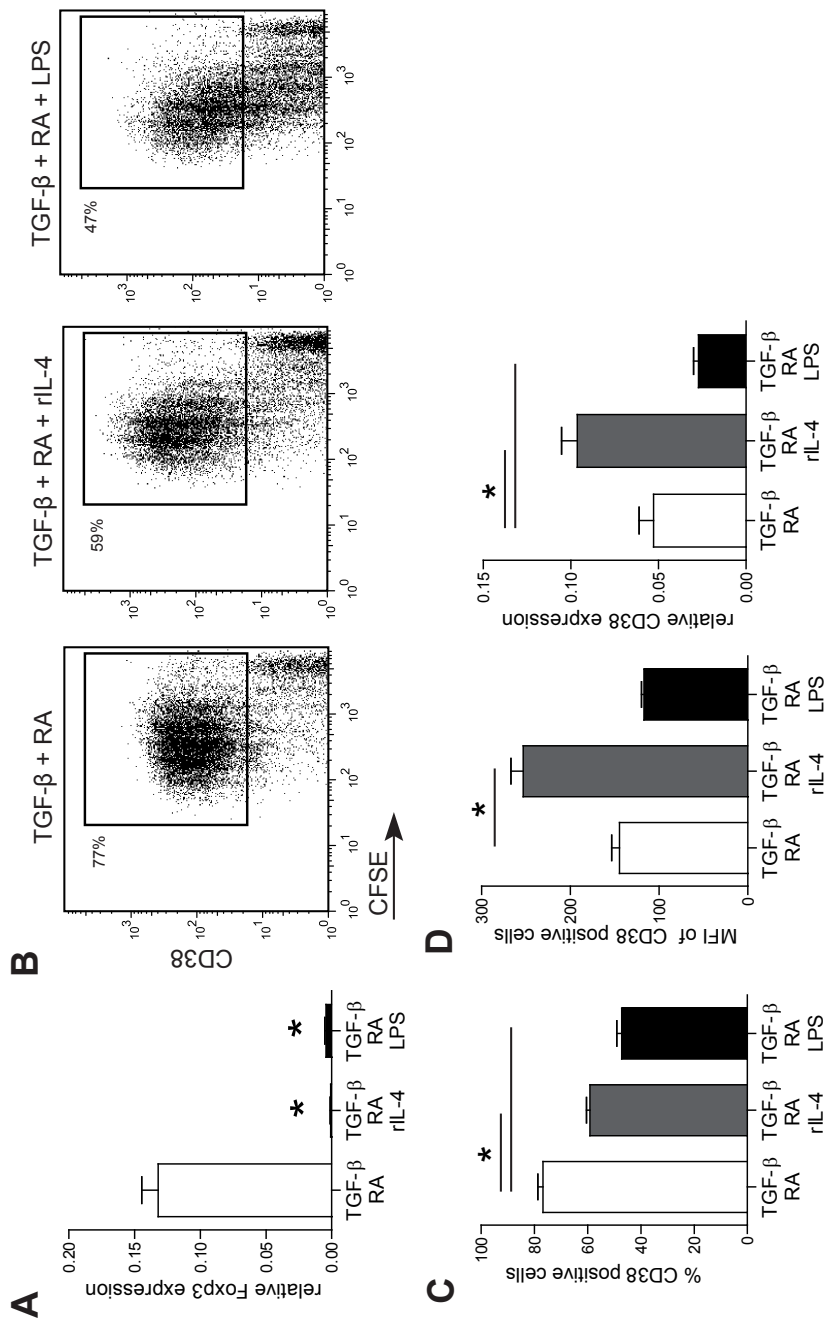
Figure 5.4 TGF-β and RA-induced CD38 is maintained when Foxp3 conversion is reduced. OVA-peptide loaded murine MLN-DC and CFSE⁺CD4⁺KJ1.26⁺ T cells were cultured with TGF-β and RA. rIL-4 and LPS were added to inhibit Foxp3⁺ differentiation. **(A)** Relative expression of Foxp3 mRNA (n=3). **(B)** CD38 expression by flow cytometry. **(C)** The percentage of CD38⁺ cells (n=3). **(D)** MFI of cells within the CD38⁺ gate and relative expression of CD38 mRNA (n=3). * Statistically significant (P<0.05). DC, dendritic cell; IL, interleukin; LPS, lipopolysaccharide; MFI, mean fluorescence intensity; MLN, mesenteric lymph nodes; OVA, ovalbumin; RA, retinoic acid; TGF, transforming growth factor.

were found mainly in the CD4$^+$CD62L$^+$CD38$^+$ cell subset (Figure 5.5 D). Most cells within both CD4$^+$CD62L^{neg} subsets were CD45RA^{neg} (Figure 5.5 D). Moreover, both CD62L^{neg}CD38$^+$ and CD62L^{neg}CD38^{neg} contained similar number of CD25$^+$ cells and had the capacity to secrete IFN-γ upon restimulation indicating that both populations consist mainly of effector memory cells (not shown). In order to determine whether the CD4$^+$CD62L^{neg}CD38$^+$ subset could be used to identify T cells with mucosal origin in the periphery, we measured expression of CCR9 and β$_7$-
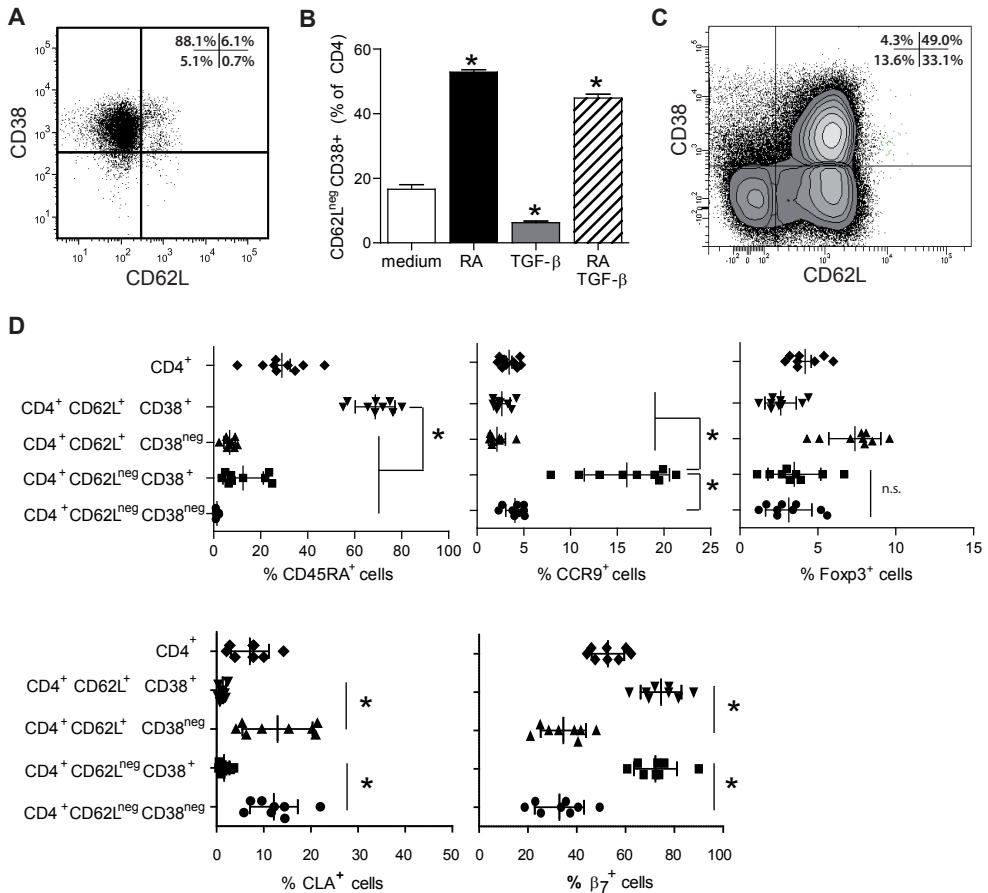
Figure 5.5 CD62L^{neg}CD38$^+$ identifies human antigen-experienced mucosal T cells. **(A)** Duodenal CD62L and CD38 expression in individuals with normal histology. Representative dotplot gated on total CD4$^+$ T cells. **(B)** PBMC from healthy individuals (n=8) were cultured with anti-CD3/anti-CD28 in the presence of TGF-β, RA or medium. The percentage of CD62L^{neg} CD38$^+$ within the CD4 population is shown. Baseline percentages of CD62L^{neg} CD38$^+$ before the start of the culture were (mean 5.69 ± SD 2.17). **(C)** Flow cytometry of CD62L and CD38 in freshly isolated (non-stimulated) peripheral blood cells (gated on CD4$^+$ cells). **(D)** Percentages of CD45RA$^+$(n=9), CCR9$^+$(n=9), Foxp3$^+$(n=9), β$_7$-integrin$^+$ (n=8) and cutaneous leukocyte-associated antigen (CLA$^+$) cells (n=8) within CD4$^+$ T-cell gate and CD62L/CD38 CD4$^+$ T-cell subsets. Error bars indicate SD. * Statistically significant (*P*<0.05). PBMC, peripheral blood mononuclear cells; RA, retinoic acid; TGF, transforming growth factor.

integrin as indicative for mucosal imprinting. There was a pronounced enrichment for CCR9$^+$ cells and β_7-integrin$^+$ cells within the CD4$^+$CD62L^{neg}CD38$^+$ population (Figure 5.5 D). Conversely, measurement of cutaneous leukocyte-associated antigen revealed that these non-mucosal cells are underrepresented in the CD4$^+$CD62L^{neg}CD38$^+$ subset (Figure 5.5 D). Foxp3$^+$ cells were present within the CD4$^+$CD62L^{neg}CD38$^+$ subset, however there was no enrichment of Foxp3$^+$ T cells when compared with the CD4$^+$CD62L^{neg}CD38^{neg} subset (Figure 5.5 D). This may reflect that peripheral Foxp3 induction occurs in mucosally imprinted (CD38$^+$) as well as non-mucosally imprinted (CD38^{neg}) effector T cells (CD62L^{neg}). In contrast, the Foxp3$^+$ population in the CD62L$^+$CD38$^+$ subset would consist of naturally-occurring Foxp3$^+$ T cells.

Gluten–specific T cells are mainly detected in the CD62L^{neg}CD38$^+$ CD4$^+$ T–cell subset

Finally, we wished to examine a human disease to demonstrate that the CD62L^{neg}CD38$^+$ T-cell phenotype can be used to detect gut-imprinted T cells. Gluten-specific T cells, restricted by the disease-associated DQ2 molecule and likely involved in the formation of coeliac lesion in the small intestine, can be cultured from the duodenal mucosa of coeliac patients but not of healthy individuals (23). In blood, these gluten-specific T cells can only be detected after a short dietary gluten challenge in CD patients that have been restricted to a gluten-free diet (17, 24). To establish that the CD62L^{neg}CD38$^+$ T-cell population indeed contains gluten-specific T cells, six treated adult CD patients were recruited and ate four slices of gluten-containing bread daily for 3 days. At day 6, CD4$^+$ PBMC were isolated and stained with both control- and gluten-tetramers (DQ2-αI and DQ2-αII), as well as antibodies against CD62L, CD38 and CCR9. In agreement with earlier observations (17), in four of the 6 patients, 0.01–0.1 % of the CD4$^+$ T cells bound the tetramers (Figure 5.6 A). In one patient, no gluten-specific cells were detected by tetramer staining. In another patient, the background staining with the control tetramer was very high precluding proper analysis. The majority of the tetramer$^+$ T cells had a CD62L^{neg}CD38$^+$ phenotype, as demonstrated in the analysis of these cells from the four remaining patients (Figure 5.6 B, C). Surprisingly, although most gluten-specific T cells were shown to express the β_7-integrin (17), CCR9 was expressed by only 10–30% of tetramer$^+$ cells (Figure 5.6 C). Taken together, these results suggest the use of the CD62L^{neg}CD38$^+$ T-cell phenotype to characterize ongoing mucosal immune responses in peripheral blood of patients with chronic intestinal disease.

No difference in the percentage of circulating CD62L^{neg}CD38$^+$ CD4$^+$ T cells in patients with chronic intestinal disease

To examine whether the percentage of circulating CD62L^{neg}CD38$^+$ CD4$^+$ T cells changed in patients with chronic intestinal disease we first included 34 patients with active CD and 21 control patients with normal duodenal histology. CD patients had a Marsh score of Marsh I (4 patients), Marsh IIIA (8 patients), Marsh IIIB (15 patients) or Marsh IIIC (7 patients). Patients
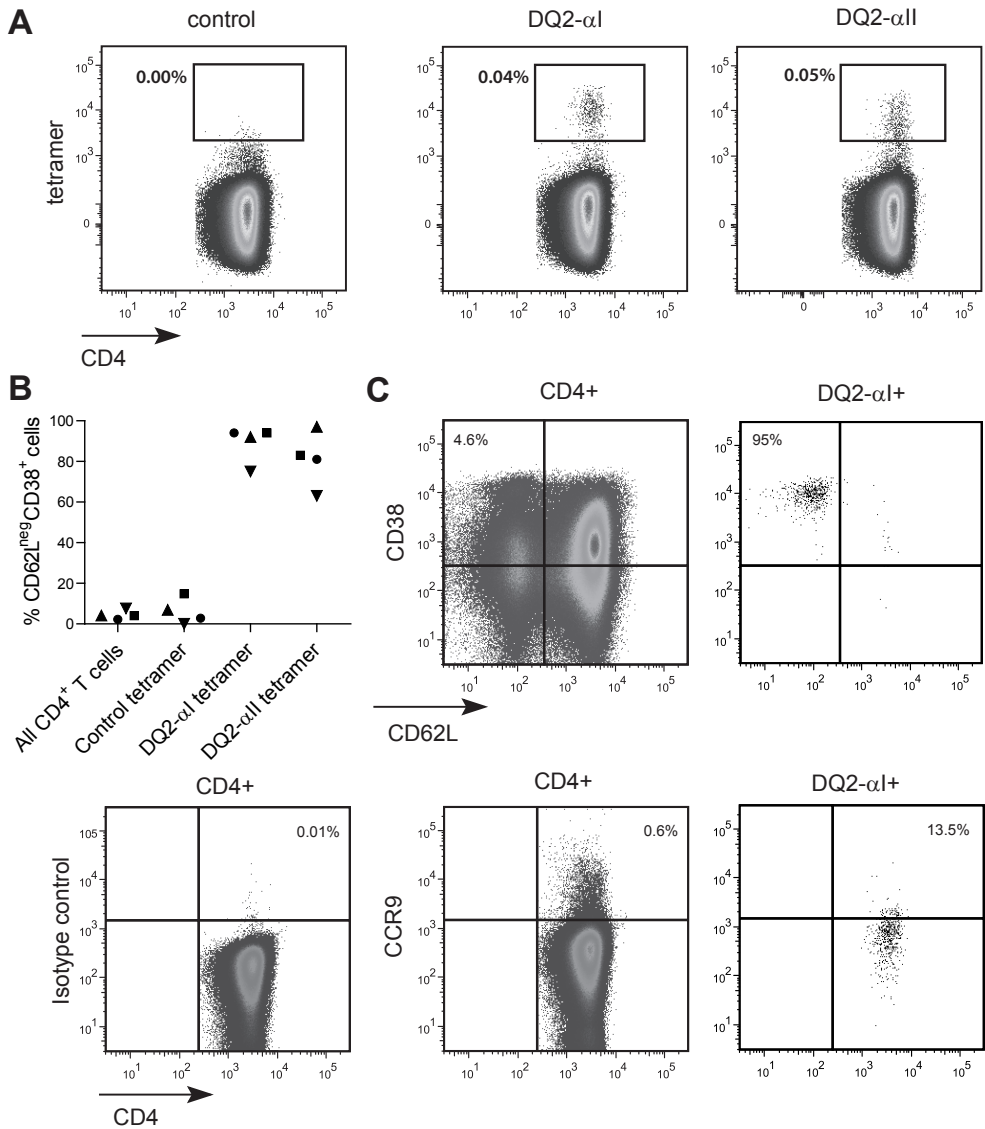
Figure 5.6 Gluten-specific T cells are mainly detected in the CD4+CD62L^neg CD38+ subset. Blood was collected from six adult CD patients after a 3-day bread challenge. CD4+ PBMC were isolated and gluten-specific T cells were detected by tetramer staining. **(A)** One representative FACS plot showing the percentage of DQ2-control tetramer, DQ2-αI and DQ2-αII positive tetramer cells within CD4+ gate. **(B)** Tetramer-positive cells of four CD patients were analyzed for CD62L and CD38. The percentages of CD62L^neg CD38+ cells within total CD4+ T-cell gate, control tetramer stained cells and DQ2-αI and DQ2-αII tetramer+ cells were calculated. **(C)** Flow cytometry of one representative patient. Staining of CD62L and CD38 of total CD4+ PBMC (upper left) and DQ2-αI tetramer+ cells (upper right). Staining of isotype control on total CD4+ PBMC (lower left), CCR9 of total CD4+ PBMC (lower middle) and DQ2-αI tetramer+ cells (lower right). * Statistically significant ($P<0.05$). CD, coeliac disease; PBMC, peripheral blood mononuclear cells.

