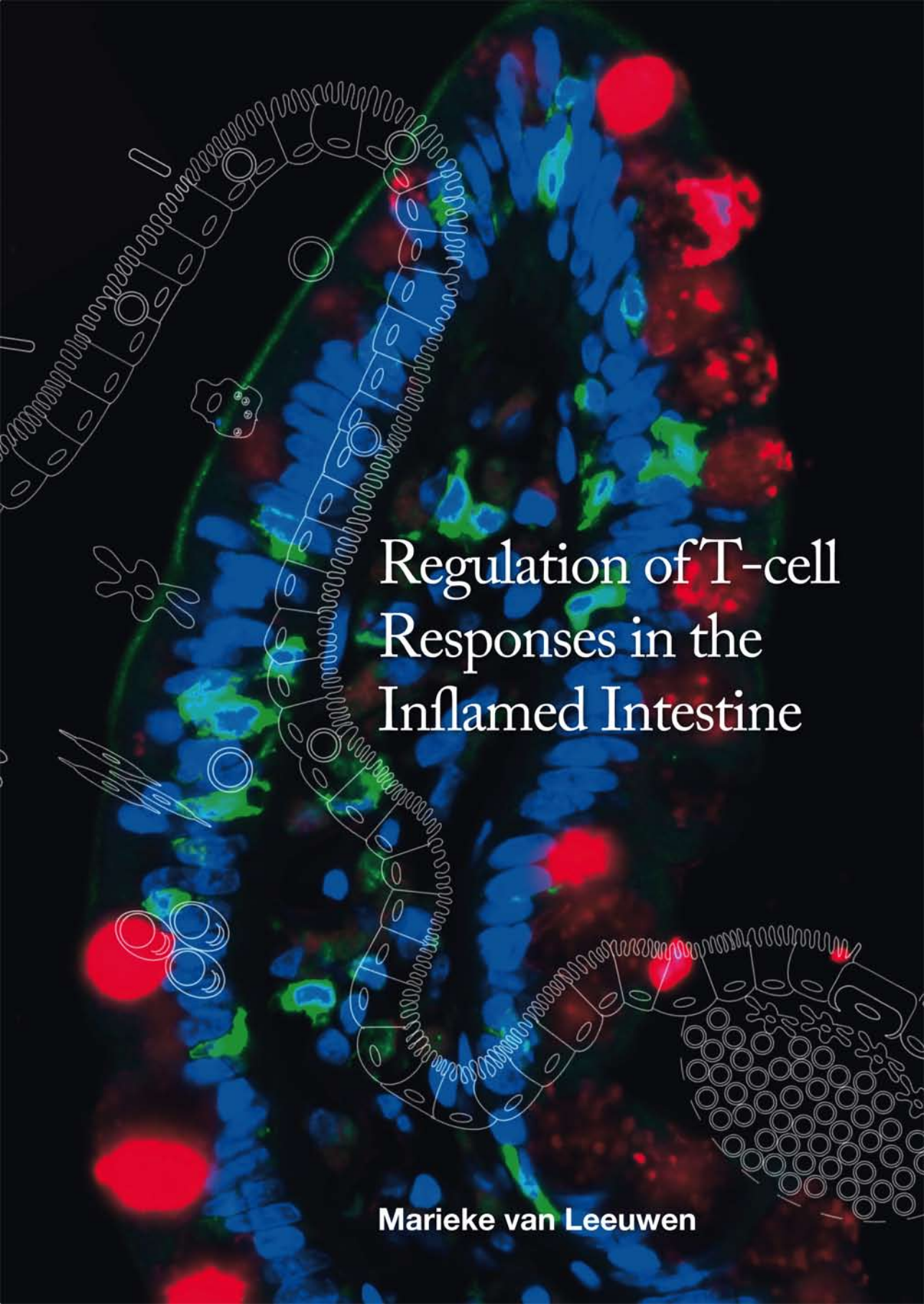

Regulation of T-cell Responses in the Inflamed Intestine

Marieke van Leeuwen

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Copromotor	Dr. J.N. Samsom

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1

General introduction and outline of the thesis

Introduction

The gastrointestinal mucosa is in direct contact with the external environment and continuously exposed to large amounts of foreign antigens from our food and microorganisms. The intestinal immune system protects the mucosal surfaces from intestinal pathogenic microorganisms and on the other hand maintains tolerance towards food antigens and non-pathogenic microorganisms, including the commensal bacteria. Mucosal tolerance is not ignorance to the antigens in the gastrointestinal mucosa or absence of an immune response, but rather a tightly regulated process that depends on the active suppression of immune responses.[1] The balance between the tailored host defense to pathogenic antigens and the maintenance of tolerance to harmless antigens provides an equilibrium that we call intestinal homeostasis.[2]

This intestinal homeostasis is disturbed when the mucosal tolerance to harmless antigens is lost leading to uncontrolled immune responses that become chronic if the antigen persists in the intestinal lumen. An example of an uncontrolled response to food antigens is celiac disease (CD), causing chronic inflammation of the small intestine in response to the food protein gluten.[3] Uncontrolled responses to commensal non-pathogenic bacteria cause inflammatory bowel disease (IBD).[4]

Organization of the intestinal immune system

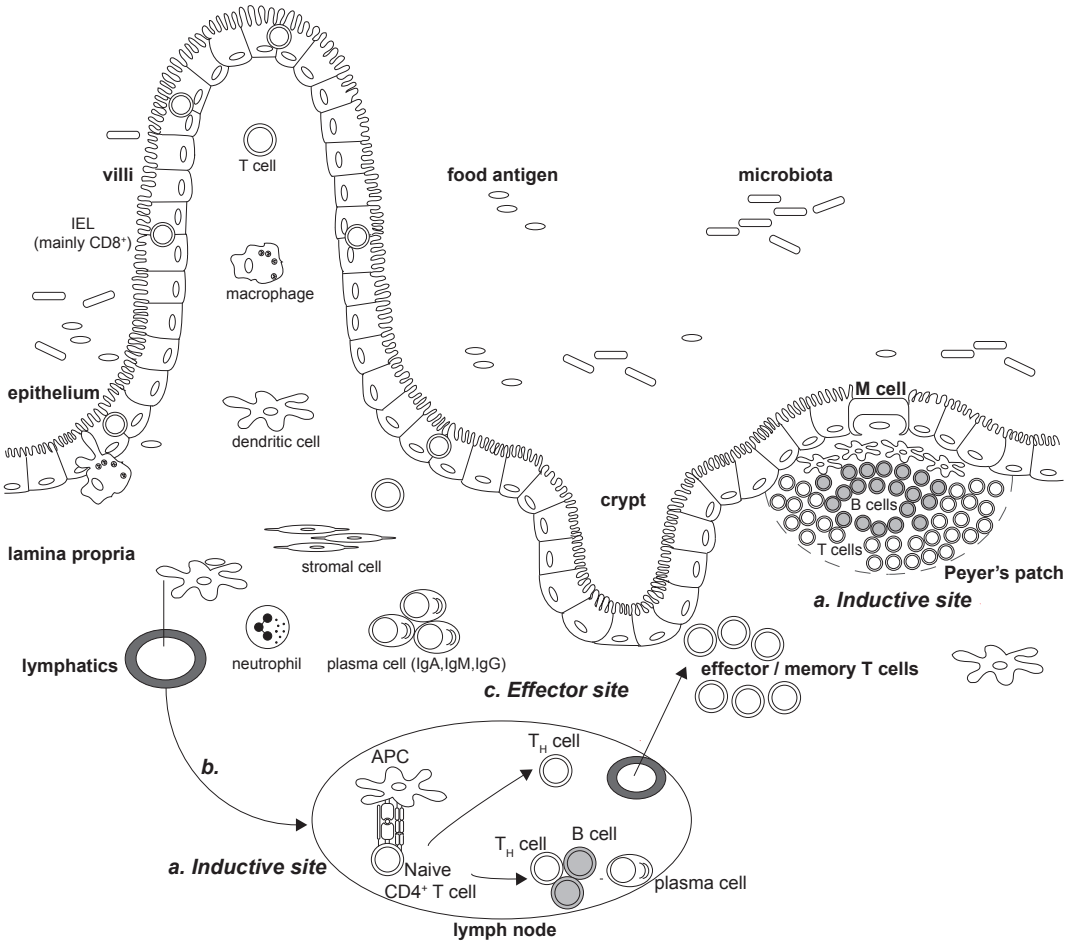
Only a single layer of epithelial cells separates the large numbers of bacteria and food particles in the intestinal lumen from the sterile environment in the intestinal tissue. To regulate the high antigenic exposure, the mucosal immune system contains the largest part of the body's immune system and is home to approximately 30×10^9 lymphocytes.[5] Antigens can be transported across the epithelium and stimulate the mucosal immune system. The lamina propria underneath the epithelium harbors large numbers of antigen presenting cells (APC), like dendritic cells (DC), and macrophages that form the first line of defense and capture and process the antigen. Residing between the immune cells of the lamina propria, tissue stromal cells are abundantly present and form the framework of the intestine.[6]

The intestinal immune system is classically divided into inductive sites and effector sites to emphasize the particular functions of both compartments. The inductive sites consist of organized gut-associated lymphoid tissues (GALT) as well as local mucosa-draining lymph nodes. The GALT consists of Peyer's patches and isolated lymphoid follicles. APC carrying the antigen migrate to the GALT and lymph nodes and secrete cytokines to initiate immune responses. Naive non-antigen experienced T- and B-cells from peripheral blood enter the GALT and mesenteric lymph nodes (MLN) and are activated by the presented antigen. In particular, the T cell receptor recognizes peptides that are derived from antigen and bound to the major histocompatibility complex (MHC) molecules on the APCs. This results in cellular activation, proliferation and differentiation of naive T cells resulting in a pool of

Figure 1 ***Organization of the intestinal immune system.***

(a) *The inductive sites consists of the intestinal immune system consist of organized gut-associated lymphoid tissues (GALT) that lie directly underneath the epithelium as well as the more distant mesenteric lymph nodes. The GALT comprises Peyer's patches and isolated lymphoid follicles. At inductive sites naive B- and T-cells differentiate upon antigen presentation by antigen presenting cells (APCs).* ***(b)*** *APCs pick up the antigen and migrate to the B- and T-cell areas in the mesenteric lymph node or Peyer's patch. Upon APC-T-cell interaction, naive T cells differentiate into effector T_H cells. Differentiated effector T_H cells will migrate to the effector sites, where they reside as memory T cells and rapidly produce cytokines upon re-encounter of their cognate antigen.* ***(c)*** *Effector sites are the sites where effector and memory T cells perform their function once they have differentiated. Effector sites are the surface epithelium, the underlying connective tissue and the lamina propria.*

APC antigen presenting cell; T_H T helper cell, IEL intra-epithelial lymphocyte

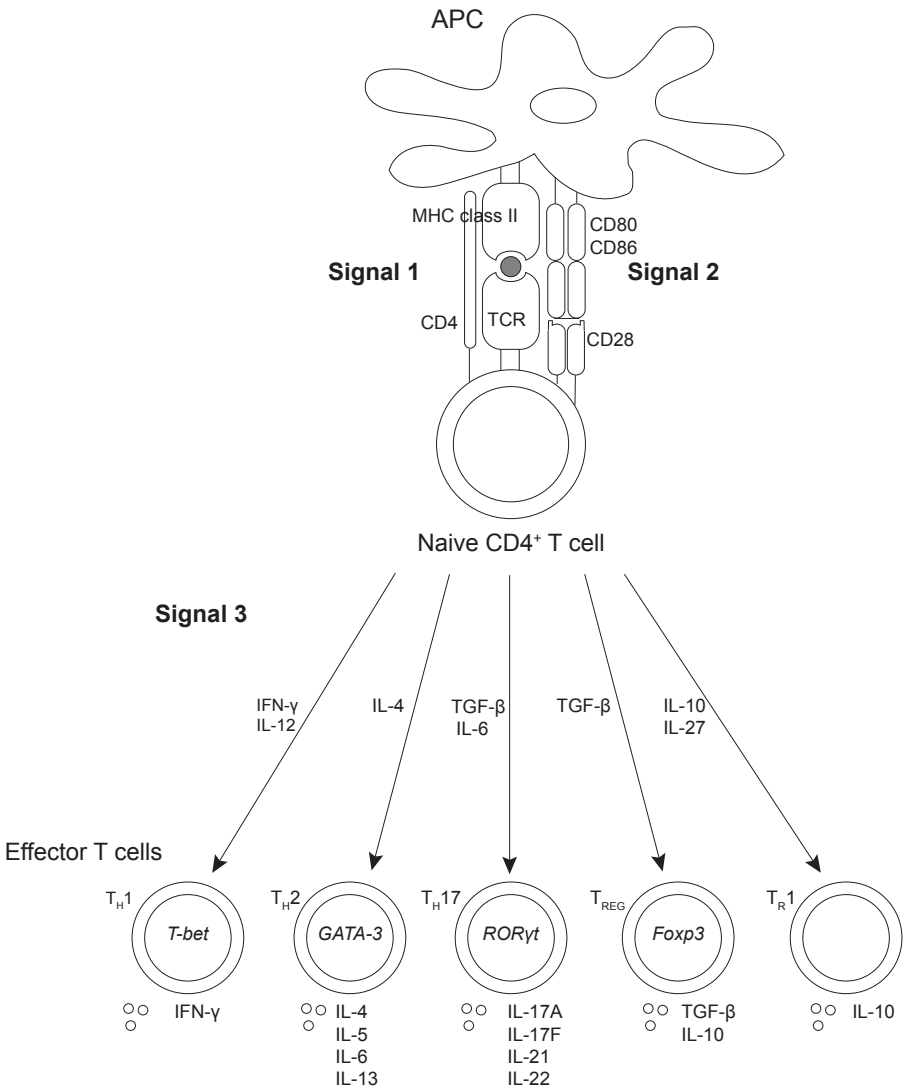


antigen-specific effector and memory T cells. After activation, a proportion of effector and memory T- and B- cells migrate towards the peripheral blood for subsequent extravasation in the intestinal lamina propria, or accumulate in between epithelial cells. These sites where differentiated antigen-specific effector cells perform their action are termed the mucosal effector sites.[7] (*Figure 1*) The migratory process of differentiated cells from the inductive site to the effector sites is directed by a specific profile of cell adhesion molecules and chemokine receptors.[7] For example, effector lymphocytes that differentiate in the MLN acquire high expression levels of the integrin $\alpha_4\beta_7$ and the chemokine receptor CCR9 that allow migration to the small intestine by binding respectively to mucosal vascular addressin (MAdCAM-1) expressed on vascular endothelial cells in the lamina propria and the chemokine (CCL-)25 expressed by epithelial cells.[8,9]

Effector T cells

Depending on the type of antigen and the microenvironmental cytokines, APCs will direct the differentiation of naive precursor T cells into different functional subsets of cytokine-producing cells. Naive T cells require three signals from the APCs for their activation and differentiation. Signal one is the engagement of the peptide-MHC complex from the APCs with the T cell receptor and is essential for activation of the naive T cell. Signal two comes from the membrane-bound costimulatory molecules, such as CD80/CD86 and CD40 on the APC that ligate CD28 and CD40L on the T cell, respectively, which promote or inhibit the survival and expansion of T cells. Besides stimulatory costimulatory molecules, like CD28 and inducible T-cell costimulator (ICOS), inhibitory molecules are expressed on the T cell membrane such as B and T lymphocyte attenuator (BTLA), programmed cell death 1 (PD1) and cytotoxic T lymphocyte antigen4 (CTLA-4). Cytokines provide a third signal that skews the differentiation of the naive T cell favoring a particular functional capacity with respect to cytokine secretion.[10, 11] (*Figure 2*) $CD8^+$ T cells recognize antigen peptides presented by MHC class I molecules, and naive $CD8^+$ T cells differentiate into cytotoxic T cells that directly kill pathogen-infected cells. $CD4^+$ T cells recognize antigen peptides presented by MHC class II molecules and naive $CD4^+$ T cells differentiate towards different effector T helper (T_H) cells, i.e. T_H1, T_H2, T_H17 and the regulatory T (T_{REG}) cells, each with a different immunological function. These T_H cells can be distinguished based on their cytokine profile and the transcription factors they express.[11] T_H1 cells differentiate in presence of the cytokines IFN-γ and interleukin (IL-)12, which promotes the expression of the transcription factor T-bet and results in the production of large amounts of the cytokine IFN-γ.[12] T_H2 cells differentiate in the presence of high levels of IL-4, which promotes the expression of the transcription factor GATA-3. T_H2 cells produce IL-4, IL-5, IL-6 and IL-13.[13, 14] When IL-6 and TGF-β are present, naive $CD4^+$ T cells develop into T_H17 cells. T_H17 cells express the transcription factor RORγt and produce IL-17A, IL-17F, IL-21 and IL-22.[15-18] (*Figure 2*)

Figure 2 ***Activation of naive CD4⁺ T helper cells by antigen presenting cells requires three qualitative signals.*** Signal 1. *Binding of the foreign-peptide:self MHC class II complex of the APC with the matching T cell receptor and the CD4 co-receptor, transmits a signal to the T cell that antigen has been encountered.* Signal 2. *The co-stimulatory signal, to be delivered by the same APC.* Signal 3. *Depending on the cytokines delivered by the APCs, promotes skewing of T-cell differentiation into specific T_H lineage with restricted functional capacity. Lineages are discriminated by a master transcription factor and their specific set of effector cytokines.*
APC antigen presenting cell; T_H T helper cell



Other proposed T_H cell subsets are T follicular helper cells (T_{FH}), which direct humeral responses via the organization of germinal centers, express BCL-6 and produce IL-21[19]; T_H9 cells which produce high levels of IL-9[20, 21] and T_H22 cells which produce IL-22.[22]

It should be noted that the categorization of T_H cells in subsets is practical for immunological research, however, as T cells with intermediate cytokine profiles are often observed it is questionable whether the defined subsets are truly separate lineages.[11] Nowadays T_H cells are seen as a more dynamic population with plasticity to adapt to the local environment.

The selective production of cytokines by the different T_H cells is functionally required as it tailors the cell to a precise immune function, for example to combat intracellular or extracellular microbes, provide help to B cells, activate cytotoxic $CD8^+$ T cells or activate innate immune cells. At the same time as generating effector T_H cells, the primary T-cell response also yields memory T cells. Memory T cells are long-lived cells that after antigen-rechallenge produce a faster and stronger response compared to the primary response.