who were diagnosed with other diseases were excluded from analysis. There were no differences in age and gender between pediatric CD patients and control patients. Upon analysis of the CD62L^{neg}CD38$^+$ CD4$^+$ mucosal T-cell population no differences in the percentages of the cells were found between pediatric CD patients and controls (Figure 5.7 A). This finding may have been expected as previous studies have shown that circulating gliadin-reactive T cells can only be detected in patients that have received a gluten challenge (17, 24).

Similarly, the percentage of CD62L^{neg}CD38$^+$ CD4$^+$ mucosal T-cells was also determined in peripheral blood of IBD patients. As such 21 Crohn's disease patients, 8 Ulcerative Colitis
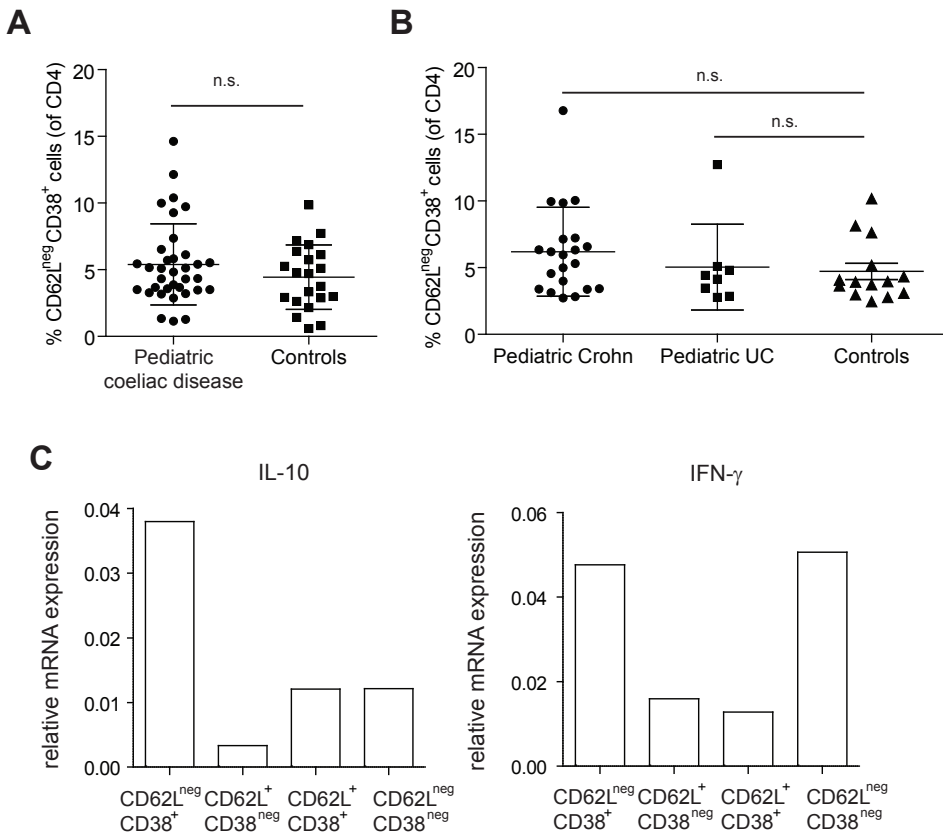
Figure 5.7 No difference in the percentage of circulating CD62L^{neg}CD38$^+$ CD4$^+$ T cells in patients with chronic intestinal disease. Peripheral blood from pediatric CD patients (A) IBD patients (B) and controls was, stained for CD4, CD62L and CD38 and subsequently analyzed by flow cytometry. **(A)** The percentage of CD62L^{neg}CD38$^+$ mucosal CD4$^+$ T cells in peripheral blood of CD patients (n=34) and controls (n=21). **(B)** The percentage of CD62L^{neg}CD38$^+$ mucosal CD4$^+$ T cells in peripheral blood of Crohn's disease (n=21), Ulcerative colitis patients (n=8) and controls (n=14). **(C)** Peripheral blood CD4$^+$ cells from a healthy donor were cell sorted into four CD62L/CD38 subsets and restimulated with PMA (0.05 µg ml^{-1}) and ionomycin (0.5 µg ml^{-1}) during 5 h. IL-10 (left) and IFN-γ (right) expression was determined relative to GAPDH. * Statistically significant (*P*<0.05). n.s. denotes non significant. CD, coeliac disease; IBD, inflammatory bowel disease; IFN, interferon; IL, interleukin; UC, ulcerative colitis.

patients and 14 age matched orthopedic control patients with no underlying inflammatory or intestinal diseases were investigated. In agreement with the data from the coeliac patients no significant differences in the percentage of CD62L^neg CD38+ CD4+ mucosal T-cells were observed (Figure 5.7 B).

From these data we conclude that the total percentage of circulating CD62L^neg CD38+ in the CD4+ T-cell pool is not an indicator of intestinal inflammation. This absence of quantitative differences in circulating lymphocytes is a phenomenon that is not restricted to intestinal disease but also seen in other chronic mucosal diseases, such as chronic pulmonary disease. However, in chronic obstructive pulmonary disease (COPD), significant advantages have been made by correlating peripheral T-cell cytokine profiles with severity of disease (25). Therefore, we examined whether sorted CD4+CD62L^neg CD38+ T cells from healthy donors exhibited detectable cytokine profiles by quantitative PCR. Indeed, in healthy controls IL-10 and IFN-γ transcripts were readily detected in highly purified CD4+CD62L^neg CD38+ T cells, demonstrating that functional analysis is feasible (Figure 5.7 C). Moreover, the new phenotype allowed us to compare CD62L^neg CD38+ mucosal effector T-cell function with peripheral CD62L^neg CD38^neg effector T-cell responses within the same individual, as upon analysis CD4+CD62L^neg CD38^neg T cells also contained IFN-γ after restimulation.

DISCUSSION

Here we identify the CD62L^neg CD38+ phenotype as a subset of CD4+ T cells that have received mucosal imprinting. Our data demonstrate that endogenous RA produced by MLN-DC is, together with TGF-β, a crucial factor in the differentiation towards the CD62L^neg CD38+ phenotype. Using a mouse model of mucosal tolerance we show that dividing T cells in the MLN are CD62L^neg CD38+, whereas this phenotype is not induced upon T-cell activation in PLN. This restricted population of mucosally-activated T cells was found to be conserved between mice and humans, as RA also induces the CD62L^neg CD38+ phenotype on human PBMC. The analysis of CD4+CD62L^neg CD38+ T cells in human peripheral blood revealed a strong enrichment of CCR9+ cells confirming mucosal imprinting. Moreover, in CD patients gliadin-specific T cells predominated in the CD4+CD62L^neg CD38+ T-cell pool. These data reveal that combined cell-surface expression of CD62L^neg and CD38 can be used to identify mucosal CD4+ T cells in peripheral blood.

Increased prevalence of T cells with either a CD62L^neg or a CD38+ phenotype in the gut has been observed in several studies (26-30). For example, it was previously shown that all intraepithelial lymphocytes in the murine small intestine, but not in the colon, have a CD62L^neg phenotype (26). Regarding CD38, it has been reported that human lamina propria T cells express CD38 at higher levels than circulating T cells (27). In addition, the majority of CD4+CD25+ T cells in the lamina propria, but not in the MLN or the spleen, lack expression of CD62L (27). It is unlikely that the CD62L^neg phenotype of lymphocytes in mucosal tissues is the result of

continuous activation as we show that T-cell activation induced by i.m. protein injection does not induce this phenotype. For CD8$^+$ T cells, CD62L expression can depend on the initial frequency of naive T cells and on the strength of the T-cell activation (31). In our *in vivo* model transfer of various numbers of T cells, resulting in different APC – T-cell ratios, did not affect CD62L expression. We argue that the mucosal microenvironmental factors RA and TGF-β, that can be secreted by mucosal DC (19, 32), modulate the expression of CD62L upon intestinal T-cell activation. In particular, Seibold et al. have demonstrated that microenvironmental factors induce shedding of CD62L in the small intestine (26).

CD38 expression could reflect a regulatory T-cell phenotype, as it is induced by RA and TGF-β, two factors that also mediate the differentiation of Foxp3$^+$ Treg cells. Indeed, we demonstrate that in mice, expression of CD38 correlates with that of Foxp3, as the percentage of CD38$^+$ cells is increased in *de novo* induced Foxp3$^+$ Treg cells when compared with Foxp3 negative cells. In agreement, CD38$^+$ T cells have suppressive capacity in vitro (29, 33). Moreover, in NOD x CD38$^{-/-}$ mice a modest reduction was detected both in the number of CD4$^+$CD25$^+$ Treg cells and Foxp3 mRNA (34). However, our data show that CD38 does not exclusively identify mucosal Treg cells, as the CD62L^{neg}CD38$^+$ phenotype is maintained when Foxp3 expression is inhibited. In particular, *in vivo* abrogation of mucosal tolerance by administration of OVA together with the adjuvant CT significantly reduced the number of Foxp3$^+$ T cells in the MLN. *In vitro* Treg cell-differentiation assays confirmed this finding, as the proportion of TGF-β and RA-induced CD38$^+$ T cells did not decrease upon complete inhibition of Foxp3 expression. Taken together, our data indicate that expression of CD38 reflects TGF-β- and RA-dependent imprinting by mucosal APC rather than a Treg-cell function. As Foxp3 was normally induced on polyclonally-stimulated CD4$^+$ T cells from CD38$^{-/-}$ mice by TGF-β and RA (data not shown), it seems unlikely that CD38 is essential for the generation of Foxp3$^+$ Treg cells *in vitro*.

Although an association between RA and CD38 was reported for human leukemia cell lines and in B cells (35-39), we are the first to report that RA induces CD38 expression in primary T cells. The role of CD38 in T-cell differentiation is unclear. While there are no published reports demonstrating a functional role for CD38 on T cells, unpublished findings show that T cells from CD38$^{-/-}$ mice do exhibit intrinsic functional defects (observations Frances Lund). Nevertheless, there is no evidence that these mice develop an intestinal pathology. It has been demonstrated that CD38 plays an important role in B-cell responses (40). Furthermore, CD38 plays a crucial role in the migration of DC (41) and therefore one could question a role for CD38 in the migration of RA- and TGF-β-imprinted CD62L^{neg}CD38$^+$ T cells to the small-intestinal mucosa. Moreover, the strength of the APC – T-cell interaction may be influenced by the expression levels of CD38 on T cells, as human CD38 associates with the T-cell receptor (42) and CD38 can be recruited to the immunologic synapse upon antigen-mediated T-cell activation (43).

Possibly, CD62L^{neg}CD38$^+$ imprinting extends to other mucosal tissues as CD62L^{neg}CD38$^+$ T cells were detected in human tonsils. Moreover, in mice four months after mucosal influenza infection all influenza-specific T cells were CD62L^{neg}CD38$^+$ (observations Frances Lund).

Additional research will address this issue and focus on the expression CD62L^{neg}CD38$^+$ on CD8$^+$ T-cell responses.

The value of defining this new subset lies in lowering the threshold for detection of luminal antigen specific T cells within the circulating CD4$^+$ T-cell pool. By selecting for CD62L^{neg}CD38$^+$ expression which comprises 5–10% of the cells within the total CD4$^+$ T-cell pool it is now possible to highly enrich for effector T cells with specificity for mucosal antigens. This is of pivotal importance for functional studies as this method removes 90–95% of CD4$^+$ non-gut imprinted effector T cells and naive T cells from the analysis. Indeed, in healthy controls IL-10 and IFN-γ transcripts were readily detected in highly purified CD62L^{neg}CD38$^+$ CD4$^+$ T cells demonstrating that functional analysis is feasible. Moreover, the new phenotype allows us to compare CD62L^{neg}CD38$^+$ mucosal effector T-cell function with peripheral CD62L^{neg}CD38^{neg} effector T-cell responses within the same individual as upon analysis CD4$^+$CD62L^{neg}CD38^{neg} T cells also contained IFN-γ after restimulation. We propose that future analysis of the anti-inflammatory and proinflammatory cytokine profile of this cellular subtype in different patient cohorts should ameliorate disease profiling in chronic intestinal disease. Currently most of these studies are performed with total PBMC. By preselecting for gut-imprinted cells instead of restimulating total PBMC the threshold of detection of T-cell responses to luminal antigens is lowered and the specificity of the readout is enhanced.

Overall better functional analysis of mucosally-imprinted T cells in the periphery may have important consequences for subtyping IBD and CD patients and help evaluating therapy responsiveness.

ACKNOWLEDGEMENTS

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CHAPTER 5

17. Raki, M., Fallang, L.E., Brottveit, M., Bergseng, E., Quarsten, H., Lundin, K.E., and Sollid, L.M. 2007. Tetramer visualization of gut-homing gluten-specific T cells in the peripheral blood of celiac disease patients. *Proc Natl Acad Sci U S A* 104:2831-2836.

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SUPPLEMENTARY FIGURES
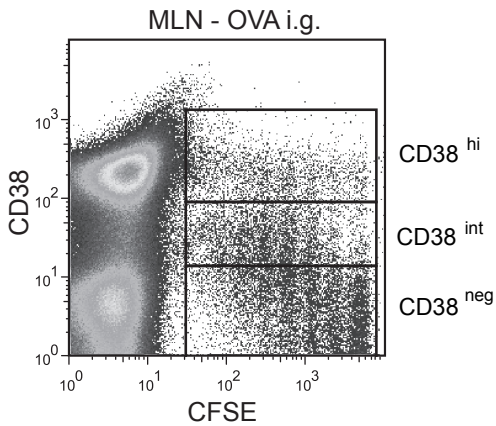
Supplementary Figure 5.1   Gating of CD38int and CD38hi T cells. 6 x 10^6 CFSE-labeled CD4$^+$KJ1.26$^+$ cells were transferred to naive BALB/c mice. One day after transfer, acceptor mice received 70 mg OVA i.g. At 72 h post OVA, single-cell suspensions of draining MLN were prepared and stained for CD38. Gating of CD38^{int} and CD38^{hi} on CFSE$^+$ CD4$^+$KJ1.26$^+$ cells was determined based on CD38 expression on non-transgenic lymphocytes (mainly B cells) within the same LN. i.g., intragastric; MLN, mesenteric lymph nodes; OVA, ovalbumin.
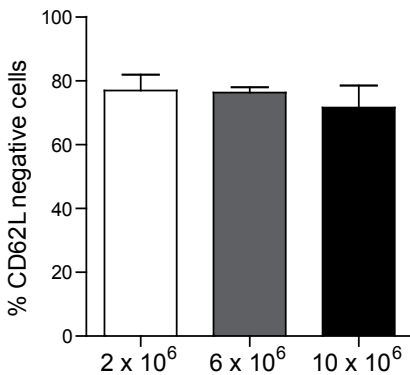
Supplementary Figure 5.2   Expression of CD62L is not dependent on DC – T-cell ratio. 2-10 x 10^6 CFSE-labeled CD4$^+$KJ1.26$^+$ cells were transferred to naive BALB/c mice. One day after transfer, acceptor mice received 70 mg OVA i.g. At 72 h post OVA, single-cell suspensions of draining MLN were prepared and the percentage of CD62L^{neg} cells within CFSE$^+$CD4$^+$ KJ1.26$^+$ cells was determined (n=3, mean ± SD). DC, dendritic cell; i.g., intragastric; MLN, mesenteric lymph nodes; OVA, ovalbumin.

PLN - OVA i.m.
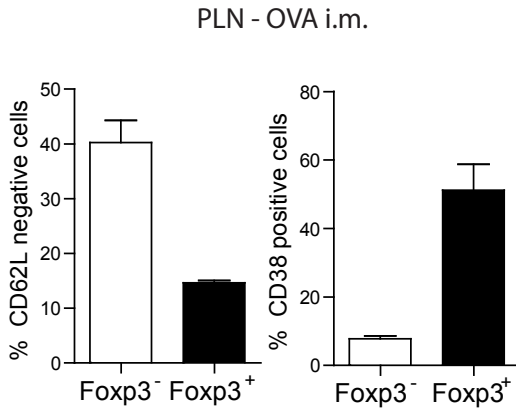
Supplementary Figure 5.3 Expression of CD38 is preferentially induced by mucosal DC in a TGF-β- and RA-dependent manner. 5 x 10^{6} CFSE-labeled CD4+KJ1.26+ cells were transferred to naive BALB/c mice. One day after transfer, acceptor mice received 400 μg OVA i.m. in each hind limb. At 72 h post OVA, single-cell suspensions of draining PLN were prepared and analyzed for expression of Foxp3, CD62L and CD38. Quantitative analysis of CD62L^{neg} and CD38+ cells within Foxp3+CD4+KJ1.26+ and Foxp3-CD4+KJ1.26+ gated cells is shown (n=3, mean ± SD). DC, dendritic cell; i.m., intramuscular; OVA, ovalbumin; PLN, peripheral lymph nodes; RA, retinoic acid; TGF, transforming growth factor.

Characterization of T–cell subsets in peripheral blood of pediatric and adult coeliac disease patients

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Marieke A. van Leeuwen, Greetje J. Tack, Chris Mulder,
Lissy de Ridder, Johanna C. Escher, Janneke N. Samsom
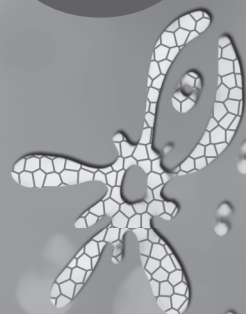
ABSTRACT

Coeliac disease (CD) is a chronic inflammation of the small intestine resulting from an intolerance to dietary gluten proteins. A central role for CD4+ T cells in the pathogenesis of CD is well established, however it is unclear why this inflammatory response is not regulated. Defects in regulatory T (Treg)-cell subsets have been proposed to be responsible for the unrestricted inflammatory T-cell response in CD. The aim of this study was to determine whether alterations in the naturally-occurring CD62L+Foxp3+ T-cell population or in mucosally-induced CD62L^{neg}CD38+ Foxp3+ Treg cells are involved in the pathogenesis of CD. Thereto, subpopulations of peripheral blood leukocytes from pediatric and adult patients were investigated by flow cytometry using surface marker expression and intracellular staining for forkhead box P3 (Foxp3). In children we compared active newly-diagnosed CD patients with non-CD control patients in different age groups. In adults, patients with active CD, treated CD and refractory CD (RCD) were compared with healthy volunteers. No differences in the percentage of CD4+ mucosally-induced CD62L^{neg}CD38+ T cells could be detected between any group of patients analyzed. Moreover, the percentages of Foxp3+ T cells within the mucosally-induced CD62L^{neg}CD38+ T-cell group were comparable between patients and controls. However, in comparison with healthy individuals, a higher percentage of circulating naturally-occurring CD62L+Foxp3+ T cells was observed in adult patients with active disease and in patients with RCD. Adult treated CD patients and newly-diagnosed untreated pediatric CD patients had percentages of Foxp3+ T cells that were similar to controls. These data indicate that the population of peripheral blood CD62L^{neg}CD38+ Foxp3+ Treg cells has a normal frequency in CD patients. We demonstrate that the loss of tolerance to gluten in CD patients is associated with changes in percentages of naturally-occurring Foxp3+ T cells in adult active CD and RCD only.

INTRODUCTION

Coeliac disease (CD) is a chronic inflammatory disease of the small intestine that develops in genetically susceptible individuals in response to the ingestion of gluten proteins from wheat, barley and rye. The disease occurs in children and adults in approximately 1% of the population and is the most common intolerance to a food protein (1). A large part of the genetic susceptibility is explained by HLA genes: over 90% of CD patients carry HLA-DQ2, while most of the HLA-DQ2-negative patients carry HLA-DQ8 (2). The association with these HLA-molecules infers a role for CD4+ T cells in the pathogenesis of the disease. Indeed, inflammatory gluten-specific CD4+ T cells that are exclusively restricted to the HLA-DQ2 or HLA-DQ8 molecules can be isolated from the small-intestinal mucosa of CD patients but not of healthy individuals (3-5). These inflammatory gluten-specific T cells produce high amounts of interferon (IFN)-γ (6, 7) and are believed to be key contributors to the development of the coeliac lesion, which is characterized by villous atrophy, crypt hyperplasia and increased numbers of infiltrating lymphocytes in both the epithelium and in the lamina propria.

Currently, the only cure for CD is a life-long gluten-free diet (GFD), resulting in complete remission and recovery of the normal intestinal architecture. However, a severe complication occurs in a small proportion of CD patients who become unresponsive to the GFD and develop refractory coeliac disease (RCD) (8). Patients are regarded as suffering from RCD when clinical

and histological symptoms persist or recur after a former good response to a strict GFD. A subgroup of RCD patients (type II), which is characterized by the presence of aberrant populations of T cells lacking the surface expression of CD3, has a high risk to develop an enteropathy-associated T-cell lymphoma.

Despite our increasing knowledge of the pathogenesis of CD, it is still unclear why tolerance to gluten is so often lost. In the healthy intestine, harmless food proteins are generally well tolerated by the intestinal immune system while an inflammatory immune response is only mounted against pathogenic microorganisms. The default immune response to orally encountered antigens is oral tolerance (9). Oral tolerance has been extensively studied in mouse models and can be mediated by the differentiation of antigen-specific forkhead box P3 (Foxp3)$^+$ regulatory T (Treg) cells (10-14). These inducible Foxp3$^+$ Treg cells differentiate from naive T cells under the control of the tolerogenic microenvironment of the gut and are different from naturally-occurring Treg cells that undergo differentiation in the thymus. A defect in the differentiation of inducible gluten-specific Treg cells is a possible explanation for the loss of tolerance to gluten in CD. Up till recently, lack of specific cell-surface markers to distinguish mucosally-induced and naturally-occurring Treg cells hampered the study of inducible antigen-specific Treg cells to food proteins.

We have recently demonstrated that mucosally-induced CD4$^+$ T cells can be identified by the expression of CD62L^{neg}CD38$^+$ (Chapter 5). The significance of this phenotype to identify mucosally-activated T cells was validated in a study of patients with CD. To allow the detection of gluten-specific T cells in peripheral blood, CD patients who are normally on a GFD diet were asked to eat four slices of gluten-containing bread daily for 3 days (15, 16). Visualization of gluten-specific T cells using tetramers revealed that the vast majority of gluten-specific T cells had a CD62L^{neg}CD38$^+$ phenotype (Chapter 5).

In this study, we characterized CD4$^+$CD62L^{neg}CD38$^+$ mucosal T cells in pediatric and adult patients with active CD, as well as adult CD patients after treatment with a GFD, adult patients with RCD and in controls. Moreover, we analyzed the percentage of Foxp3$^+$ Treg cells in the general CD4$^+$ T-cell population and within the CD62L^{neg}CD38$^+$ mucosal T-cell subset.

METHODS

Patients

Pediatric patients who underwent an esophago-gastro-duodenoscopy (EGD) with suspicion of CD in the Erasmus MC – Sophia Children's Hospital, Rotterdam, The Netherlands, were approached for participation in our study. Children with biopsy-proven CD were included in the patient group whereas children with a normal intestinal histology were included in the control group. Patients diagnosed with other diseases were excluded from the study.

Adult patients with active CD, treated CD (i.e. CD patients responding to a GFD) and RCD (i.e. CD patients not responding to a gluten-free diet) from the VUMC, Amsterdam, The

Netherlands were included in the study. In our analyses, we have chosen to combine patients with RCD type I (7/11) and RCD type II (6/11). However, it should be noted that the clinical features of these two disease subtypes are very different. All RCD patients had an earlier diagnosis of RCD and had a history of treatment with immunomodulatory drugs, including cladribine or 6-thioguanine.

The studies were approved by the local medical ethical committee, and all participants or parents of participants gave written informed consent before enrollment.

White blood cell count

Peripheral blood samples were collected by means of venipuncture in 3 ml EDTA vacutainer tubes (BD-Pharmigen, Woerden, The Netherlands). For the pediatric patients, blood was obtained from the peripheral infuse line that was used for administration of anesthetics during the EGD procedure. The total number of white blood cells (WBC) per liter peripheral blood was determined with a Coulter counter (Coulter Electronics, Mijdrecht, The Netherlands).

Flow cytometry

After erythrocyte lysis, whole blood samples were stained for flow cytometry using monoclonal antibodies against CD3 (HIT3α), CD4 (RPA-T4), CD38 (HIT2), CD62L (DREG-56, all BD-Pharmingen), CD45RA (MEM-56, Invitrogen, Breda, The Netherlands), Foxp3 (PCH101, EMELCA Bioscience, Bergen op Zoom, The Netherlands), CCR9 (248621, R&D Systems, Abingdon, UK) and appropriate isotype controls. Flow-cytometric analysis was performed on a FACScanto (BD-Biosciences).

Statistics

Linear regression analysis was performed using Prism software (GraphPad, Software Inc). Differences between groups were analyzed using the Mann Whitney U test. $P<0.05$ was considered statistically significant.

RESULTS

Analysis of CD4$^+$CD62L^{neg}CD38$^+$ and Foxp3$^+$ T cells in peripheral blood of pediatric CD patients and controls

Blood was drawn from children aged 1 to 17 years who underwent an EGD with a suspicion of CD. We included 34 patients with active CD and 21 control patients with normal duodenal histology. CD patients were diagnosed with Marsh I (4 patients), Marsh IIIA (8 patients), Marsh IIIB (15 patients) or Marsh IIIC (7 patients; Table 6.1). Patients who were diagnosed with other diseases were excluded from analysis. There were no differences in age and gender between pediatric CD patients and control patients (Table 6.1). The group of CD patients had a significantly higher WBC count in comparison with the control group (Figure 6.1).

Table 6.1 Demographic features of pediatric coeliac disease patients and controls

		Pediatric CD	Controls
Number		34	21
Age in years, mean (SD)		5.5 (4.5)	5.9 (4.2)
Boys, % (n)		29 (10)	33 (7)
Marsh Score, % (n)	Marsh I	12 (4)	N/A
	Marsh II	0 (0)	N/A
	Marsh IIIA	24 (8)	N/A
	Marsh IIIB	44 (15)	N/A
	Marsh IIIC	21 (7)	N/A

CD, coeliac disease; N/A, not applicable.
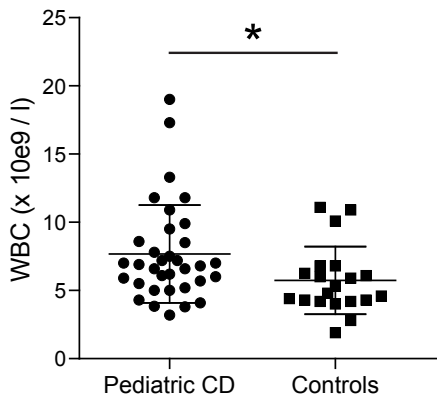
Figure 6.1 White blood cell count is increased in pediatric CD patients. WBC counts were determined in peripheral blood from pediatric CD patients (n=34) and controls (n=21). * Statistically significant (P=.020, Mann Whitney test). CD, coeliac disease; WBC, white blood cell.

As the distribution of lymphocyte subsets in peripheral blood varies with age (17), we first determined whether the distribution of CD4+ T cells within the CD62L and CD38 T-cell subsets varied with age. Gating of CD4+ T cells based on the expression of CD62L and CD38 was performed as shown in Figure 6.2 A. CD4+CD62L+CD38+ T cells gradually decreased from a very high percentage (85.6% ± SD 5.02) in children between 1 and 2 years of age to a much lower and more variable percentage in children over the age of 6 (56.6% ± SD 18.02, Figure 6.2 B). The majority of CD4+ T cells in the CD62L+CD38+ T-cell subset expressed CD45RA (data not shown), indicating that this subset mainly consists of naive T cells. The relative decrease in CD62L+CD38+ T cells was associated with an increase in the percentage of CD62L^{neg}CD38^{neg} and CD62L+CD38^{neg} T cells, which both significantly increased with age (Figure 6.2 B). In contrast, the population of CD4+CD62L^{neg}CD38+ mucosal T cells was not subjected to age-related changes (Figure 6.2 B). Therefore, no age distinction was made for further analysis of the CD4+CD62L^{neg}CD38+ cells.