Regulatory T cells

Besides activating T_H cells, T_{REG} cells can be generated that limit the extent of the immune response.[23, 24] T_{REG} cells are defined by their functional capacity to suppress effector T-cell responses.[25, 26] T_{REG} cells mediate their effects either in a contact-dependent fashion or by secreting cytokines IL-10 and or TGF-β that can inhibit T-cell proliferation.[27] Peripheral T_{REG} cells have different developmental origins. One subset of T_{REG} cells differentiates in the thymus; these are called the thymus-derived or natural T_{REG} cells. These natural T_{REG} cells express the transcription factor forkhead box P3 (Foxp3) and primarily play a role in maintaining self-tolerance and preventing auto-immunity.[25,28] Peripherally-induced or adaptive T_{REG} cells differentiate from naive $CD4^+$ T cells in secondary lymphoid organs or mucosa-associated lymphoid tissue.[29] The intestinal immune system potently supports the generation of induced T_{REG} cells. T_{REG} cells induced within intestinal lymphoid compartments receive homing receptors, which direct these cells to the gut lamina propria where they expand and locally suppress immune responses.[30]

Several subsets of induced T_{REG} cells have been defined, of which induced $Foxp3^+$ T_{REG} cells[31] and $Foxp3^{neg}$ IL-10-producing type 1 regulatory (Tr1) cells[32] are of special importance in the intestine.[33] The differentiation of induced T_{REG} cell involves activation of naive T cells in the peripheral LN in the presence of transforming growth factor (TGF)-β for $Foxp3^+$ T_{REG} cells and IL-27 for Tr1 cells.[34] Differentiated $Foxp3^+T_{REG}$ cells express TGF-β or IL-10 or both. Tr1 cells have immunosuppressive capacity by production of IL-10 but do not express Foxp3. (*Figure 2*) Tr1 cells were first described as IL-10- and IFN-γ-producing T cells that arose *in vitro* after culture with IL-10.[35] The term Tr1 cells is used for all IL-10-secreting T cell populations

with regulatory activity of which the induction is IL-10 dependent.[32] The majority of the T_{REG} cells in the intestine are Foxp3$^+$ but in the human immune system there is currently no reliable marker that fully distinguishes between natural and peripherally-induced T_{REG} cells within this pool.

Oral tolerance

Mucosal tolerance to harmless intestinal antigens is dependent on active suppression of antigen-specific immune responses. The term mucosal or oral tolerance is classically defined as a state of reduced immunologic responsiveness to an antigen induced by oral feeding of that antigen.[36,37] Interestingly, after developing an adaptive tolerogenic T_{REG}-cell response to the soluble antigen, the immune response not only provides tolerance in the intestinal tissue but also suppresses peripheral challenges with the same antigen.

The major mechanisms of the acquired immune system that induce mucosal tolerance are anergy of antigen-specific T cells, deletion of antigen-specific T cells and generation of antigen-specific T_{REG} cells.[38,39] The relative contribution of each of these mechanisms in physiological tolerance to normal food antigens is still unclear, although it has been suggested that single high dosages of soluble antigen may favor anergy and deletion whereas low dosages more effectively induce T_{REG}-cell differentiation.[1,40]

The differentiation of mucosal T_{REG} cells from precursor naive T cells depends on the presentation of orally encountered antigens by APCs. Multiple inductive sites contribute to mucosal T_{REG}-cell differentiation. Within 24 hours after feeding of the soluble egg-white protein ovalbumin T_{REG} cells arise in the Peyer's patches and within 48 hours functional T_{REG} cells have differentiated in the draining MLN.[41] Surgical removal of the MLN impairs the induction of tolerance to ovalbumin, whereas removal of the Peyer's patches did not affect tolerance induction demonstrating that the MLN is the crucial inductive site for oral tolerance.[42,43] A third category of inductive sites that contributes to tolerance comprises systemic sites as the liver and spleen. Antigens that cross the epithelial layer, may enter the circulation and reach the liver, via the portal vein, where oral tolerance can be induced.[44] In addition, we have shown that tolerance to the harmless food protein gluten is induced in the spleen of mice that transgenically express HLA-DQ2 and a gluten-specific humanized T-cell receptor.[45] As gluten is less soluble than ovalbumin and has a very different protein structure it is possible that depending on the nature of the harmless antigen, oral tolerance may arise in a different preferential inductive site.

The mucosal environment of the gut is often denoted as immune privileged as it imposes induction of tolerance to harmless luminal antigens, which would have evoked an immune response if they had been encountered via an systemic route. In recent years some of the pathways that regulate oral tolerance, in particular the generation of induced T_{REG} cells, have been identified. The most well known players in creating the tolerogenic environment are locally acting cytokines, enzymes and metabolites

(e.g. TGF-β, IL-10, cyclooxygenase- (COX-) 2 and retinoic acid) as well as a variety of locally adapted cell types (e.g. DC, macrophages, stromal cells and epithelial cells). TGF-β and IL-10 are anti-inflammatory cytokines that are abundantly expressed in the intestine and produced by many immune cells. Both cytokines are relevant in regulating T-cell responses but can be produced by many cell types in the intestine. TGF-β is a suppressor of T_H1 and T_H2 differentiation and drives the differentiation of naive T cells into T_{REG} cells that prevent autoimmunity. However, in the presence of IL-6, TGF-β has also been found to promote the differentiation of naive T cells into proinflammatory T_H17 cells, which promote autoimmunity and inflammation.[18, 46] Defects of TGF-β lead to severe multi-organ inflammation and early death in mice.[47, 48]

Different $CD4^+$ T_H cell subsets can produce IL-10, including T_H1, $Foxp3^+T_{REG}$, T_H3, T_H17 and Tr1 cells.[49] IL-10 deficiency leads to chronic intestinal inflammation in mice[50] and human[51] and exogenous IL-10 could restore intestinal homeostasis in mice with colitis[52]. In contrast to promoting tolerance to intestinal bacteria, the role for IL-10 appeared to be dispensable for the induction of oral tolerance to ovalbumin.[53] Whether IL-10 is required to maintain the ovalbumin-specific T-cell response during homeostasis in the lamina propria remains to be established.

Another regulatory player in the intestine is COX-2 that synthesizes prostaglandins from arachidonic acid. Stromal cells and a subset of mucosal DC constitutively express COX-2. Inhibiting COX-2 in mice led to loss of tolerance to dietary antigen.[54] Moreover, it was shown that this inhibition was associated with enhanced T-cell-derived IL-4 production and defective T_{REG}-cell differentiation in the MLN, indicating that COX-2 in the MLN-DC is required to control tolerance.[55]

Particular mucosal DC subtypes have intrinsic regulatory properties when compared to non-mucosal DC. For example, DC expressing the integrin CD103 continuously migrate from the lamina propria and Peyer's patches to the MLN.[56] The $CD103^+$ DC induce expression of gut-homing receptors on T cells and induce the differentiation of inducible $Foxp3^+$ T_{REG} cells in the MLN.[57, 58] Compared to splenic DC, mucosal DC promote the differentiation of naive T cells into $Foxp3^+$ T_{REG} cells and are less able to induce T_H17 cells.[59] In the lamina propria, under influence of epithelial cells and possibly stromal cells, DC adopt functional characteristics that are specific for their intestinal mucosal environment. For example, $CD103^+$ DC in the intestinal lamina propria have acquired the enzyme retinal dehydrogenase which has the capacity to metabolize dietary vitamin A into retinoic acid.[60] $CD103^+$ DC-derived retinoic acid can act as a cofactor in the TGF-β-mediated differentiation of naive $CD4^+$ T cells in T_{REG} cells.[30] Other factors present in the mucosa may also contribute to the production of retinoic acid by DC, such as macrophage colony-stimulating factor, IL-4 and ligands for Toll-Like Receptor 2.[61, 62]

Intestinal epithelial cells promote the tolerogenic phenotype of DC via the release of molecules such as the cytokine thymic stromal lymphopoietin (TSLP), TGF-β and retinoic acid.[63, 64]

Retinoic acid not only enhances T_{REG}-cell induction but also induces the expression of the gut homing receptors CCR9 and $\alpha_4\beta_7$ integrin on activated T cells. In addition, it was shown that mucosal DC-derived retinoic acid and TGF-β induced differentiating $CD4^+$ T cells adopt a $CD62L^{neg}CD38^+$ phenotype in the MLN. This mucosally-imprinted $CD62L^{neg}CD38^+$ phenotype is retained by T cells in the circulation thus allowing to distinguish these cells from other $CD4^+$ effector T cells in peripheral blood. In agreement with their microenvironmental imprinting $CD4^+CD62L^{neg}CD38^+$ T cells have enriched expression of the gut-homing CCR9 and β7 integrin. This mucosal imprinting not only occurs in mice as $CD62L^{neg}CD38^+CD4^+$ T cells can be identified in humans.[65]

In addition to the lamina propria, stromal cells in the mucosa-draining lymph nodes form a tolerogenic environment by expressing retinoic acid converting enzymes.[66] It was shown that resident stromal cells in the MLN were essential for the induction of CCR9 expression on T cells.[67]

All the above-mentioned factors contribute to effective regulation of mucosal tolerance by T cells. To what extent Tr1 cells or T_{REG} cells or both are pivotal for intestinal homeostasis is not fully understood. The role of Foxp3 expressing T_{REG} cells in oral tolerance is best characterized. Interestingly, on the basis of murine models it has been hypothesized that natural T_{REG} cells are dispensable for protein specific oral tolerance.[68] In contrast, oral tolerance to the dietary protein ovalbumin could not be generated in murine models without induced T_{REG} cells,[69, 70] suggesting that inducible T_{REG} cells are essential for mucosal tolerance. The lack of specific markers for Tr1 cells has hampered assessment of their role in oral tolerance. In mice it was shown that Tr1 cells inhibited T-cell transfer colitis via an IL-10 dependent manner.[35] Moreover, as multiple $CD4^+$ effector T_H cell subsets can produce IL-10 in a specific phase of differentiation there is a possibility that the Tr1-cell function was attributed to effector T_H cells that had temporarily downregulated proinflammatory cytokine release but maintained their IL-10 production.[71]

After differentiation, T cells home back to the lamina propria by expressing gut-homing receptors. It was shown that expression of $\alpha_4\beta_7$ and CCR9 on T cells were essential to induce tolerance.[72, 73] However oral tolerance induction in the CCR9 deficient mice was influenced by the composition of microbiota.[74] IL-10 produced in the lamina propria is needed to drive or maintain $Foxp3^+$ T_{REG} cells. Under influence of IL-10 produced by $CXCR1^{high}$ macrophages in the lamina propria T_{REG} cells undergo secondary expansion.[70] In addition, IL-10 production by $CD11b^+$ myeloid cells is needed to maintain Foxp3 expression in the colon when inflammation occurs.[75]

Mucosal tolerance of the intestine is best demonstrated by experiments examining the tolerance to dietary proteins. Because commensal bacterial antigens are likely encountered in the context of these same tolerogenic responses, it has been proposed that the gastrointestinal tract also promotes the differentiation of T_{REG} cells specific for commensal bacterial antigens. Indeed, in specific-pathogen-free mice the repertoire T

cell antigen receptor (TCR) expressed by colonic T_{REG} cells was shown to be different from the TCR repertoire used by T_{REG} cells in other organs. These colonic T_{REG} TCR were responsive to stimulation with commensal bacteria from the mice and transfer of the T_{REG} TCR in thymocytes did not drive thymic T_{REG}-cell selection arguing that common colonic T_{REG} TCRs do not derive from natural T_{REG}. The latter data argue that colonic T_{REG} cell differentiate in response to the microbiota of the host. Currently, several bacterial antigens that support such differentiation of microbiota-specific T_{REG} cells have been identified.[76,77]

Breakdown of intestinal homeostasis

Celiac disease

In the 1940s, the Dutch pediatrician Willem-Karel Dicke discovered that CD was caused by intake of gluten and in consequence Dicke introduced the gluten-free diet.[78] Gluten proteins can be found in wheat, barley and rye. CD is the most common intolerance to a food-protein and occurs in adults and children in approximately 1-2% of the population. CD patients typically present with diarrhea, weight loss, steatorrhea and symptoms of malnutrition like anaemia and vitamin deficiencies.[79]

Immunologically, CD is driven by an inflammatory T-cell response to dietary gluten proteins resulting in a small intestinal enteropathy in genetically predisposed individuals.[80] The small intestinal enteropathy is characterized by villous atrophy, crypt hyperplasia and increased numbers of infiltrating lymphocytes in epithelium and the lamina propria. CD lesions are histopathologically classified by the Marsh score (Marsh 0: normal small intestinal mucosa, Marsh 1: increased numbers of intra-epithelial lymphocytes (IEL), Marsh 2: increased numbers of IEL with crypt hyperplasia, Marsh 3: increased numbers of IEL, crypt hyperplasia and villous atrophy).[81,82] There is a strong genetic susceptibility for CD since virtually all CD patients carry the MHC class II HLA-DQ2 gene and most of the HLA-DQ2-negative patients carry HLA-DQ8.[79] In the context of HLA-DQ2 and DQ8 molecules, $CD4^+$ T cells recognize deamidated gluten epitopes.[83-86] Inflammatory gluten-specific $CD4^+$ T cells can be isolated from the small intestinal mucosa of CD patients but not from healthy individuals with the same genotype.[87] The inflammatory gluten-specific $CD4^+$ T cells, producing the pro-inflammatory cytokines IFN-γ and IL-21, are thought to play a role in the mucosal damage in CD patients.[88,89] The small intestinal mucosa of CD patients is characterized by the presence of high levels of pro-inflammatory cytokines, such as IFN-α [90], IL-15 [91], IL-17 [92,93], IL-18 [94] and IL-21 [95] that may disrupt the tolerogenic environment of the gut. The expression levels of the anti-inflammatory cytokines IL-10 [96,97] and TGF-β [98] are not decreased in the mucosa of CD patients. From mouse models we know that the adaptive inflammatory immune response alone is not sufficient to induce disease. Transgenic mice that carry HLA-DQ2/DQ8 on

the APCs and have humanized gluten-specific CD4+ T cells do not develop CD pathology upon gliadin feed[45, 99], but instead gluten-specific tolerance was mounted in the spleen where suppressive IL-10-secreting type 1 regulatory T (Tr1) cells were formed.[45] This emphasizes the importance of other factors, like the innate immune system or environmental triggers, in the breach of tolerance to gluten.

Currently, for CD patients the only treatment consists of a life-long gluten-free diet, resulting in complete remission and recovery of the normal small intestinal architecture.[87, 88] However, a severe complication occurs in a small proportion of CD patients who become unresponsive to the gluten-free diet and develop refractory CD.[100, 101] In addition a subgroup of refractory CD patients, denoted as type II, have a higher risk to develop an enteropathy-associated T-cell lymphoma.[102]

Despite the increased knowledge on the CD pathogenesis it is still unclear why tolerance to gluten is so often lost and why some people with an HLA-DQ2 genotype develop CD while others do not. It is remarkable that 25% of the western European population is HLA-DQ2 positive and only 1% will develop CD.

Inflammatory bowel disease

Inflammatory bowel disease (IBD) is a chronic relapsing disease driven by an inflammatory T-cell response to intestinal microbiota and consists of two entities, namely Crohn's disease (Crohn) and ulcerative colitis (UC). The incidence of IBD in the United States and Europe is approximately 5-15 per 100,000/year.[103] Most IBD patients have clinical manifestations like diarrhea, rectal bleeding, abdominal pain and growth failure when affected as a child. Crohn typically results in lesions that show a transmural inflammation and can affect every part of the gastrointestinal tract. UC lesions are more superficial and inflammation is invariably restricted to the colon. Unlike in UC, inflammatory lesions in Crohn may be patchy and segmental. IBD has no currently available cure and life-long immune suppressive therapy and surgical interventions may be required.[104, 105]

The precise etiology of IBD remains unknown, but three interacting factors are thought to play a causative role. These three factors are genetic susceptibility, microbiota and the intestinal immune system.[106] It is hypothesized that IBD stems from an inappropriate exaggerated T-cell response to a subset of harmless commensal bacteria that arises in a genetically predisposed host.[104] Furthermore, there is strong evidence that the induction of IBD involves genetic defects in mucosal barrier function and the innate immune system.[107] These genetic defects are thought to lead to impaired clearance of bacteria that have penetrated the epithelial layer, which in turn activates the adaptive immune system. In these patients insufficient host defense to microbiota may cause IBD.

CD4+ T cells play a key role in the pathogenesis of IBD as high numbers of T cells infiltrate the inflamed mucosa, the secretion of large amounts of T-cell-derived cytokines and the requirement for T cells in various animal models of chronic intestinal inflammation.[108, 109] However, the best proof of involvement of the adaptive immune

system in IBD comes from effective T-cell suppressing therapy in the majority of patients using biological agents such as azathioprine or monoclonal antibodies against tumor necrosis factor alpha (TNFα) which elicits T-cell apoptosis.[110] Traditionally it was believed that UC en Crohn exhibited different immunological patterns. It was thought that immune responses in UC has bias towards a T_H2 profile with increased expression of IL-13 while Crohn was characterized by a T_H1 profile with increased expression of IFN-γ and IL-12.[111-113] Recent studies suggest a more complex overlapping immune response with a role for T_H17 cells in both diseases. T_H17 cells produce the pro-inflammatory cytokine IL-17 and are closely associated with the cytokine IL-23.[114] IL-23 signaling in T_H17 cells can enhance their pathogenic activity and promote the production of proinflammatory cytokines and suggest that IL-23-dependent signaling in T_H17 cells is important for their pathogenic activity.[115] In murine IBD models, depletion of IL-23 caused a reduced T cell dependent colitis in T-cell transfer models of IBD.[116, 117] Genome wide association studies demonstrated that IL-23 receptor coding variants might contribute to both Crohn and UC susceptibility.[118] T_H1 and T_H17 cells are important players in intestinal inflammation as they are responsible for the pro-inflammatory cytokine release and tissue damage.[109, 119]

In a healthy situation the inflammatory T-cell response towards commensal bacteria are kept in check through active regulation by T_{REG} cells and anti-inflammatory cytokines like IL-10 and TGF-β. Indeed, in mice and human deletions and loss of function mutations in the *Foxp3* gene result in fatal inflammatory disease including colitis.[120, 121] The intestinal pathology seems to result from inability to regulate the $CD4^+$ effector T-cell responses.[121] The critical role of the cytokines IL-10 and TGF-β as immunosuppressive cytokines has been demonstrated in animal models; for example mice with defects in the IL-10 or TGF-β regulation have aberrant T-cell response to commensal bacteria leading to severe intestinal inflammation of the intestine.[50] In humans, mutations in the *IL-10* gene or genes coding for proteins in the IL-10 signaling pathway lead to loss of IL-10 function and cause severe intractable enterocolitis in infants and small children.[51] In addition to this, single nucleotide polymorphisms in the *IL-10* gene region are associated with IBD.[122]

Despite an evident connection between IBD and T-cell responses it needs further investigation how an immunological response to commensals could cause IBD and how in homeostasis commensal-specific T cells are regulated.