A

B
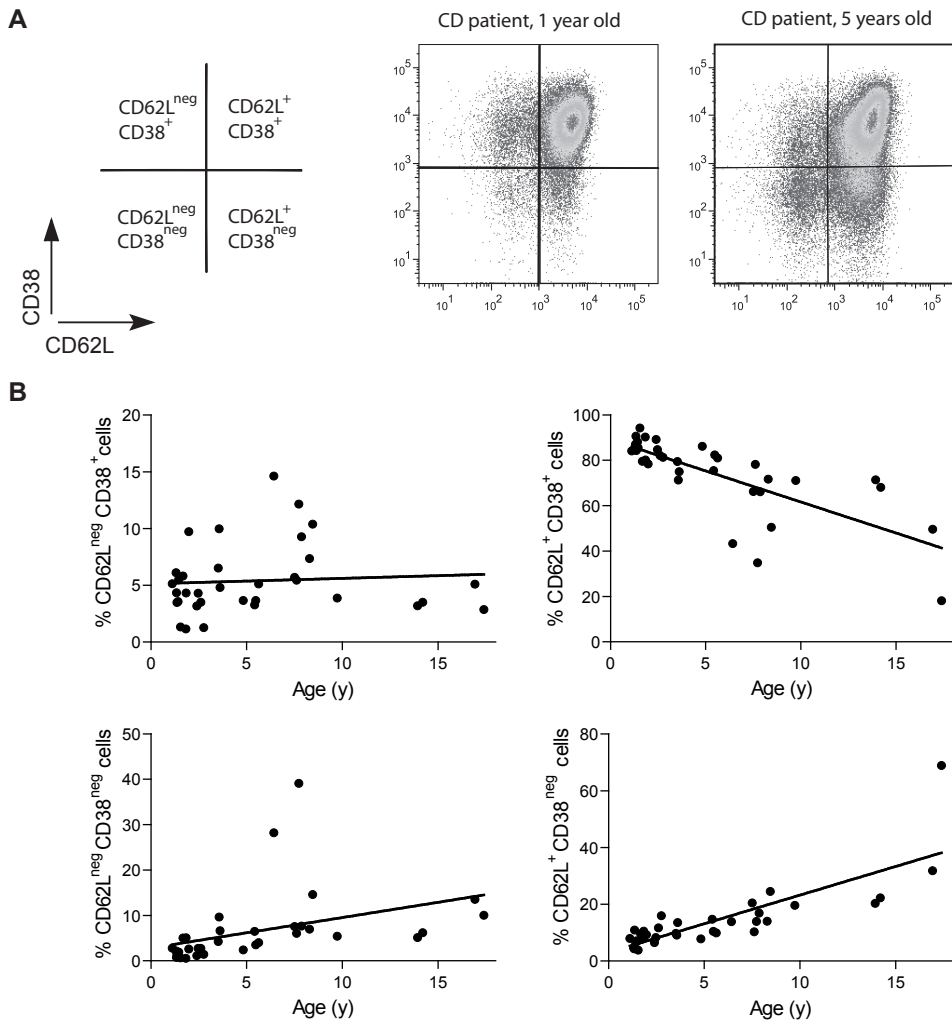
Figure 6.2 Expression of CD62L and CD38 on CD4+ peripheral blood lymphocytes changes with age. Peripheral blood was obtained from pediatric CD patients and stained for CD4, CD62L and CD38 for flow-cytometric analysis. **(A)** Gating of CD4+CD62L/CD38 T-cell subsets. Two representative density plots of CD patients aged 1 and 5 years old are shown. **(B)** The percentages of CD4+ T cells in each of the four CD62L/CD38 T-cell subsets were calculated. The proportion of CD62L^{neg}CD38+ CD4+ T cells does not change with age (r^2=.00, P=.692). The CD62L+CD38+ CD4+ T-cell subset decreases with age (r^2=.55, P<.0001), to the benefit of both the CD62L+CD38^{neg} and CD62L^{neg}CD38^{neg} CD4+ T-cell subsets (r^2=.64, P<.0001 and r^2=.15, P=.021, respectively). CD, coeliac disease.

In Chapter 5 we reported no differences in the percentage of thee CD4+CD62L^{neg}CD38+ mucosal T-cell population between pediatric CD patients and controls. We have previously demonstrated that expression of the gut-homing chemokine receptor CCR9 is highly enriched in the CD4+CD62L^{neg}CD38+ T-cell population in peripheral blood of healthy adults (Chapter 5). Flow-cytometric analysis revealed that this also applied to cells from pediatric CD patients

as well as to the controls (data not shown) and that the percentage of CCR9$^+$ cells within the CD4$^+$CD62L^{neg}CD38$^+$ T-cell subset does not differ between the two groups (Figure 6.3 A). We next determined the expression of CD45RA to determine whether CD62L^{neg}CD38$^+$ T cells from pediatric patients and controls differ in the proportion of naive (CD45RA$^+$) and memory (CD45RA$^-$) T cells. However, as shown in Figure 6.3 B, a mean of 20% naive T cells was observed in CD62L^{neg}CD38$^+$ T cells from both CD patients and controls.

No differences in Foxp3 expression in the CD62L^{neg}CD38$^+$ mucosal T-cell subset were detected, indicating that there is no defect in the frequency of mucosally-induced Treg cells in peripheral blood of pediatric CD patients (Figure 6.3 C). In addition, we observed no differences in Foxp3$^+$ cells as a percentage of the total CD4$^+$ T-cell population, indicating that the naturally-occurring Foxp3$^+$ Treg-cell population is also not decreased in pediatric CD patients (Figure 6.3 D).
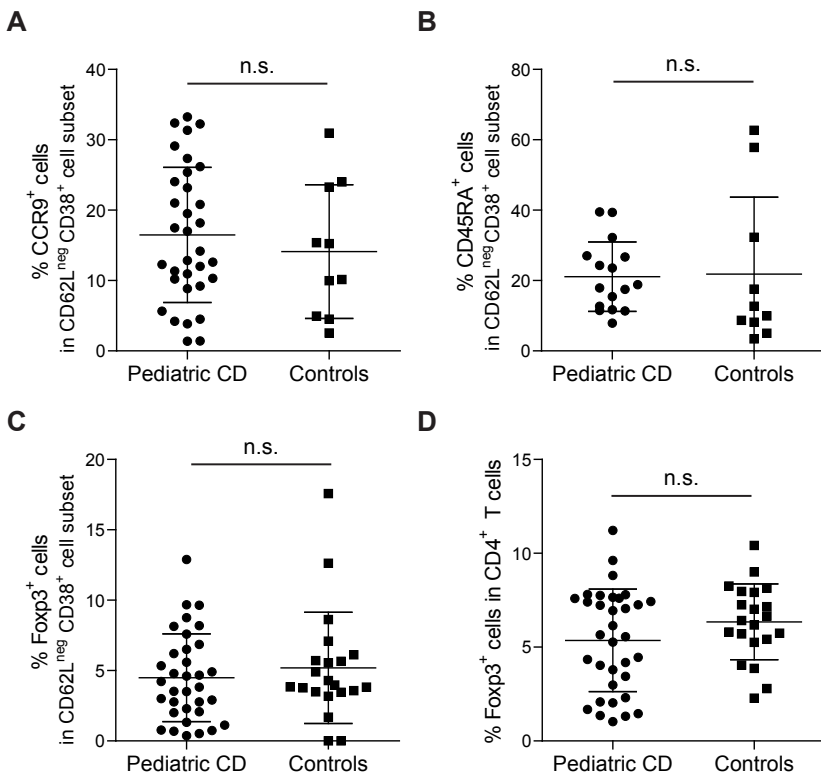
Figure 6.3 Analysis of T-cell subsets in pediatric CD patients and controls. Peripheral blood from pediatric CD patients and controls was, after erythrocyte lysis, stained for CD4, CD62L, CD38, CCR9, CD45RA and Foxp3 and analyzed by flow cytometry. **(A)** The percentage of cells staining positive for CCR9 within the CD62L^{neg}CD38$^+$ CD4$^+$ T-cell subset, no differences between pediatric CD patients (n=33) and controls (n=10). **(B)** Percentage of naive (CD45RA$^+$) cells within CD62L^{neg}CD38$^+$ CD4$^+$ T-cell is similar in pediatric CD patients (n=16) and controls (n=10). **(C)** The percentage of adaptive (mucosally induced) Foxp3$^+$CD62L^{neg}CD38$^+$ in peripheral blood is similar in pediatric CD patients (n=34) and in controls (n=21). **(D)** Analysis of total Foxp3$^+$ cells (gated on CD4$^+$ lymphocytes) shows no differences between pediatric CD patient group (n=34) and control group (n=21). n.s. not significant (Mann Whitney test). CD, coeliac disease.

Analysis of CD4$^+$CD62L^{neg}CD38$^+$ and Foxp3$^+$ T cells in peripheral blood of adult patients with active CD, treated CD or RCD and controls

Peripheral blood was obtained from adult patients with active CD (n=4), treated CD (n=14), patients diagnosed with RCD (n=13) and healthy controls (n=14). Demographic features of the patient groups and controls are depicted in Table 6.2. The active CD group consisted of three patients with newly-diagnosed CD and one patient who was diagnosed earlier with CD but was non-compliant with the GFD. All treated CD patients consumed a GFD for at least 6 months and, as a result, their Marsh scores had improved from Marsh IIIA-C at the time of diagnosis to a Marsh 0 or 1 at the time of blood sampling for this study. Patients whose condition was not responding to a GFD and who were diagnosed with RCD type I or type II were included in the RCD group. The latest available Marsh scores of RCD patients varied from Marsh 0 to Marsh IIIB (see Table 6.2). Restored mucosal architecture was seen in RCD patients who received treatment with immunomodulatory drugs, such as cladribine. No differences in the WBC count were detected between the different patient groups and controls (data not shown).

Similar to our findings with pediatric CD patients, we did not observe any significant differences in the percentage of total CD4$^+$ T cells nor in the distribution of CD4$^+$ T cells within the four CD62L/CD38 T-cell subsets in any of the treatment groups (Figure 6.4). As such, the frequency of mucosally-induced CD62L^{neg}CD38$^+$ T cells is not increased in CD patients with active small-intestinal inflammation. Moreover, no differences in the expression of CCR9 were observed (data not shown). However, it was noticed that the percentage of naive CD45RA$^+$CD62L$^+$CD38$^+$ CD4$^+$ T cells was noticeably lower in patients with RCD in comparison with healthy controls, possibly as a consequence of the treatment that these patients received (Figure 6.5).

Strikingly, a higher percentage of Foxp3$^+$ T cells was observed in the total CD4$^+$ T-cell population in patients with active CD and in patients with RCD in comparison with healthy controls (Figure 6.6). This difference was not observed for the percentage of CD4$^+$Foxp3$^+$ T cells in treated CD patients (Figure 6.6). Analysis of the different CD62L/CD38 T-cell subsets

Table 6.2 Demographic features of adult coeliac disease patients and healthy controls

		Active CD	Treated CD	Refractory CD	Healthy Controls
Number		4	14	13	14
Age in years, mean (SD)		46.5 (19.6)	47.5 (16.5)	62.6 (11.6)	36.5(11.5)
Male, % (n)		50 (2)	29 (4)	46 (6)	29 (4)
Marsh Score, % (n)	Marsh 0	0 (0)	50 (7)	46 (6)	N/D
	Marsh I	25 (1)	36 (5)	23 (3)	N/A
	Marsh II	0 (0)	0 (0)	0 (0)	N/A
	Marsh IIIA	0 (0)	0 (0)	15 (2)	N/A
	Marsh IIIB	50 (2)	0 (0)	8 (1)	N/A
	Marsh IIIC	0 (0)	0 (0)	8 (1)	N/A
	Unknown	25 (1)	14 (2)	0 (0)	N/A

CD, coeliac disease; N/D, not determined; N/A, not applicable.

revealed that this increase in circulating CD4$^+$Foxp3$^+$ cells was explained by higher proportions of Foxp3$^+$ cells in both CD62L-positive cell subsets, but not in the CD62L-negative T-cell subsets (Figure 6.6). As naturally-occurring Treg cells are characterized by a high surface expression of CD62L, these data infer that the increase in Foxp3 in CD patients may be caused by changes in the naturally-occurring Treg-cell population but not by induction of mucosally-derived Foxp3$^+$CD62L^{neg}CD38$^+$ Treg cells.
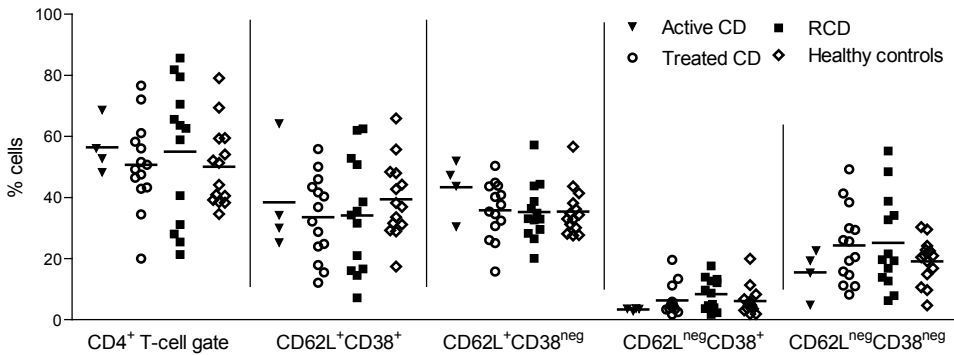
Figure 6.4 Analysis of CD4$^+$ CD62L/CD38 T-cell subsets in adult CD patients. Whole peripheral blood samples were obtained from adult patients with active CD (n=3), CD patients responding to a GFD (treated CD, n=11), patients with RCD (n=11) and healthy controls (n=14). After erythrocyte lysis, cells were stained for CD3, CD4, CD62L and CD38 and the percentages of total CD4$^+$ T cells (within CD3$^+$ T-cell gate) and of the different CD62L/CD38 T cells (within CD4$^+$ T-cell gate) were determined by flow cytometry. No significant differences between the patient groups and controls could be detected. CD, coeliac disease; GFD, gluten-free diet; RCD, refractory coeliac disease.
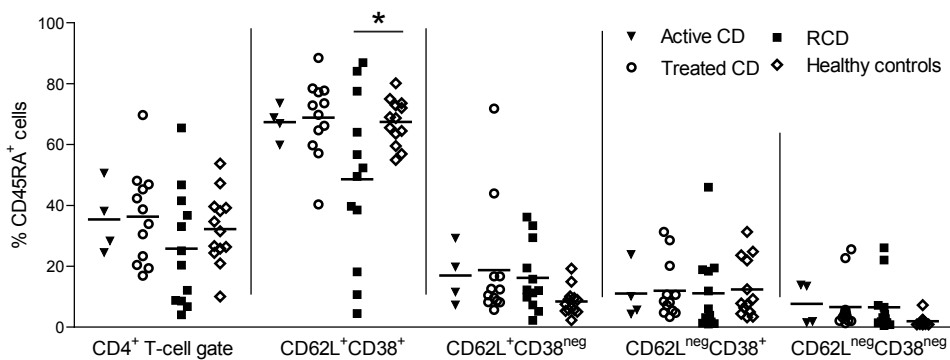
Figure 6.5 CD4$^+$CD62L$^+$CD38$^+$CD45RA$^+$ naive T cells are decreased in patients with RCD. Blood from adult CD patients and controls was obtained as described in Figure 6.4 and co-stained for CD45RA to identify naive T cells. The percentage of CD45RA$^+$ naive T cells in the CD62L$^+$CD38$^+$ CD4$^+$ T-cell subset was significantly lower in patients with RCD when compared with healthy controls (*P=.027, Mann Whitney test). CD, coeliac disease; RCD, refractory coeliac disease.