Aim and outline of the thesis

The focus of this thesis lies on identifying immune processes that regulate T-cell responses in the intestine. As described above, T cells play an important role in the development of CD and IBD. Balance between the function of T_{REG} cells and the $CD4^+$ effector T cells is crucial for intestinal homeostasis. Better knowledge on T-cell responses is essential for understanding the etiology and pathophysiology of intestinal

inflammatory diseases and for the development of novel therapies. The general aim of this thesis is to provide further insight into defective regulation of T-cell responses leading to intestinal inflammation.

Decreased numbers of T_{REG} cell populations could be an explanation for the loss of tolerance in intestinal inflammation. In **chapter 2**, we investigated whether numeric deficiency of mucosally-induced ($CD62L^{neg}CD38^{+}Foxp3^{+}$) or natural ($CD62L^{+}Foxp3^{+}$) T_{REG} cells can be detected in peripheral blood of CD patients. We compared peripheral blood cells from pediatric, adult treated CD and adult refractory CD patients with non-CD controls. In addition, frequencies of $Foxp3^{+}$ cells were investigated in tissue biopsies of pediatric and adult CD patients.

Although IL-10 is known to be crucial for maintaining intestinal immune homeostasis, its mechanisms of action in the human intestine are not well studied. In **chapter 3** we investigated the functional consequences of defective IL-10 signaling on innate cells and effector T cells. We describe a now 11-year-old patient who developed early-onset enterocolitis due to a mutation in the IL-10 receptor gene. We have identified mechanisms by which IL-10 regulates immune responses and maintains intestinal immune homeostasis.

Inflammatory $CD4^{+}$ T-cell responses to dietary gluten cause CD pathology. In **chapter 4**, the role of the pro-inflammatory $CD4^{+}$ T-cell derived cytokines IL-21 and IL-17A in the onset of CD and severity of lesion formation is described. For this we studied expression of these cytokines in cells from peripheral blood and mucosal tissue using a cohort of new-onset pediatric CD patients with different histopathological scores. In addition, we tested whether specific microbial stimulation leads to IL-21 or IL-17A production in $CD4^{+}$ T cells.

Duodenal myofibroblasts are abundantly expressed in the lamina propria, however their phenotype and immune function have not been studied. In **chapter 5** we report that duodenal myofibroblasts are immunologically active. Using isolated duodenal myofibroblasts from CD and non-CD patients we aimed to clarify the mode of activation, their cytokine en chemokine profile and their interaction with T cells.

In **chapter 6**, we aimed to identify the mechanisms by which orally administered gliadin elicits gliadin-specific T-cell proliferation and differentiation of Tr1 cells in the spleen. In particular, we investigated the role of macrophages in this process. We used transgenic mice expressing HLA-DQ2 and a gluten-specific humanized T-cell receptor to study the mechanisms of immune response to gluten feed in mucosa-associated lymphoid tissue and the spleen.

Finally, **chapter 7** provides a general discussion of the data described in this thesis.

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2

Changes in natural Foxp3^{+}Treg but not mucosally-imprinted CD62L^{neg}CD38^{+}Foxp3^{+}Treg in the circulation of celiac disease patients

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ABSTRACT

BACKGROUND Celiac disease (CD) is an intestinal inflammation driven by gluten-reactive $CD4^+$ T cells. Due to lack of selective markers it has not been determined whether defects in inducible regulatory T (T_{REG}) cell differentiation are associated with CD. This is of importance as changes in numbers of induced T_{REG} cells could be indicative of defects in mucosal tolerance development in CD.

Recently, we have shown that, after encounter of retinoic acid during differentiation, circulating gut-imprinted T cells express $CD62L^{neg}CD38^+$. Using this new phenotype, we now determined whether alterations occur in the frequency of natural $CD62L^+Foxp3^+$ T_{REG} or mucosally-imprinted $CD62L^{neg}CD38^+Foxp3^+$ T_{REG} cells in peripheral blood of CD patients. In particular, we compared pediatric CD, aiming to select for disease at onset, with adult CD.

METHODS Cell surface markers, intracellular Foxp3 and Helios were determined by flow cytometry. Foxp3 expression was also detected by immunohistochemistry in duodenal tissue of CD patients.

RESULTS In children, the percentages of peripheral blood $CD4^+Foxp3^+$ T_{REG} cells were comparable between CD patients and healthy age-matched controls. Differentiation between natural and mucosally-imprinted T_{REG} cells on the basis of CD62L and CD38 did not uncover differences in Foxp3. In adult patients on gluten-free diet and in refractory CD increased percentages of circulating natural $CD62L^+Foxp3^+$ T_{REG} cells, but normal mucosally-imprinted $CD62L^{neg}CD38^+Foxp3^+$ T_{REG} cell frequencies were observed.

CONCLUSIONS Our data exclude that significant numeric deficiency of mucosally-imprinted or natural $Foxp3^+$ T_{REG} cells explains exuberant effector responses in CD. Changes in natural $Foxp3^+$ T_{REG} cells occur in a subset of adult patients on a gluten-free diet and in refractory CD patients.

Introduction

Celiac disease (CD) is a chronic inflammatory disease of the small intestine that develops in genetically susceptible individuals in response to the ingestion of gluten from wheat, barley and rye. Inflammatory gluten-specific $CD4^+$ T cells that are restricted to HLA-DQ2 or HLA-DQ8 molecules can be isolated from the small intestinal mucosa of CD patients but not from healthy individuals.[1, 2] These inflammatory gluten-specific T cells produce large amounts of interferon-γ[3] and are expected to be key contributors to intestinal tissue damage. Currently, the only treatment 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 celiac disease (RCD). RCD is defined by the identification of malabsorption and persisting duodenal villous atrophy, despite adherence to a GFD and absence of other enteropathies.[4, 5] A subgroup of RCD patients, denoted as type II, have aberrant populations of T cells lacking the surface expression of CD3 rendering these patients at high risk to develop an enteropathy-associated T-cell lymphoma (EATL).[6]

Despite our increasing knowledge of the pathogenesis of CD, it is still unclear why oral tolerance to gluten is so often lost and why the excessive pro-inflammatory T-cell response in CD is not suppressed by a regulatory T-cell response. Several regulatory T (T_{REG}) cell subsets have been described to be important for immune tolerance. On the basis of their origin they can be divided in thymus-derived natural T_{REG} cells and the peripherally induced T_{REG} cells.[7] Both subsets share the transcription factor forkhead box P3 (Foxp3). Natural T_{REG} cells primarily maintain tolerance to self-antigen and prevent autoimmunity.[8] On the basis of murine models it has been postulated that natural T_{REG} cells are dispensable for protein specific oral tolerance.[9] In contrast, induced T_{REG} cells that have differentiated from naive T cells in the tolerogenic environment of the gut draining lymph nodes can mediate protein specific oral tolerance in these models.[10-14] Currently, it is technically impossible to study gluten-specific T_{REG} in patients. However, changes in peripheral blood $Foxp3^+$ T_{REG} cells and lamina propria $Foxp3^+$ cells have been reported in CD patients. Most studies describe an increase of $Foxp3^+$ cells in CD patients compared to controls either in peripheral blood [15] or in the small intestinal mucosa.[16-20] However, in other studies, no difference in $Foxp3^+$ cells was observed between CD patients and controls.[21-23] As CD patient populations with different ages and clinical status were investigated and because of the large variability in data we hypothesize that changes in $Foxp3^+$ T cells may be transient and possibly related to a particular subset of CD patients.

Earlier studies have not investigated whether the increase of circulating $Foxp3^+$ T_{REG} cells occurred in the natural T_{REG} cell or induced T_{REG} cell population. This is of importance as changes in numbers of induced T_{REG} cells could be indicative of defects in mucosal tolerance development in CD. Previously, there was a lack

of cell surface markers to distinguish mucosally-imprinted T_{REG} cells from natural T_{REG} cells. Recently, we have demonstrated that mucosally-imprinted $CD4^+$ T cells can be identified by the expression of $CD62L^{neg}CD38^+$.[24] In mice, this mucosal $CD62L^{neg}CD38^+$ T cell phenotype is efficiently induced during differentiation in the gut-draining lymph node, which can be mimicked by differentiation in the presence of retinoic acid (RA) and TGF-β. For human $CD4^+$ T cells,[24] RA alone is sufficient for $CD62L^{neg}CD38^+$ induction. In human peripheral blood these mucosally-imprinted $CD4^+CD62L^{neg}CD38^+$ T cells have enriched expression of the gut-homing chemokine receptor C-C chemokine receptor type 9 (CCR9) and β7-integrin whereas expression of the skin-homing marker cutaneous leukocyte-associated antigen is almost absent.[24] Crucially, staining of peripheral blood from CD patients who underwent a challenge revealed that virtually all gluten-specific DQ2 tetramer-positive T cells had the $CD62L^{neg}CD38^+$ phenotype.[24] With this new set of markers we can now distinguish between the mucosally-imprinted $Foxp3^+$ T cells and the non-mucosally-imprinted cells.

In this study, we determined whether alterations in the frequency of circulating mucosally-imprinted $CD62L^{neg}CD38^+Foxp3^+$ T_{REG} cells are detected in CD patients. We analyzed the percentage of $Foxp3^+$ T_{REG} cells in the whole circulating $CD4^+$ T cell population and within the mucosally-imprinted $CD62L^{neg}CD38^+$ population. In view of the variability in the results of previous $Foxp3^+$ quantification we focused our analysis on a relatively homogenous patient population of pediatric untreated CD patients aiming to select for disease at onset. For comparison we analyzed adult patients with RCD, adult patients receiving a GFD and compared them with healthy adult controls.

Patients and methods

Patients (see Table 1 and 2)

Pediatric patients who underwent an esophagogastroduodenoscopy (EGD) with suspicion of CD were approached for participation in our study at the Erasmus Medical Centre – Sophia Children' s Hospital, Rotterdam, The Netherlands. After diagnosis, those with biopsy-proven CD and positive autoantibodies (n=34) or with a Marsh score 1-2, positive autoantibodies and response to GFD (n=2) were included in the patient group (n=36). Whereas children with a normal intestinal histology and negative autoantibodies were included in the control group (n=20). Patients diagnosed with other diseases were excluded from the study. Adult patients with treated CD (i.e. CD patients responding to a GFD) and RCD (i.e. CD patients not responding to a GFD) from the VUMC, Amsterdam, The Netherlands were included in the study. The studies were approved by the medical ethical committee of the Erasmus Medical Centre and all participants or parents of participants gave written informed consent before enrollment.

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), CCR9 (248621, R&D Systems, Abingdon, UK). Intracellular staining was performed with the Foxp3 staining buffer kit, according to manufacturer's protocol (eBioscience), followed by Foxp3 (PCH101, EMELCA Bioscience, Bergen op Zoom, The Netherlands), Helios (Biolegend, San Diego, US) and the appropriate isotype controls. Flow cytometric analysis was performed on a FACSCanto™II (BD-Biosciences).

Immunohistochemistry

Immunohistochemical Foxp3 stainings were performed on paraffin-embedded duodenal biopsies as described previously.[25] Prior to staining the samples were blocked with 10% normal human serum in a solution containing Tris buffer (10 mM), EDTA (5mM), NaCl (0.15 M), gelatine (0.25%) and Tween-20 (0.05%). Tissue sections were incubated overnight using the primary antibody to Foxp3 (eBioscience) or isotype control mouse IgG_1. Sections were incubated with VECTASTAIN ABC Elite Kit (Vector Laboratories) and stained with biotinylated horse-anti-mouse (Vector Laboratories) as described previously.[25]

Table 1 *Demographic features of pediatric celiac disease (CD) and controls*

		Pediatric CD	Controls
Number		36	20
Age in years, mean		5.9	6.1
Male, n (%)		n=9 (25%)	n=8 (40%)
Marsh score, n (%)	Marsh 0	n=0 (0%)	n=20 (100%)
	Marsh 1	n=2 (6%)	n=0 (0%)
	Marsh 2	n=0 (0%)	n=0 (0%)
	Marsh 3A	n=9 (25%)	n=0 (0%)
	Marsh 3B	n=17 (47%)	n=0 (0%)
	Marsh 3C	n=8 (22%)	n=0 (0%)

Cytokine analysis

IL-15 concentrations in plasma were analyzed using an enzyme-linked immunosorbent assay set (BD Biosciences) according to the manufacturer's instructions.

Statistics

Linear regression analysis was performed using Prism software (GraphPad, Software Inc) the Kruskal-Wallis one-way analysis of variances. Differences between multiple groups were first evaluated by ANOVA using the Kruskal-Wallis test. Differences between groups were analyzed using the Mann-Whitney *U* test. $P < 0.05$ was considered statistically significant.

Table 2 *Demographic features of adult celiac disease (CD) patients and controls.*

		Refractory CD	Treated CD	Healthy controls
Number		14	13	14
Age in years, mean (SD)		60.5 (11.0)	46.1 (14.7)	36.5 (11.5)
Male, n (%)		6 (43%)	6 (55%)	4 (29%)
Positive antibodies				
(EmA,tTG), n (%)		1 (7%)	0 (0%)	not detected
Marsh score, n (%)	Marsh 0	6 (43%)	7 (54%)	not determined
	Marsh 1	3 (21%)	6 (46%)	
	Marsh 2	0 (0%)		
	Marsh 3A	2 (14%)		
	Marsh 3B	2 (14%)		
	Marsh 3C	1 (7%)		
Diagnosis, n (%)	RCD 1	7 (50%)		
	RCD 2	5 (35%)		
	EATL	3 (21%)		
Treatment, n (%)	No	0 (0%)	0 (0%)	14 (100%)
	GFD	13 (93%)	13 (100%)	
	Immuno-regulatory drugs	9 (64%)	0 (0%)	
	Unknown	1 (7%)	0 (0%)	

CD, celiac disease; EmA, anti-endomysial antibodies; tTG anti-tissue transglutaminase antibodies; RCD, refractory celiac disease; EATL, enteropathy-associated T cell lymphoma; GFD, gluten free diet.

Results

Pediatric patients

As we hypothesize that changes in numbers of circulating Foxp3$^+$ T cells may be transient and possibly different in pediatric and adult CD, we first collected a well-defined cohort of newly diagnosed pediatric patients. Blood was drawn from children aged 1 to 17 years who underwent an EGD with a suspicion of CD that were not on a GFD. After diagnosis, those with biopsy-proven CD and positive autoantibodies (n=34) or with a Marsh score 1-2, positive autoantibodies and response to GFD (n=2) were included in the patient group (n=36). Whereas children with a normal intestinal histology and negative autoantibodies were included in the control group (n=20). CD patients were diagnosed with Marsh 1 (2 patients), Marsh 3A (9 patients), Marsh 3B (17 patients) or Marsh 3C (8 patients). Patients who were diagnosed with other diseases were excluded from analyses. There was no difference in age between pediatric CD patients and control patients. Demographic features are summarized in *Table 1*.

No difference in numbers of circulating T_{REG} cells or mucosally-imprinted T_{REG} cells between pediatric CD patients and controls

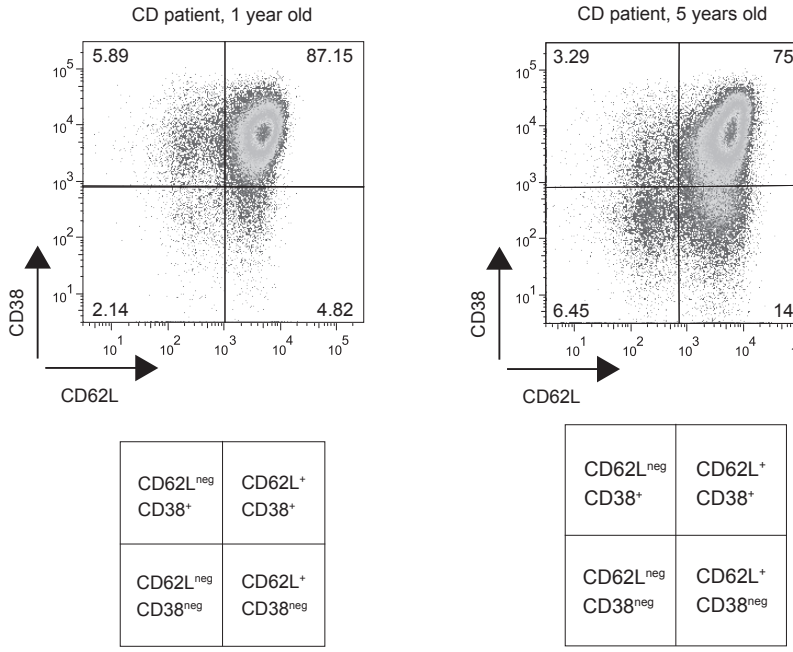
Previously, we have established that mucosally-imprinted T cells in peripheral blood can be identified by the expression of CD62L^{neg}CD38$^+$.[24] As this is the first cohort of CD patients in which the phenotype of the mucosal the CD62L^{neg}CD38$^+$ population was extensively analyzed we controlled for several other markers to validate the use of the CD62L^{neg}CD38$^+$ phenotype in this cohort. It is known that the distribution of lymphocyte subsets in peripheral blood varies with age.[26] Therefore, 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 1a*. 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 5 (56.6% ± SD 18.02, *Figure 1b*). In contrast to the other CD62L/CD38 subsets, the population of CD4$^+$CD62L^{neg}CD38$^+$ mucosally-imprinted T cells was not subjected to age-related changes (*Figure 1b*). Hence, no age distinction was made for analysis of the pediatric CD patient group for the CD4$^+$CD62L^{neg}CD38$^+$ cells. In addition, no differences were found between pediatric patients and controls in the overall percentage of mucosally-imprinted CD62L^{neg}CD38$^+$ T cells within the total CD4 population (*Figure 2a*). The percentages of gut-homing CCR9 positive cells did not differ between patients and controls (*Figure 2b*). The frequency of naive CD45RA$^+$ cells within the mucosal population was also similar in both groups (*Figure 2c*). It should be noted that the group of CD patients had a significantly higher WBC count in comparison with the control group (*Figure 2d*). However, all WBC subpopulations

were slightly increased and no selective enhancement of $CD3^+$ or $CD4^+$ cells was seen in patients when compared to controls (data not shown).