CHAPTER 6
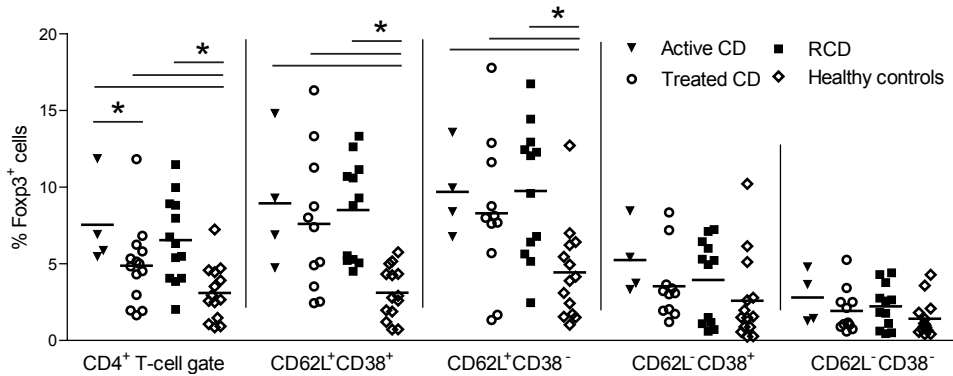
Figure 6.6 Increased proportion of Foxp3^{+} in adult patients with active or refractory CD. Blood was obtained from adult CD patients and controls as described in Figure 6.4 and co-stained for Foxp3. Patients with active CD had a significantly higher percentage of Foxp3^{+} cells within the total CD4^{+} T-cell gate (P=0.01) and in the CD62L^{+}CD38^{+} and CD62L^{+}CD38^{neg} T-cell subsets (P<0.01 and P=0.02, respectively) in comparison with healthy controls. Patients with RCD had a significantly increased proportion of Foxp3 cells in total CD4^{+} T cells (P<0.005), CD62L^{+}CD38^{+} T cells (P<0.0005) and CD62L^{+}CD38^{neg} T cells (P=0.005). The percentage of Foxp3^{+} cells in blood of treated CD patients was significantly higher in comparison with healthy individuals within total CD4^{+} T cells (P=0.03), CD62L^{+}CD38^{+} cells (P=0.01) and in CD62L^{+}CD38^{neg} cells (P=0.02), but the percentage of CD4^{+}Foxp3^{+} T cells was significantly lower than observed in patients with active CD (P=0.04). Differences between other groups did not reach significance. Data analyzed with Mann Whitney test. CD, coeliac disease; RCD, refractory coeliac disease.

DISCUSSION

We have recently identified that expression of CD4^{+}CD62L^{neg}CD38^{+} on CD4^{+} T cells distinguishes mucosally-activated T cells (Chapter 5) in peripheral blood. To explore whether a defect in the differentiation of inducible gluten-specific Treg cells explains the loss of tolerance to gluten in CD, we characterized the peripheral blood CD62L^{neg}CD38^{+} mucosal T-cell population in pediatric CD, adult CD and controls. Our findings demonstrate no substantial differences in the percentage of CD4^{+}CD62L^{neg}CD38^{+} mucosal T cells between CD patients and controls. In all groups, this subset had a similar frequency of naive versus memory T cells and had equal percentages of cells expressing the gut-homing receptor CCR9. Moreover, the proportion of CD62L^{neg}CD38^{+} mucosal T cells that expressed the regulatory T-cell transcription factor Foxp3 did not differ between CD patients and controls, implicating that the frequency of mucosally-induced Foxp3^{+} Treg cells in peripheral blood is not altered in the CD patients. In contrast, we did observe a higher percentage of naturally-occurring CD62L^{+}Foxp3^{+} T cells in peripheral blood of adult patients with active or refractory CD. In children however, we did not detect a difference in the circulating Foxp3^{+} T cells between pediatric CD and control patients.

We report that increased numbers of circulating CD4^{+} Foxp3^{+} T cells are characteristic for adult CD but not pediatric CD. Changes in peripheral blood Foxp3^{+} cells in CD have been reported earlier. In particular, Frisullo et al. (18) observed a higher percentage of CD4^{+}CD25^{+}Foxp3^{+} T

cells in peripheral blood of adult patients with untreated CD when compared with CD patients on a gluten-free diet. However, in contrast to our study, no significant difference was detected between the percentage of CD4+CD25+Foxp3+ T cells from untreated patients and healthy controls. In a second study, no differences in circulating Foxp3+ T cells were observed between healthy controls, CD patients responding to a GFD and RCD patients (19). To date no data are available regarding the percentage of Foxp3+ T cells in peripheral blood of children with CD. However, at the mRNA level, peripheral blood mononuclear cells from pediatric CD patients on a GFD showed a higher spontaneous and phytohemagglutinin-induced expression of Foxp3 in comparison with both pediatric type 1 diabetes patients and non-diseased children (20). In addition, increased numbers of Foxp3+ T cells have been found in small-intestinal biopsies from pediatric patients with CD (21, 22). The large variability in these data infer that the changes in circulating Foxp3+ T cells may be transient and possibly related to a particular state of inflammation in CD patients. As increases in peripheral Foxp3+ T cells have also been reported for patients with malignant cancers (23, 24), primary Sjogren's Syndrome and rheumatoid arthritis (25), Psoriasis (26) and systemic sclerosis (27), we argue that a non-specific inflammatory mediator can cause this effect. As such, it is of note that the increased percentage of Foxp3+ cells in our cohort had a naive CD62L+ phenotype which has been described for Foxp3 cells that are expanded with inflammatory cytokines such as interleukin (IL)-15 or IL-2 (28, 29). Such Foxp3+ T-cell induction after cytokine stimulation is not associated with an increase in regulatory capacity (28). This agrees with earlier reports that, in human CD4+ T cells, Foxp3 does not exclusively identify T cells with regulatory function (30, 31).

Thus, we conclude that our observation that CD4+Foxp3+ T-cell numbers are increased in adult CD and RCD are of importance because it may help to discern a particular phase of inflammation. However, whether the changes reflect altered immune regulation is difficult to establish. In particular this is due to the fact that the T-cell receptor specificity of peripheral blood CD4+Foxp3+ T cells is very broad. This may account for contradictory reports describing that the suppressive function of CD4+CD25+ Treg cells from CD patients is impaired in one study (32), but not in another study (18).

As it has been demonstrated that gluten-specific T-cell responses can only be visualized with DQ2-gluten peptide tetramers in peripheral blood of CD patients after a short-term dietary-gluten challenge, it can be expected that gluten-specific regulatory cells reside in only a very small subset of peripheral cells (16). Therefore, we specifically analyzed the small subpopulation of CD4+CD62L^neg CD38+ mucosal T cells which strongly enriches for the disease-relevant gluten-specific T cells (Chapter 5). We clearly demonstrate that there are no differences in both the relative distribution of CD4+CD62L^neg CD38+ T cells and no difference in expression of Foxp3, CCR9 and CD45RA within this subset in our cohort. These data establish that there is no overt defect in the composition of the mucosal T-cell population in peripheral blood of patients with pediatric CD, adult CD or RCD. Next we aim to assess whether there are functional differences between CD62L^neg CD38+ T cells from CD patients and healthy individuals. Preliminary

data revealed that, in comparison with CD62L^{neg}CD38^{neg} and CD62L$^+$CD38^{neg} T cells, circulating CD62L^{neg}CD38$^+$ T cells produce very high levels of IFN-γ and IL-10 upon *in vitro* restimulation (data not shown). Future experiments will be performed to assess whether the balance between the proinflammatory cytokine IFN-γ and the immunosuppressive IL-10 is disturbed in patients with active CD. This is of importance as IL-10 production by gluten-specific T cells from CD patients on a GFD has been suggested to inhibit gluten-specific inflammatory T-cell responses (33).

We conclude that the population of mucosal CD62L^{neg}CD38$^+$ Foxp3$^+$ Treg cells has a normal frequency in blood of CD patients. Moreover, the loss of tolerance to gluten in CD patients is associated with changes in percentages of naturally-occurring Foxp3$^+$ Treg cells in adult active CD and RCD only but not in pediatric active CD.

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7

General discussion

INTRODUCTION

The intestines are continuously exposed to foreign antigens, including food proteins, antigens derived from the commensal bacterial flora and from pathogens. As a consequence of this permanent antigenic pressure, the intestinal immune system has evolved as a highly-regulated organization that can mount both protective immune responses against potential pathogens as well as tolerogenic immune responses to harmless proteins. Different subsets of regulatory T (Treg) cells have been shown to play a crucial role in the maintenance of this complex balance between immunogenic and tolerogenic responses in the intestines. Mucosally-induced antigen-specific Treg cells, that differentiate under the control of the tolerogenic microenvironment of the gut, are key players in the establishment of tolerance to harmless antigens encountered via the mucosal surfaces of the gastrointestinal tract. Local tolerogenic dendritic cells (DC) have been recognized as important controllers in the induction of mucosal Treg-cell subset. In particular, CD103^{+} mucosal DC are efficient inducers of *de novo* forkhead box P3 (Foxp3)[+] Treg-cell differentiation (1). Moreover, several mucosal DC populations have been shown to induce the expression of interleukin (IL)-10-producing Tr1 cells (2-4). It has recently been demonstrated that these tolerogenic mucosal DC can in turn be conditioned by local microenvironmental factors, such as epithelial-cell derived transforming growth factor (TGF)-β and retinoic acid (RA) (5, 6).

Given the abundant presence of immunosuppressive factors such as TGF-β, IL-10 and RA in the intestines, as well as the redundancy in immunoregulatory mechanisms, it is difficult to dissect how tolerance to gluten is broken in patients who develop coeliac disease (CD). CD is the most frequent intolerance to a dietary protein. It is largely unknown why this disease develops so frequently and why gluten, out of so many food proteins, causes a food intolerance.

However, research in the past years has yielded important clues, which are discussed below.

Genetics & coeliac disease

First, it has become evident that genetic susceptibility is a key determinant in the development of CD. The strong impact of a genetic component has been demonstrated by a much higher concordance rate of CD in monozygotic twins (83-86%) than in dizygotic twins (17-20%) (7, 8). HLA-DQ2 and HLA-DQ8 molecules are the main risk factor for the development of CD and are estimated to account for at least 40% of the genetic contribution (9). Over 90% of all patients with CD carry HLA-DQ2, while most of the remaining patients carry HLA-DQ8 (10). The mechanism underlying the genetic predisposition of these HLA molecules in the pathogenesis of CD is well understood. Both HLA-DQ2 and HLA-DQ8 have characteristic binding motifs that allow an efficient interaction with deamidated gluten peptides, and inflammatory gluten-reactive CD4^{+} T cells that are exclusively restricted to these HLA molecules play a central role in disease pathogenesis (11).

Recently, a major focus of CD research has been the analysis of genome-wide association studies to find an explanation for the remaining non-HLA heritability. A number of additional candidate genes has been identified that contribute to disease heritability, although they only explain a rather small part of the additional heritability of CD (12-14). Together with the fact that the majority of HLA-DQ2 or HLA-DQ8 individuals do not develop CD, these data indicate that other, environmental, factors must be involved.

What is so particular about gluten?

Features in the structure of gluten itself, the single environmental factor that causes the disease, may already provide several indications that could explain a failure of tolerance induction for this particular food protein. It has been demonstrated that antigens that induce oral tolerance are generally soluble, whereas antigens that are particulate in nature are likely to induce immunity (15). In contrast to most food proteins, gluten proteins are insoluble in water and contain disulfide and hydrogen bonds, resulting in the assembly of stable gluten aggregates (16). Moreover, these proline-rich aggregates are resistant to degradation by gastrointestinal enzymes so that large gluten complexes reach the lumen of the gut (17). Furthermore, incomplete degradation may increase immunostimulatory capacities of gluten proteins. It has been demonstrated that the gluten peptides can activate the innate immune system and may thus contribute to the development of gluten-induced enteropathy independently of their capacity to bind HLA-DQ2/DQ8 and activate gluten-specific T cells. Maiuri et al. demonstrated that the gluten p31-43 peptide elicits the production of the proinflammatory cytokine IL-15 by lamina propria macrophages and DC in biopsies of CD patients (18). More recently, it was demonstrated that gluten peptides can even trigger an IL-15-mediated innate immune response in individuals without CD (19). Since the first publication by Maiuri et al., evidence is accumulating that gluten can induce the maturation of DC (20-25), thereby reinstructing previously tolerogenic DC to stimulate the differentiation of proinflammatory T helper 1 (Th1) cells. In agreement with these findings, it was demonstrated that DC isolated from the coeliac mucosa have a non-tolerogenic phenotype, as indicated by high surface expression of CD80, CD86 and CD83, increased expression of interferon (IFN)-α and decreased levels of IL-10 and TGF-β transcripts (26).

Loss of the tolerogenic intestinal microenvironment in coeliac disease

The intolerance to gluten proteins in CD patients may also be explained by perturbations in the intestinal microenvironment. The small-intestinal mucosa of CD patients is characterized by the presence of high levels of proinflammatory cytokines, such as IFN-α (27), IL-15 (28, 29), IL-18 (30) and IL-21 (31), that may interfere with the tolerogenic microenvironment of the gut. Perhaps surprisingly, the expression levels of the anti-inflammatory cytokines TGF-β and IL-10 are not decreased in the mucosa of patients with active CD. Several groups have performed immunohistochemical staining of intestinal biopsies and observed an intense TGF-β staining in the epithelium and lamina propria of CD patients, that was comparable with controls (32-34).

In addition, the inflamed intestine of untreated CD contains high levels of IL-10-producing T cells that was similar to (35) or even increased in comparison with treated or healthy controls (36, 37). Despite the fact that these anti-inflammatory cytokines are not decreased in active CD, a relative lack due to the massive increase in proinflammatory cytokines or defective signaling may contribute to the loss of gluten tolerance in coeliac disease. For example, exogenous IL-10 was shown to suppress the gluten-specific inflammatory T-cell response in *ex vivo* culture of biopsies from CD patients, suggesting that the IL-10 levels in the coeliac mucosa are insufficient to downregulate ongoing inflammation and that administration of exogenous IL-10 may contribute to reset the balance between tolerance and immunity (37). Moreover, IL-10 is associated with disease remission as IL-10-producing gliadin-specific Tr1-cell clones inhibited the proliferation of gliadin-specific T-cell clones (38) and these findings may implicate the involvement of IL-10-producing Tr1 cells in response to dietary proteins.

It has recently been demonstrated that TGF-β signaling is impaired in CD patients and that intestinal T cells isolated from active CD patients are unresponsive to exogenous TGF-β signals (34). Moreover, it was found that this defective TGF-β signaling is the result of the high levels of IL-15 in the coeliac mucosa, which directly impede TGF-β-signaling through inhibition of the Smad3 pathway (34). Thus, IL-15-mediated inhibition of TGF-β-signaling might be an important mechanism that causes abrogation of oral tolerance to gluten and promotes the development of intestinal inflammation in CD. A direct association between the presence of proinflammatory cytokines and the loss of intestinal homeostasis has been suggested by several studies. Cammarota et al. reported the onset of CD in two patients with chronic hepatitis C that received treatment with IFN-α (39). Moreover, overexpression of IL-15 in mice results in the development of small-intestinal pathology that resembles features of human CD (40, 41).

Viral infections

Precise characterization of the pattern of intestinal inflammation that is associated with abrogation of mucosal tolerance in CD yielded possible factors that trigger the perturbed cytokine environment in this disease. An obvious candidate of course is gluten itself, which has been shown to activate cells of the innate immune system and mediate secretion of proinflammatory cytokines (18, 19).

An interesting hypothesis is that viral infections might trigger the onset of CD. It has been reported that repeated infections by rotavirus, a double-stranded RNA virus, promote the onset of CD (42). An important role in the early innate anti-viral immune response is attributed to IFN-α, IL-15 and IL-21 (43), the same cytokines that are implicated in the pathogenesis of CD. It can therefore be envisioned that the high levels of these proinflammatory cytokines in CD are the consequence of a viral infection (44). Moreover, a gastrointestinal infection that is associated with increased intestinal permeability, proinflammatory cytokines and activated APC may lead to enhanced presentation of gluten peptides and an increased risk of developing CD, at least in genetically susceptible individuals. Taken together, these findings demonstrate that,

in addition to genetic risk factors and the ingestion of gluten, other environmental factors that disturb the tightly controlled regulatory mechanisms in the mucosal microenvironment may play a role in the onset of CD.

THIS THESIS

How do the findings presented in this thesis advance our understanding of the loss of tolerance to gluten in patients with CD?

Compartmentalization of the intestinal immune system

First, the mechanisms underlying the induction of mucosal tolerance differ in different regions of the intestine. In **chapter 3**, our data show that uptake of the protein ovalbumin (OVA) via the colonic mucosa resulted in T-cell differentiation in the iliac lymph nodes (ILN), but not in the small-intestine draining MLN, indicating that there is regional compartmentalization within the intestinal immune system. We demonstrated that this compartmentalization has important consequences for the outcome of the induced OVA-specific T-cell response, as a much lower proportion of adaptive Foxp3+ T cells was induced by colonic protein application in comparison to protein feed. The reduced Foxp3 induction upon colonic protein application could likely be explained by intrinsic differences in the resident CD103+ DC subset between the ILN and the MLN, as we observed that, unlike CD103+ DC in MLN, ILN-derived CD103+ DC expressed only very low levels of the vitamin A metabolizing enzyme RALDH2. In contrast, the CD103+ DC from ILN expressed high levels of IL-10. Mucosal DC have long been recognized as important controllers of mucosal tolerance, but only recently has been proposed that these tolerogenic DC are steered by the local microenvironment. It has been demonstrated that the epithelial-cell derived factors thymic stromal lymphopoietin (TSLP), TGF-β and RA can condition mucosal DC into a non-inflammatory state (5, 6, 45). However, little is known about what drives the release of these factors by epithelial cells and whether there are differences between epithelial cells in different regions of the intestines. Possibly, luminal antigens in turn condition the intestinal epithelial cells and, in this way, different regulatory systems arise in the proximal and distal intestines. Indeed, it has been demonstrated that luminal antigens derived from commensal bacteria can regulate intestinal immune responses (46-49). In addition, dietary elements such as vitamin A may also influence epithelial cells in the small intestine.

In addition to the data presented in **chapter 3**, regional differences in IL-10-producing and Foxp3+ Treg cells have been observed in the intestines under steady-state conditions (50). In particular, it was demonstrated Foxp3- IL-10-producing Tr1 cells are presented in high numbers in the small-intestinal mucosa, whereas in the colon all IL-10-producing T cells co-express Foxp3 (50). This may in particular be relevant to the regulation of gluten-induced immune responses as we have shown in **chapter 4** that, in mice, gluten-specific Tr1-like cells are induced that secrete high levels of IL-10 but lack the expression of Foxp3. However, more knowledge regarding the differences

in IL-10-producing and Foxp3^{+} Treg-cell subsets in the small and large intestine is required for a better understanding for the loss of mucosal tolerance. These differences will be important to take into account for our understanding of diseases restricted to a specific part of the gastrointestinal tract, such as the small instestine for CD or the large intestine in ulcerative colitis.

Cyclooxygenase-2

Cyclooxygenase-2 (COX-2) is the inducible isoform of the COX enzymes that catalyze the formation of prostaglandins from arachidonic acid. Whereas the COX-1 isoform is constitutively active in most tissues, COX-2 is induced by proinflammatory cytokines and microbial agents and has therefore long been recognized as a proinflammatory enzyme (51). However, in the intestines, the expression of COX-2 is constitutive (52) and contributes to the maintenance of mucosal homeostasis. Although COX-2 deficient mice do not show any spontaneous inflammation of the gut, these mice have an increased susceptibility to chemically-induced colitis that is associated with an inability to produce the COX-2-dependent metabolite prostaglandin E_2 (PGE$_2$) (53). In agreement, a deficiency in EP4, one of the G-coupled PGE$_2$ receptors, also causes increased susceptibility to chemically-induced colitis, whereas deficiency in other prostaglandin receptors did not have this effect (54).

In addition, treatment of mice with the selective COX-2 inhibitor NS-398 resulted in loss of tolerance to fed proteins (55, 56). In **chapter 2** we have provided a mechanism for the loss of tolerance to dietary proteins in the absence of COX-2. Our data showed that selective inhibition of COX-2 during protein feed was associated with an enhanced T-cell-derived IL-4 production and a defective induction of Treg cells in the gut-draining MLN. Crucially, IL-4 was shown to be directly responsible for the loss of oral tolerance as treatment of mice with anti-IL-4 antibodies prior to COX-2 inhibition and protein feed restored oral tolerance. In agreement with earlier reports (57-61), we showed that IL-4 directly inhibited Foxp3^{+} Treg-cell differentiation. Thus, COX-2 promotes mucosal Treg-cell differentiation through downregulation of IL-4-producing Th2-like cells. In addition, we and others have demonstrated that the COX-2-dependent metabolite PGE$_2$ can also directly induce Foxp3 expression in T cells (62-64). Taken together, these studies demonstrate a crucial role for COX-2-dependent arachidonic acid metabolites in maintaining both small-intestinal and colonic homeostasis.

Whether defects in the COX-2 pathway may also contribute to the loss of tolerance to gluten in CD patients is unknown. CD is mediated by the activation of gluten-specific CD4^{+} T cells that secrete high amounts of IFN-γ. Therefore, at first thought, the high levels of IL-4 that are produced in the absence of COX-2 argue against a role for the enzyme in the formation of this highly-inflammatory gluten-specific T-cell response. However, Newberry et al. have demonstrated that the administration of COX-2 inhibitors simultaneous with protein feed resulted in pathological changes in the proximal small intestine that resemble human CD, including villus blunting and increased proliferation of epithelial crypt cells resulting in crypt expansion (56). Moreover, a decrease in villus height was also observed in HLA-DQ8 transgenic

(Tg) mice that were immunized with gliadin and treated with the COX inhibitor indomethacin (65). Thus, these finding indicate that there may be a role for COX-2 in preventing the gluten-induced enteropathy in CD patients.

Systemic monitoring of T-cell tolerance

Despite our growing knowledge concerning the induction of oral tolerance to food proteins and tolerogenic nature of the intestinal microenvironment, it is still unclear why the ingestion of gluten proteins results in the proliferation of inflammatory IFN-γ-producing T cells instead of T cells with regulatory function in CD patients. One of the aims of this thesis was to assess whether the gluten-specific inflammatory T-cell response in CD patients could develop due to a defect in the Treg-cell population. Thereto, we determined whether alterations in Foxp3$^+$ Treg-cell populations in peripheral blood of patients with CD could be involved in the pathogenesis of CD.

In **chapter 5** we have demonstrated that CD4$^+$ T cells that have been activated in the gut-mucosal draining lymphoid tissue can be characterized in the periphery by the expression of CD62L^{neg}CD38$^+$. This CD4$^+$CD62L^{neg}CD38$^+$ T-cell phenotype was shown to be imprinted by mucosal DC and depended on the production of RA and the presence of TGF-β (Figure 7.1). Importantly, CD4$^+$CD62L^{neg}CD38$^+$ specifically identified T cells of mucosal origin in both mice and humans. We have validated the significance of this phenotype to identify mucosally-activated T cells in a study of patients with CD. Using HLA-DQ2 gluten-peptide specific tetramers it has previously been demonstrated that gluten-specific T cells can only be detected in very low numbers in the peripheral blood of CD patients after a short-term dietary gluten challenge (66). Our findings (**chapter 5**) demonstrate that the vast majority of these tetramer$^+$ gluten-specific T cells were CD4$^+$CD62L^{neg}CD38$^+$. As the CD4$^+$CD62L^{neg}CD38$^+$ T-cell subset comprises only a very small proportion of all circulating CD4$^+$ T cells in healthy volunteers (5.69% ± SD 2.17, **chapter 5**), we argued that this small subset of T cells strongly enriches for disease-relevant T cells in patients with chronic inflammatory diseases of the small intestine and that analysis of the CD4$^+$CD62L^{neg}CD38$^+$ T-cell population will be a powerful tool to characterize defects in gluten-specific T-cell regulation in CD.

Therefore, we specifically analyzed the CD4$^+$CD62L^{neg}CD38$^+$ T-cell subset in paediatric and adult patients with active CD, as well as adult CD patients after treatment with a gluten-free diet, adult patients with refractory coeliac disease (RCD) and in controls. The results in **chapter 6** clearly demonstrate that we did not observe any differences in both the relative distribution of CD4$^+$CD62L^{neg}CD38$^+$ T cells and in the expression of Foxp3 within this subset between any of the groups of patients analyzed. Future research will be conducted to assess whether there are functional differences between CD4$^+$CD62L^{neg}CD38$^+$ T cells from CD patients and healthy individuals.
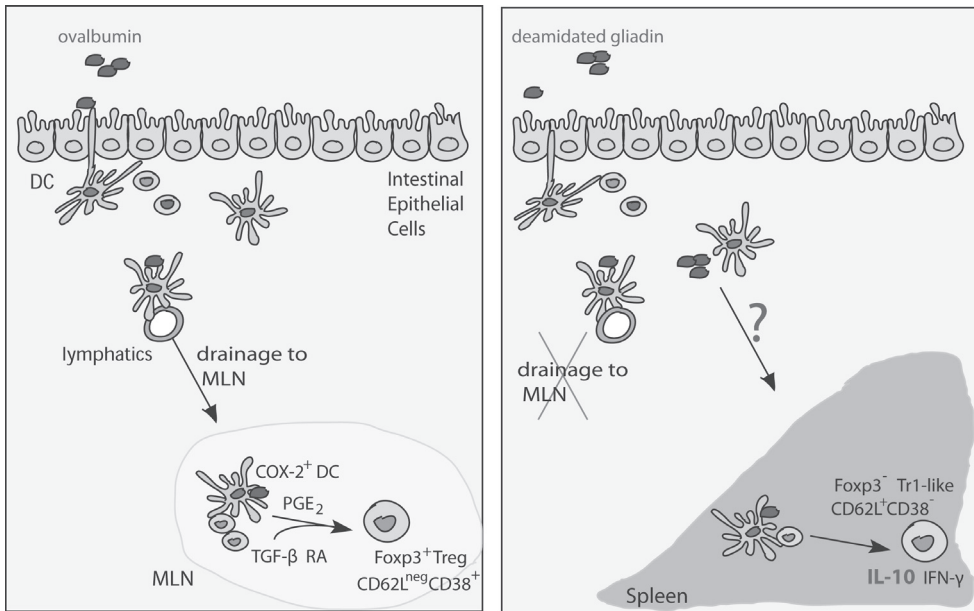
Figure 7.1 Schematic comparison of the tolerogenic T-cell responses to ovalbumin and deamidated gliadin. Orally-applied OVA is taken up by mucosal DC that migrate to the gut-draining MLN, resulting in the differentiation of mucosally-induced OVA-specific Treg cells. Differentiating MLN Treg cells *de novo* expressed Foxp3. The induction of these cells was shown to be dependent on the expression of COX-2 in mucosal DC (chapter 2). In contrast, the oral administration of deamidated gliadin did not induce a vigorous T-cell response in the draining MLN, but instead, T cells proliferated in the spleen. Splenic differentiating gliadin-specific T cells did not express Foxp3, and were characterized by the secretion of IFN-γ and IL-10. As these cells exhibited regulatory cell function, splenic gliadin-specific T cells resemble Tr1 cells. COX-2, cyclooxygenase 2; DC, dendritic cell; IFN, interferon; IL, interleukin; MLN, mesenteric lymph nodes; OVA, ovalbumin; PGE_2, prostaglandin E_2; RA, retinoic acid; TGF, transforming growth factor; Tr1, type 1 regulatory T cell; Treg, regulatory T cell.

Mouse model: gluten-specific T-cell responses in the healthy intestine

Knowledge about the regulation of gluten-specific immune response in the healthy intestine is scarce. Most experimental data on the pathogenesis of CD are obtained from studies in patients with already fully established disease, obtained with *in vitro* culture experiments of intestinal biopsies from patients with active disease or using gluten-reactive T-cell clones that were generated from these biopsies. However, these models are unsuitable to study the regulation of gluten-specific T-cell responses during intestinal homeostasis and the early events that may lead to the development of coeliac disease. To address these issues, we used newly-generated transgenic mice expressing HLA-DQ2 and a gluten-specific T-cell receptor from a CD patient. In **chapter 4**, we described that feeding these mice deamidated gliadin induces tolerance, but that the induction of tolerance to gliadin bypasses the 'classical rules' of oral tolerance in the gut-associated lymphoid tissue. Instead, deamidated gliadin feed induced dominant

proliferation of gliadin-specific T cells in the spleen and very little in the gut-draining MLN. Perhaps as a consequence of the activation outside the tolerogenic microenvironment of the gut, the differentiating gliadin-specific T cells lacked the expression of Foxp3 and could instead be characterized by the secretion of high amounts of IFN-γ and IL-10. These IFN-γ- and IL-10-producing T cells resembled Tr1 cells as they were shown to possess regulatory function in a delayed type hypersensitivity model. Therefore, we hypothesize that CD may develop as a result of a dysbalance between tolerogenic IL-10-producing Tr1-like cells and inflammatory IFN-γ-producing T-cell responses. As we observed that circulating CD4$^+$CD62L^{neg}CD38$^+$ T cells have an increased potential over other the CD62L/CD38 T-cell subsets to produce both cytokines upon *in vitro* stimulation, it is of interest to determine whether the balance between IL-10- and IFN-γ-producing T cells within this mucosal T-cell population is altered in patients with ongoing CD.

Role for regulatory T-cell subsets

An attractive hypothesis is that defects in mucosal Treg-cell populations contribute to the pathogenesis of CD. On the basis of our results in **chapter 4**, we favor the hypothesis that IL-10-producing Tr1 cells are crucial in preventing the development of inflammatory gluten-specific T-cell responses. This is supported by the finding that IL-10-producing gluten-specific Tr1-cell clones that could suppress inflammatory gluten-specific T-cell responses can be isolated from the mucosa of treated CD patients (38).

In contrast, we did not find evidence for a role for Foxp3$^+$ Treg cells in tolerance to orally-administered deamidated gluten in the HLA-DQ2.glutenTCR Tg mouse model. Our data agree with a study by de Kauwe et al. (67), who also found no increase in Foxp3$^+$ cells after gluten ingestion in glutenTCR Tg mice. Moreover, we did not detect any changes in the proportion of Foxp3$^+$ cells within the CD62L^{neg}CD38$^+$ mucosal T-cell population between our cohorts of CD patients and controls (**chapter 6**), supporting the thought that tolerance to gluten may be regulated by different mechanisms, perhaps through the induction of IL-10-dependent Tr1 cells. Whether CD62L^{neg}CD38$^+$ IL-10-producing Tr1 cells may be involved in human CD will be the focus of future research.