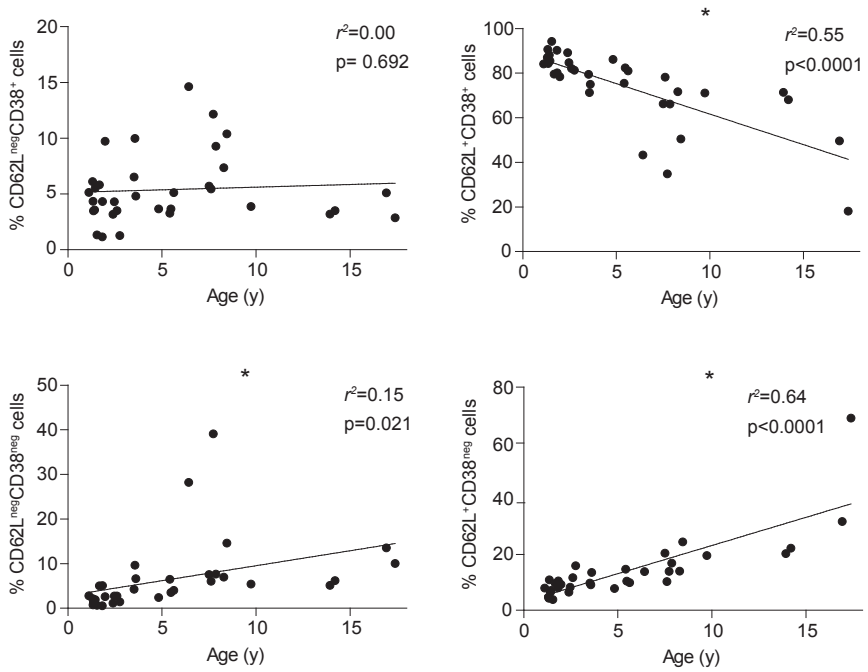
To establish whether overall differences in the frequency of $Foxp3^+$ cells were detectable we determined the percentage of $Foxp3^+$ T cells within the total $CD4^+$ T cell population. No difference in the percentage of $Foxp3^+$ cells within the total $CD4^+$ T cell population was detected between the pediatric CD patient group and the control group (*Figure 2e*). As only 4% of peripheral blood $CD4^+$ T cells have the $CD62L^{neg}CD38^+$ mucosal phenotype the sensitivity of detection of differences in the mucosally-imprinted Foxp3 population is greatly enhanced. Therefore in our pediatric CD cohort we determined whether these circulating mucosal T_{REG} cells were altered in frequency. Despite a clear detectable population of $Foxp3^+$ within the $CD62L^{neg}CD38^+$ population, no difference in the percentage of Foxp3 was detected in

Figure 1 ***Distribution of CD62L/CD38 subsets within CD4⁺ peripheral blood lymphocytes; changes with age.*** *Peripheral blood from pediatric CD patients was stained for flow cytometric analysis.* ***(a)*** *Representative gating strategy for analysis of CD62L/CD38 subsets within the CD4⁺ T cell population. Two representative CD62L/CD38 analyses of peripheral blood CD4⁺ T cells in CD patients aged 1 and 5 years old.* ***(b)*** *The percentages of cells in each of the four CD62L/CD38 T-cell subsets were calculated (Kruskal-Wallis test). CD, celiac disease.*

1a



1b



the CD62L^{neg}CD38^{+} mucosally-imprinted T cell subset upon comparison of pediatric CD with controls (*Figure 2f*). In addition, no difference was found in the percentage of CCR9^{+} cells within the mucosally-imprinted CD62L^{neg}CD38^{+} Foxp3^{+} T cell subset from patients (26.5% ± SD 13.7) when compared to controls (31.0% ± SD 25.4). These data infer that patient and control mucosally-imprinted T_{REG}s should have similar capacity to receive CCR9-mediated CCL25 signals. Also no differences in the percentages of Foxp3 were observed in the other three CD62L and CD38 subsets of pediatric CD patients versus controls (data not shown).

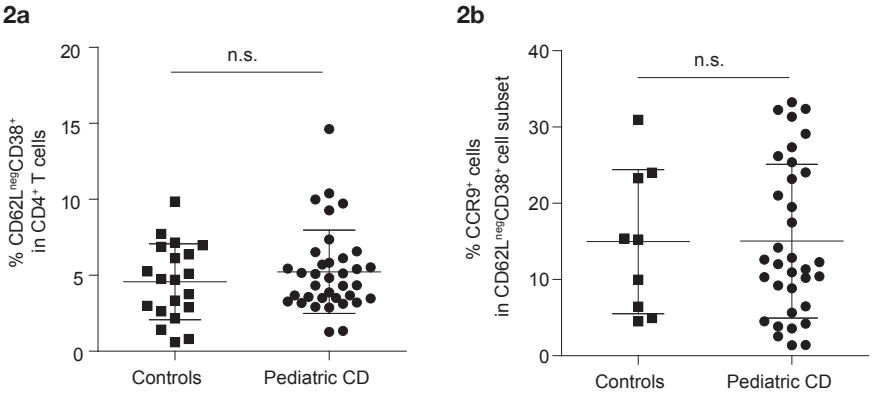
Overall, this demonstrates that in a well-defined patient cohort of pediatric CD patients no difference in the frequency of total or mucosally-imprinted Foxp3^{+} T cells can be observed.

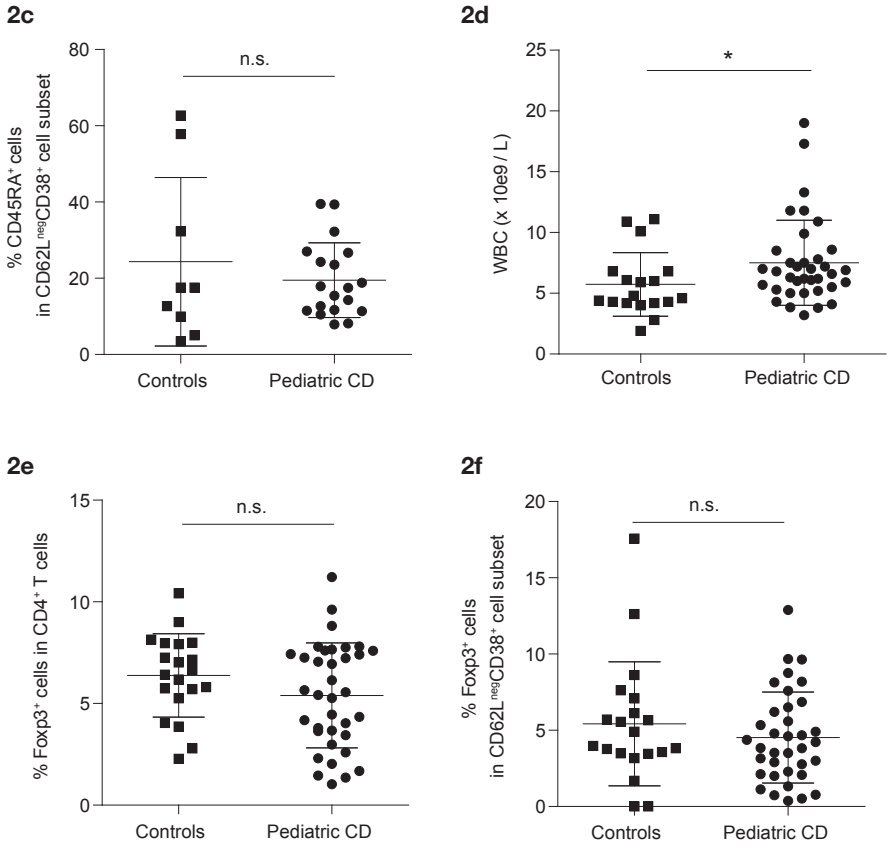
Adult patients

Having established that significant numeric deficiency of mucosal or systemic Foxp3^{+} T cells cannot explain the exuberant effector response in untreated pediatric CD, we hypothesized that alterations in Foxp3^{+} T cell frequencies may occur in more chronic forms of disease or only in adult patients. As our pediatric cohort contained only patients with active disease before diagnosis and before GFD, we collected peripheral blood of patients with a severe form of CD that is refractory to a GFD (RCD) (n=14) and adult patients with treated CD (n=13). Adult patient blood was

compared with blood of healthy volunteers (n=14). Demographic features of the adult patient groups and controls are depicted in *Table 2*. All treated CD patients were on a GFD for at least 6 months and, as a result of this treatment, their Marsh scores had improved from Marsh 3A-C at the time of diagnosis to a Marsh 0 or 1 and autoantibody concentrations were negative at the time of blood sampling. Patients who were not responding to a GFD and diagnosed with RCD type I or type II were included in the RCD group. As shown in *Table 2*, the RCD patient group consisted of a heterogeneous population of patients. All RCD patients had an earlier diagnosis of RCD and had a history of treatment with immunomodulatory drugs, including cladribine or 6-thioguanine. The latest available Marsh scores of RCD patients varied from Marsh 0 to Marsh 3C. Mucosal healing was seen in RCD patients who received treatment with immunomodulatory drugs, such as cladribine. Three patients with an EATL were also included. No differences in the WBC count were detected between the different patient groups and controls (data not shown).