However, we did observe an association between CD and alterations in Foxp3$^+$ T-cell subsets. Adult patients with active CD or RCD had a higher percentage of circulating CD62L$^+$Foxp3$^+$ T cells in comparison with healthy individuals (**chapter 6**). These changes in Foxp3$^+$ cells were not observed in adult patients with treated CD or in pediatric CD patients. Increased levels of Foxp3$^+$ cells in peripheral blood of CD patients have been reported in one other study (68). Moreover, it has been reported that Foxp3$^+$ T cells are increased in the mucosa of pediatric CD patients (69, 70).

However, as Foxp3 does not exclusively identify T cells with regulatory cell function in humans (71, 72) and as increases in peripheral Foxp3$^+$ T-cells have also been reported for patients with malignant cancers (73, 74), primary Sjogren's Syndrome and rheumatoid arthritis (75), Psoriasis (76) and systemic sclerosis (77), we argue that the increase in Foxp3 may reflect

a particular stage of chronic inflammation. In agreement with this hypothesis, it has recently been demonstrated that the proinflammatory cytokine IL-15, which is abundantly expressed in CD, can stimulate the induction of Foxp3 in human T cells (78, 79) without increasing cellular regulatory capacity (79). In addition, IL-15 was shown to interfere with the suppressive functions of Treg cells by inducing the resistance of effector T cells to suppression by Treg cells (78).

Taken together, these results support a role for IL-10-producing Tr1 cells, but provide no evidence for Foxp3+ Treg cells, in the maintenance of oral tolerance to gluten.

CONCLUDING REMARKS

The findings in this thesis provide important insights that add to our understanding of tolerance to mucosally-encountered protein antigens. Our data point out that multiple, in part redundant, mechanisms which are all under the control of the local microenvironment contribute to the establishment of tolerance in the gastrointestinal tract. Not only have we demonstrated that such differences occur in different regions of the intestines, our comparison of two TCR Tg mouse models, one specific for OVA and one for deamidated gliadin, also revealed that oral tolerance may be induced differently for different food proteins. These findings should be taken into account when developing new strategies that aim at restoring mucosal tolerance in patients with inflammatory diseases of the intestines, such CD or inflammatory bowel diseases.

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8

Samenvatting
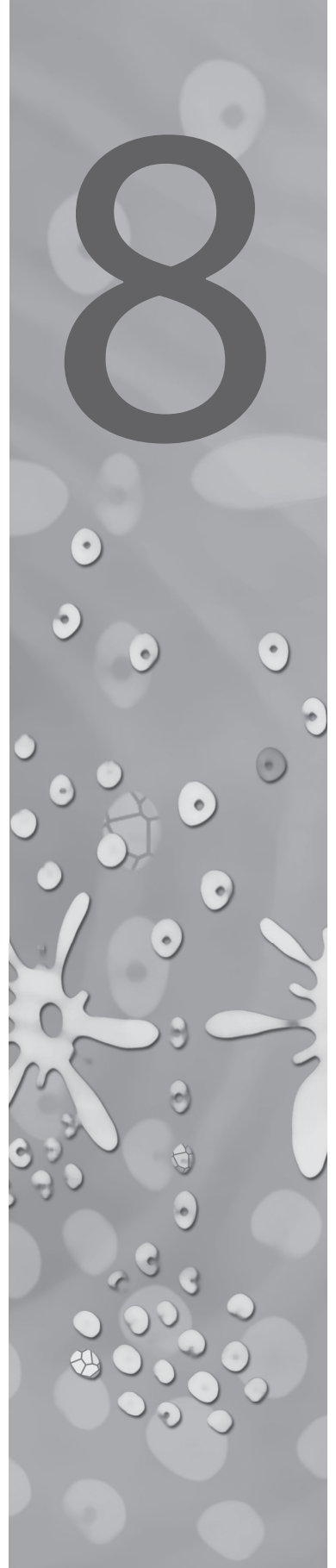
Het maag-darmstelsel

De belangrijkste taak van het maag-darmstelsel is het verteren en het opnemen van voedings-stoffen. Naast voedingsstoffen, komen in de darm echter nog tal van andere stoffen binnen, waaronder ook schadelijke organismen zoals ziekteverwekkende bacteriën of virussen. De darm moet steeds beslissen welke stoffen gewenst zijn en moeten worden opgenomen, en welke stoffen juist moeten worden tegengehouden. Deze beslissing wordt gemaakt door het immuunsysteem in de darm. Om de beslissing tussen 'goed' (onschadelijk) en 'fout' (gevaarlijk) steeds adequaat te kunnen maken, is het niet vreemd dat het immuunsysteem in de darm het meest complexe immuunsysteem van ons lichaam is. Verreweg de meeste cellen van het immuunsysteem bevinden zich dan ook in de darm.

Het immuunsysteem

Het immuunsysteem wordt in gang gezet wanneer lichaamsvreemde stoffen (zogenaamde antigenen) door witte bloedcellen worden herkend. De eerste witte bloedcellen van het immuunsysteem in de darm die een antigeen zullen tegenkomen, zijn zogenaamde dendritische cellen. Dendritische cellen danken hun naam aan de vorm die deze cellen hebben. Ze worden gekenmerkt door uitlopers ('dendrieten') waarmee ze in staat zijn om tussen de laag cellen die de darm bekleedt ('epitheelcellen') door te steken en zich zo toegang te verschaffen tot de inhoud van de darm (het 'lumen'). Dendritische cellen hebben als taak om antigenen op te nemen en deze op hun celoppervlak te presenteren. Daarom worden dendritische cellen ook wel antigeen-presenterende cellen genoemd. Zodra dendritische cellen een antigeen hebben opgenomen bewegen ze zich via de lymfevaten naar de lymfeklieren, waar ze de antigenen aanbieden aan een ander type witte bloedcel: de T-cel (of T-lymfocyt).

Wanneer de T-cel het antigeen herkent, leidt dit ertoe dat de T-cel wordt geactiveerd. De geactiveerde T-cel zal zich dan vermenigvuldigen en een afweerreactie in gang zetten. Het type afweerreactie dat geïnduceerd wordt, hangt af van de signalen die het antigeen afgeeft en van de omgevingsfactoren waaronder de dendritische cel en de T-cel elkaar tegenkomen. Een ziekteverwekkende bacterie bijvoorbeeld geeft zoveel gevaarsignalen af aan het immuunsysteem dat de alarmbellen gaan rinkelen. De dendritische cel die bacterie-antigenen heeft opgenomen zal het gevaar herkennen en doorgeven aan de T-cel, met als gevolg dat er een ontstekingsreactie in gang wordt gezet die moet leiden tot het opruimen en het doden van de bacterie.

Zo'n agressieve ontstekingsreactie is echter niet wenselijk bij onschuldige lichaamsvreemde stoffen, zoals voedingsstoffen. Gelukkig gaan, als alles goed gaat, de alarmbellen van het immuunsysteem in de darm niet af bij een voedselantigeen. Integendeel: als een dendritische cel een voedselantigeen opneemt en deze vervolgens presenteert aan de T-cel, zal de T-cel ervoor zorgen dat een agressieve reactie van het immuunsysteem wordt onderdrukt. Er worden T-cellen gevormd die de functie hebben om een afweerreactie te remmen. Deze T-cellen worden regulatoire T-cellen genoemd.

Mucosale tolerantie

Het bijzondere aan het immuunsysteem van de darm is dat alle lichaamsvreemde stoffen, mits ze geen overduidelijke gevaarsignalen afgeven, in principe worden gedoogd. Dit proces wordt mucosale tolerantie genoemd (naar de 'mucosae': de slijmvliezen van de darm). Bij de totstandkoming van mucosale tolerantie speelt de vorming van regulatoire T-cellen een heel belangrijke rol. Ook is de omgeving van groot belang. Eerder onderzoek heeft aangetoond dat in de slijmvliezen van de darm veel factoren aanwezig zijn die de cellen van het immuunsysteem leren om tolerant te zijn tegen onschuldige antigenen. Deze factoren stimuleren de ontwikkeling van regulatoire T-cellen en helpen om agressieve afweerreacties te voorkomen. Een aantal belangrijke factoren zijn zogenaamde cytokines, waaronder transforming growth factor-β (TGF-β) en interleukine-10 (IL-10), maar ook het vitamine A afbraakproduct retinolzuur speelt hierbij een grote rol.

Indien er ergens in het proces van mucosale tolerantie iets mis gaat kan dit leiden tot ongewenste afweerreacties tegen onschadelijke stoffen, bijvoorbeeld in patiënten met een voedselallergie.

Coeliakie

Coeliakie is een ziekte van de dunne darm die ontstaat door een intolerantie of onverdraagzaamheid tegen gluten, de belangrijkste eiwitten in tarwe. Bij patiënten met coeliakie veroorzaakt het eten van voedsel dat gluten bevat een chronische ontsteking van de dunne darm, met als gevolg dat de darmvlokken beschadigd raken. Door deze beschadiging, genaamd vlokatrofie, wordt het normaal zeer grote oppervlak van de darm sterk gereduceerd en kunnen voedingsstoffen minder goed worden opgenomen. Patiënten met coeliakie kunnen last hebben van buikpijn, diarree, ondervoeding, vermoeidheid, botontkalking en onvruchtbaarheid en hebben een vergroot risico op het ontwikkelen van darmkanker.

Coeliakie komt in Nederland voor bij ongeveer 1 op de 100 mensen. Het is daarmee de meest voorkomende aandoening die wordt veroorzaakt door een voedseleiwit. Het is niet duidelijk waarom zoveel mensen coeliakie ontwikkelen en waarom juist gluten voor de problemen zorgen. Wel is duidelijk dat er een sterke associatie is met zogenaamde HLA-genen. Deze HLA-genen spelen een rol bij het presenteren van antigenen aan T-cellen. Vrijwel alle patiënten met coeliakie hebben het HLA-DQ2- of het HLA-DQ8-gen. Maar daartegenover staat dat 30% van de Nederlanders wel HLA-DQ2 of HLA-DQ8 heeft maar geen coeliakie. Andere factoren spelen dus ook een rol.

Dit proefschrift

Dit proefschrift heeft zich gericht op de vraag waarom mucosale tolerantie ontregeld is in patiënten met coeliakie. Hoofdstuk 1 geeft een algemene inleiding over wat er tot nu toe bekend is over mucosale tolerantie, de vorming van regulatoire T-cellen en de rol van darmspecifieke factoren hierin.

Om te begrijpen waarom mucosale tolerantie tegen gluten mislukt in patiënten met coeliakie, moesten we eerst meer te weten komen over welke factoren in de darm nodig zijn voor de totstandkoming van tolerantie en hoe deze factoren hun werk doen. Om dit te onderzoeken is gebruik gemaakt van een muismodel. Onderzoek voorafgaand aan dit proefschrift heeft aangetoond dat muizen na het eten van het kippeneiwit ovalbumine tolerant worden voor dit eiwit. Ook was bekend dat wanneer je muizen tijdens het eten van ovalbumine behandelt met een remmer voor het eiwit cyclooxygenase-2 (COX-2) in de darm, de muizen niet langer tolerant worden voor het kippeneiwit. COX-2 is dus belangrijk voor de totstandkoming van mucosale tolerantie. In **hoofdstuk 2** van dit proefschrift is onderzocht hoe COX-2 bijdraagt aan de ontwikkeling van mucosale tolerantie in muizen. Er is aangetoond dat wanneer COX-2 werd geremd, er geen regulatoire T-cellen gevormd werden. Door het ontbreken van de regulatoire T-cellen werd een ontstekingsreactie niet onderdrukt. Tegelijkertijd zagen we dan ook dat er meer T-cellen werden gevormd die het ontstekingscytokine interleukine-4 (IL-4) maken. Deze ontstekingsfactor IL-4 zorgde er direct voor dat er geen regulatoire T-cellen gevormd werden en dat er daardoor ook geen tolerantie ontwikkeld kon worden. Samengevat is in **hoofdstuk 2** aangetoond dat COX-2 op twee manieren belangrijk is voor de totstandkoming van mucosale tolerantie. Ten eerste stimuleert COX-2 de ontwikkeling van regulatoire T-cellen in de darm. Ten tweede voorkomt het de activatie van ontstekings-T-cellen die IL-4 maken. Vervolgonderzoek zal moeten uitwijzen of een verminderde functie van COX-2 wellicht een rol speelt in patiënten met coeliakie.

Hoewel er tot nu toe veel bekend was over hoe mucosale tolerantie tot stand komt in de dunne darm, wisten we nog niet of dit op een vergelijkbare manier gebeurt in de dikke darm. Dit is met name van belang voor patiënten met een chronische darmontsteking, zoals de ziekte van Crohn. Deze ziekte ontstaat door het verlies van mucosale tolerantie tegen de normaal gesproken gezonde bacteriën in de darm. In de ziekte van Crohn zijn in sommige patiënten zowel delen van de dunne als de dikke darm ontstoken, terwijl de ziekte zich in andere patiënten beperkt tot alleen de dikke darm. Waarom dit zo verschilt tussen patiënten is onduidelijk. In **hoofdstuk 3** zijn belangrijke verschillen aangetoond in de totstandkoming van mucosale tolerantie tussen de dunne darm en de dikke darm. Het is gebleken dat dendritische cellen die eiwitten (antigenen) opnemen in de dikke darm naar een andere lymfeklier migreren dan dendritische cellen in de dunne darm. De dendritische cellen uit de dunne en uit de dikke darm bieden hun antigeen dus in verschillende lymfeklieren aan T-cellen aan. Als gevolg hiervan is er een verschil in de locatie waar de T-cellen geactiveerd worden. Omdat de omgeving voor een groot deel bepaalt wat voor soort immuunreactie er moet komen, kan dit belangrijke consequenties hebben voor welk type T-cellen er worden gevormd. De resultaten van ons onderzoek hebben aangetoond dat er zowel in de lymfeklieren van de dunne darm als in de lymfeklieren van de dikke darm, regulatoire T-cellen worden gevormd. Echter, er lijken proportioneel minder regulatoire T-cellen te worden gevormd wanneer eiwitten worden opgenomen in de dikke darm. Een mogelijke oorzaak voor dit verschil hebben we gevonden in de dendritische cellen. Het is gebleken dat

dendritische cellen in de lymfeklieren bij de dunne darm andere genen aanzetten dan de dendritische cellen in de dikke darm.

In **hoofdstuk 4** is een nieuw muismodel ontwikkeld om te kunnen onderzoeken hoe het immuunsysteem in de darm reageert op gluten. Hiervoor zijn muizen gebruikt die net als de meeste mensen met coeliakie het HLA-DQ2-gen hebben. Ook hebben we ervoor gezorgd dat alle T-cellen in deze muizen specifiek kunnen reageren op gluteneiwitten. Deze muizen hebben het HLA-DQ2-gen en gluten-speficieke T-cellen, maar zijn desondanks gezond en krijgen geen coeliakie na het eten van gluten. De bevindingen in **hoofdstuk 4** tonen aan dat deze muizen 'gewoon' mucosale tolerantie ontwikkelen tegen gluten. Wel is gebleken dat mucosale tolerantie tegen gluten heel anders tot stand komt dan tegen het kippeneiwit ovalbumine. De 'klassieke' vorming van regulatoire T-cellen in de darm zoals we zien in muizen na het eten van ovalbumine vindt echter niet plaats wanneer we muizen gluten voeren. Opvallend was dat het eten van gluten in deze muizen leidt tot activatie van T-cellen in de milt, en dus niet in de darm of in de lymfeklieren in de buurt van de darm. Bovendien zagen de geactiveerde T-cellen in de milt er heel anders uit dan we gewend waren. Het bleek dat deze T-cellen zowel ontstekingsremmende (IL-10) als ontstekingsstimulerende (interferon-γ, afgekort IFN-γ) cytokinen maken. Op basis van de resultaten in **hoofdstuk 4** denken we dat een verstoring in de balans tussen het ontstekingsremmende IL-10 en het ontstekinsstimulerende IFN-γ één van oorzaken kan zijn voor het ontwikkelen van coeliakie.

In **hoofdstuk 5** is gekeken naar de mogelijkheid om op basis van het uiterlijk van T-cellen een onderscheid te kunnen maken tussen T-cellen die zijn geactiveerd in de darm en T-cellen die elders in het lichaam zijn gevormd. Omdat verschillende T-cellen veel op elkaar lijken was het tot dusver lastig om een dergelijke onderverdeling te maken. Het is gebleken dat factoren die in de darm zorgen voor een tolerante omgeving ook hun stempel drukken op het uiterlijk van de T-cel. De factoren TGF-β en retinolzuur spelen hierbij een belangrijke rol. Samen zorgen ze ervoor dat T-cellen die in hun aanwezigheid worden geactiveerd, kunnen worden herkend aan de hand van "CD62L^{neg}CD38$^+$" op het celoppervlak. In het muismodel is gebleken dat T-cellen met CD62L^{neg}CD38$^+$ alleen ontstaan wanneer eiwitten via de darm binnen komen, en niet wanneer eiwitten via een andere route worden toegediend aan de muis. In patiënten met coeliakie hebben we gecontroleerd of T-cellen met CD62L^{neg}CD38$^+$ ook gebruikt kunnen worden om deze ziekte beter te begrijpen. Het is gebleken dat het merendeel van de T-cellen die specifiek reageren op gluten inderdaad CD62L^{neg}CD38$^+$ op het celoppervlak hebben.

Om die reden is in **hoofdstuk 6** gekeken of er verschillen zijn tussen CD62L^{neg}CD38$^+$ T-cellen in het bloed van patiënten met coeliakie en in het bloed van gezonde personen. Echter, CD62L^{neg}CD38$^+$ T-cellen waren in gelijke percentages aanwezig in zowel kinderen als volwassen met coeliakie en in gezonde personen. Ook was er geen verschil in de expressie van Foxp3, een eiwit dat kenmerkend is voor regulatoire T-cellen, in de CD62L^{neg}CD38$^+$ T-cellen van patiënten met coeliakie en van gezonde personen. Wel is gebleken dat er een toename is van het totale aantal cellen dat Foxp3 tot expressie brengt in volwassen patiënten met coeliakie.

Of deze toename van Foxp3 iets zegt over een verstoorde regulatie van de immuunrespons in deze patiënten zal verder onderzocht moeten worden.

Samengevat laten de studies in dit proefschrift zien dat de totstandkoming van mucosale tolerantie een complex proces is dat op verschillende niveaus gereguleerd wordt. Hierbij speelt een aantal lokale factoren uit de darm, zoals de cytokinen TGF-β en IL-10 en retinolzuur, een cruciale rol. We hebben aangetoond dat mucosale tolerantie bereikt kan worden door een aantal verschillende type immuunresponsen. De gegevens in dit proefschrift leveren een belangrijke bijdrage aan de kennis van het verloop van de ziekte coeliakie. Mogelijk kunnen de resultaten in dit proefschrift in de toekomst een bijdrage leveren aan de ontwikkeling van nieuwe therapieën voor patiënten met coeliakie of met andere chronische ontstekingsziekten in de darm.

List of abbreviations
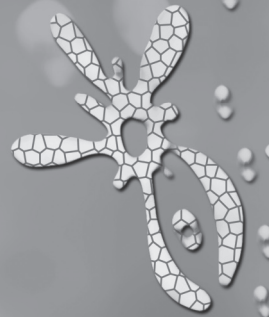
APC	Antigen presenting cell
CD	Coeliac disease
CFSE	5,6-carboxy-succinimidyl-fluoresceine-ester
COX-2	Cyclooxygenase-2
CT	Chymotrypsin (chapter 4)
CT	Cholera toxin (chapter 5)
DC	Dendritic cell
DTH	Delayed type hypersensitivity
Foxp3	Forkhead box P3
GALT	Gut-associated lymphoid tissue
GFD	Gluten-free diet
IBD	Inflammatory bowel diseases
IEC	Intestinal epithelial cell
IEL	Intraepithelial lymphocyte
IFA	Incomplete Freund's adjuvant
IFN	Interferon
i.g.	Intragastric
IgA	Immunoglobulin A
IL	Interleukin
ILN	Iliac lymph nodes
i.m.	Intramuscular
i.r.	Intrarectal
LAP	Latency associated peptide
LPS	Lipopolysaccharide
M cell	Microfold cell
MFI	Mean fluorescence intensity
MLN	Mesenteric lymph node
NK cell	Natural killer cell
NKT cell	Natural killer T cell
OVA	Ovalbumin
PBMC	Peripheral blood mononuclear cells
PGE_2	Prostaglandin E_2
PLN	Peripheral lymph node
PP	Peyer's patches
R	Receptor
TCR	T-cell receptor
Tg	Transgenic

TG2	Transglutaminase 2
TGF	Transforming growth factor
Th cell	T helper cell
Tr1 cell	Type 1 regulatory T cell
Treg cell	Regulatory T cell
TSLP	Thymic stromal lymphopoietin
RA	Retinoic acid
RALDH	Retinal dehydrogenase
RCD	Refractory coeliac disease
WBC	White blood cell

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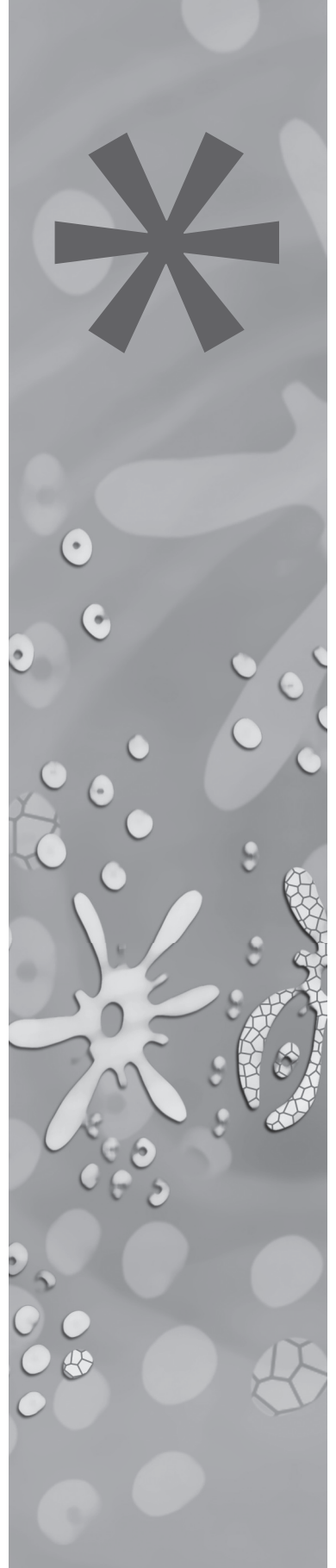
Zo dat is af!!! En hoewel er maar één naam op de voorkant van dit boekje staat, is het natuurlijk niet alleen mijn verdienste. De afgelopen vijf jaar hebben heel veel mensen een bijdrage geleverd aan dit proefschrift en ik ben al die mensen daarvoor enorm dankbaar.

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Fleur

About the author

CURRICULUM VITAE

Marie Fleur du Pré was born on May 4th, 1981 in Delft, The Netherlands. In 1999, she completed secondary school at the Gymnasium Haganum in Den Haag, and, in 2000, she moved to Utrecht to study Biology at Utrecht University. After obtaining her Bachelor degree in Biology in 2003, she started with the master program Infection and Immunity. During her master education she completed a research project at the Eijkman-Winkler Institute for Microbiology and Inflammation of the University Medical Center Utrecht, under the supervision of Dr. Frank Coenjaerts and Prof. dr. Andy Hoepelman. The second lab rotation was performed under the supervision of Prof. dr. Douglas Robinson in the group of Leukocyte Biology at the National Heart and Lung Institute, Imperial College, London, UK. Fleur received the Master of Science degree in October 2005. In December that year, she started as a PhD student in the Laboratory of Pediatric Gastroenterology and Nutrition of the Erasmus MC, Rotterdam, under the supervision of Dr. Janneke Samsom, Prof. dr. Edward Nieuwenhuis and Prof. dr. Bert van der Heijden. The research was concluded in July 2010, and the results are described in this thesis. From July 2010 to January 2011, Fleur was on a sabbatical travelling around South America. From April 2011, she will be working as a Post-doc in the lab of Prof. dr. Ludvig Sollid at the Centre for Immune Regulation, Institute of Immunology, of the Oslo University Hospital – Rikshospitalet in Oslo, Norway.

LIST OF PUBLICATIONS

Anderson, A.E., Mackerness, K.J., Aizen, M., Carr, V.A., Nguyen, D., Du Pre, F., Durham, S.R., and Robinson, D.S. 2009. Seasonal changes in suppressive capacity of CD4$^+$ CD25$^+$ T cells from patients with hayfever are allergen-specific and may result in part from expansion of effector T cells among the CD25$^+$ population. *Clin Exp Allergy* 39:1693-1699.

du Pré, M.F.*, Broere, F.*, van Berkel, L.A., Garssen, J., Schmidt-Weber, C.B., Lambrecht, B.N., Hendriks, R.W., Nieuwenhuis, E.E., Kraal, G., and Samsom, J.N. 2009. Cyclooxygenase-2 in mucosal DC mediates induction of regulatory T cells in the intestine through suppression of IL-4. *Mucosal Immunol* 2:254-264.

du Pré, M.F., and Samsom, J.N. 2010. Adaptive T-cell responses regulating oral tolerance to protein antigen. *Allergy* 2010 DOI:10.1111/j.1398-9995.2010.02519.x.

du Pré, M.F., van Berkel, L.A., Ráki, M., van Leeuwen, M.A., de Ruiter, L.F., Broere, F., ter Borg, M.N.D., Lund, F.E., Escher, J.C., Lundin, K.E.A., Sollid, L.M., Kraal, G., Nieuwenhuis, E.E.S., and Samsom, J.N. 2011 CD62L^{neg}CD38$^+$ expression on circulating CD4$^+$ T cells identifies mucosally differentiated cells in protein fed mice and in human celiac disease patients and controls. *Am J Gastroenterol, accepted for publication january 2011*.

du Pré, M.F., ter Borg, M.N.D., van Berkel, L.A., Lindenbergh-Kortleve, D., Torp Jensen, L., Kooy-Winkelaar, Y., Koning, F., Boon, L., Nieuwenhuis, E.E.S., Sollid, L.M., Fugger, L.*, and Samsom, J.N.* Tolerance to ingested deamidated gliadin in mice is maintained by splenic Tr1-like cells. *Submitted*.

*These authors contributed equally to the work.

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PHD PORTFOLIO

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Presentations	
Oral, Dutch Society for Gastroenterology (NVGE) Spring Meeting, Veldhoven *De novo FoxP3 expression in mucosal regulatory T cells that are generated through mucosal antigen application*	2007
Oral, Annual Meeting Dutch Society for Immunology (NVVI), Noordwijkerhout *Retinoic-acid controlled Cyclooxygenase-2 mediates induction of regulatory T cells in the intestine*	2007
Oral, Molecular Medicine Day (MolMed), Rotterdam *Retinoic-acid controlled Cyclooxygenase-2 mediates induction of regulatory T cells in the intestine*	2008
Oral, World Immune Regulation Meeting, Davos, Switzerland *Retinoic-acid controlled Cyclooxygenase-2 mediates induction of regulatory T cells in the intestine*	2008
Oral, Digestive Disease Week, San Diego, USA *Retinoic-acid controlled Cyclooxygenase-2 mediates induction of regulatory T cells in the intestine*	2008
Oral, Meeting of the European Mucosal Immunology Group (EMIG), Milan, Italy *Retinoic-acid controlled Cyclooxygenase-2 mediates induction of regulatory T cells in the intestine (presented by Janneke Samsom)*	2008
Oral, Annual Meeting Dutch Society for Immunology (NVVI), Noordwijkerhout *Retinoic-acid induced CD38 expression on T cells reflects mucosal imprinting*	2008
Poster, Dutch Society for Gastroenterology (NVGE) Spring Meeting, Veldhoven *Retinoic-acid induced CD38 expression on T cells reflects mucosal imprinting*	2009
Oral, International Coeliac Disease Symposium, Amsterdam *Localization and phenotype of the gluten-specific T cell response after oral gluten intake in mice (presented by Janneke Samsom)*	2009
Oral, Digestive Disease Week, Chicago, USA *Localization and phenotype of the gluten-specific T cell response after oral gluten intake in mice (presented by Janneke Samsom)*	2009
Poster, International Congress of Mucosal Immunology (ICMI), Boston, USA *Retinoic-acid induced CD38 expression on T cells reflects mucosal imprinting*	2009

Presentations	Year
Oral, Annual Meeting Dutch Society for Immunology (NVVI), Noordwijkerhout *Expression of CD4$^+$CD62L^{lo}CD38$^+$ identifies mucosal T cells in peripheral blood*	2009
Oral, Annual Meeting Dutch Society for Immunology (NVVI), Noordwijkerhout *A novel mouse model for coeliac disease reveals that gluten feed does not abide the rules of oral tolerance*	2009

International conferences	
5th Meeting of the European Mucosal Immunology Group (EMIG), Prague, Czech Republic	2006
2nd World Immune Regulation Meeting (WIRM), Davos, Switzerland	2008
Digestive Disease Week 2008, San Diego, USA	2008
13th International Coeliac Disease Symposium, Amsterdam, the Netherlands	2009
14th International Congress of Mucosal Immunology (ICMI), Boston, USA	2009

Seminars and workshops	
2nd Symposium and Masterclasses on Mucosal Immunology (MolMed)	2006
Molecular Diagnostics (MolMed)	2007
Browsing Genes and Genomes with Ensembl (MolMed)	2007
3rd Symposium and Masterclasses on Mucosal Immunology (MolMed)	2008

Grants	
Dutch Society for Immunology (NVVI) Young Investigator Travel Grant	2008
Dutch Society for Gastroenterology (NVGE) Travel Grant	2008, 2009

Award	
Best Workshop Presentation Award, World Immune Regulation Meeting (WIRM), Davos, Switzerland	2008

About the author

NEDERLANDSE
Coeliakie
VERENIGING

De Nederlandse Coeliakie Vereniging (NCV) is de snelst groeiende patiëntenvereniging van Nederland en heeft inmiddels meer dan 11.000 leden. Het doel van de vereniging is het leven met een glutenvrij dieet te vergemakkelijken en de belangen van coeliakiepatiënten te vertegenwoordigen. Tevens spant de NCV zich in om het wetenschappelijk onderzoek naar coeliakie en dermatitis herpetiformis te stimuleren (www.glutenvrij.nl).