Figure 2 ***No differences in numbers of circulating Treg cells or mucosally-imprinted Treg cells between pediatric CD patients and controls. (a)*** *The percentage of CD62L^{neg}CD38^{+} mucosally-imprinted T cells were observed within the peripheral blood CD4^{+} T cell population of CD patients (n=36) and controls (n=20).* ***(b)*** *The percentage of CCR9^{+} cells within the CD62L^{neg}CD38^{+}CD4^{+} T cell subset in pediatric CD patients (n=34) and controls (n=9).* ***(c)*** *Percentages of naive (CD45RA^{+}) cells within CD62L^{neg}CD38^{+}CD4^{+} T cell in pediatric CD patients (n=19) and controls (n=9).* ***(d)*** *WBC counts per liter peripheral blood for pediatric CD patients (n=36) and controls (n=20).* ***(e)*** *The frequency of total Foxp3^{+} cells (gated on CD4^{+} lymphocytes) in pediatric CD patients (n=36) and controls (n=20).* ***(f)*** *The percentage of mucosally-imprinted Foxp3^{+}CD62L^{neg}CD38^{+} cells in pediatric CD patients (n=36) and in controls (n=20). * Statistically significant (P<0.05), n.s. not significant (Mann-Whitney U test). CD, celiac disease.*



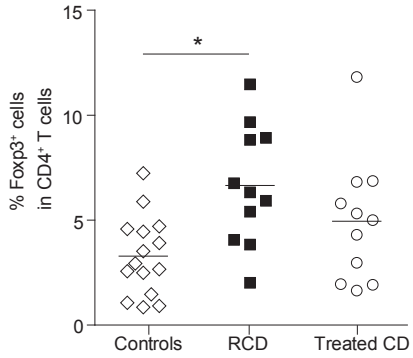


Increased numbers of circulating natural T_{REG} cells in adult RCD and treated CD compared to controls

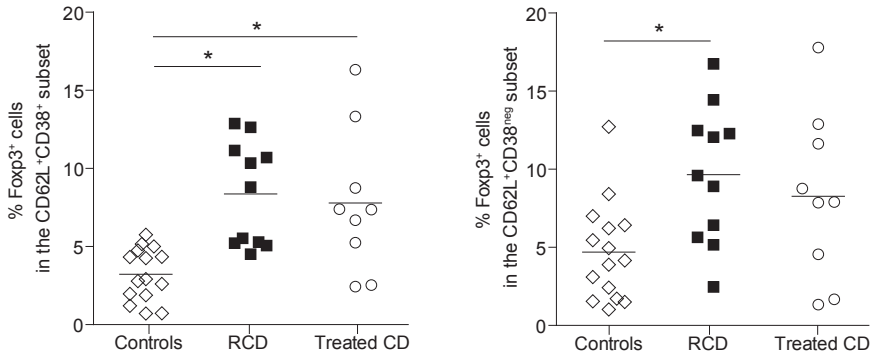
Strikingly, a higher percentage of circulating Foxp3$^+$ T cells was observed in the total CD4$^+$ T cell population in patients with RCD in comparison with healthy controls (*Figure 3a*). Analysis of the different CD62L/CD38 T cell subsets in RCD patients revealed that this increase in circulating CD4$^+$Foxp3$^+$ cells was explained by higher proportions of Foxp3$^+$ cells in both CD62L$^+$ cell subsets suggestive of a more naive phenotype (*Figure 3b*). Moreover, no changes were observed in the mucosally-imprinted (CD62L^{neg}CD38$^+$) or in the other memory T cell containing CD62L^{neg} T cell subset (*Figure 3c*). In treated CD patients, the percentage of Foxp3$^+$ cells was significantly increased in the CD62L$^+$CD38$^+$ subset, a subset which contains about 70% CD45RA$^+$ cells.[24] Together, these data infer that the increase in Foxp3 in RCD and treated CD patients can be attributed to changes in the natural T_{REG} cell population but not to changes in percentages of the mucosally-imprinted Foxp3$^+$CD62L^{neg}CD38$^+$ T_{REG} cells.

Figure 3 ***Increased numbers of circulating natural Treg cells in adult RCD and treated CD compared to controls.*** *Peripheral blood was obtained from adult patients with RCD (n=14), CD patients responding to a GFD (treated CD, n=13) and healthy controls (n=14).* ***(a)*** *The percentages of Foxp3 cells within the total CD3$^+$CD4$^+$ T cells and within the different CD4$^+$CD62L/CD38 T cells were determined.* ***(a)*** *Percentage of Foxp3$^+$ cells within the total CD4$^+$ T cell gate.* ***(b)*** *The percentage of Foxp3$^+$ T cells in the CD62L$^+$CD38$^+$ and CD62L$^+$CD38^{neg} subset.* ***(c)*** *The percentage of Foxp3$^+$ in the mucosally-imprinted CD62L^{neg}CD38$^+$ T cells or in the CD62L^{neg}CD38^{neg} T-cells in patient groups and controls.* ***(d)*** *Lymphocytes of 4 RCD patients were gated on CD3$^+$CD4$^+$ T cells and Foxp3 and Helios positivity were analyzed. The large majority of the Foxp3$^+$ cells co-expressed Helios.* ***(e)*** *Representative dot plots of Foxp3 and Helios expression (left panel) and isotype controls (right panel) are shown. Data analyzed Mann-Whitney U test. CD, celiac disease; RCD, refractory celiac disease.*

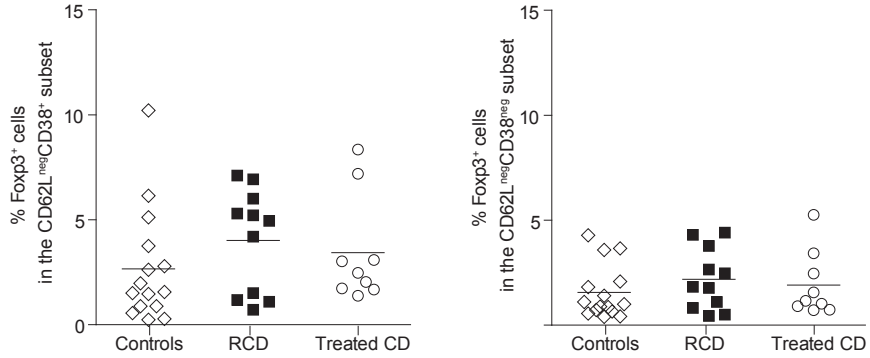
3a



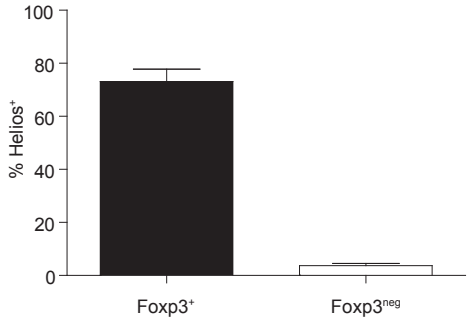
3b



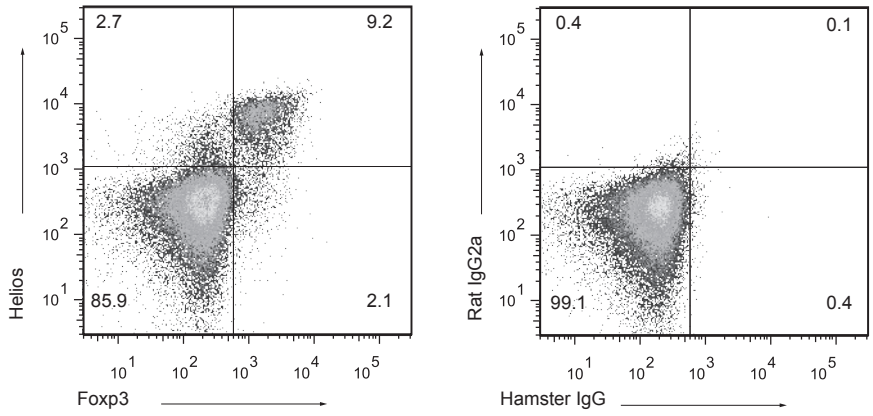
3c


% Foxp3+ cells in the CD62L negCD38+ subset
15
10
5
0
Controls
RCD
Treated CD
% Foxp3+ cells in the CD62L negCD38neg subset
15
10
5
Controls
RCD
Treated CD

3d


% Helios+
100
80
60
40
20
0
Foxp3+
Foxp3neg

3e


2.7
9.2
85.9
2.1
Helios
Foxp3
0.4
0.1
99.1
0.4
Rat IgG2a
Hamster IgG

Recent studies have reported that expression of the transcription factor Helios is heterogeneous in Foxp3$^+$ T_{REG} cells.[27,28] Helios is a marker for T-cell activation and in Foxp3$^+$ cells Helios expression may select for more proliferative cells that secrete low levels of effector cytokines.[29,30] Therefore, we examined Foxp3 and Helios expression in CD3$^+$CD4$^+$ T cells in peripheral blood of 4 RCD patients. The Foxp3$^+$ cells virtually all co-expressed Helios (*Figure 3d*). Representative flow cytometric dot plots of Foxp3 and Helios expression and the isotype negative controls are shown in *Figure 3e*.

These data established that numeric increases in systemic Helios$^+$ Foxp3$^+$ T cells, but not mucosally-imprinted Foxp3 are associated with more chronic forms of disease in adult patients.

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 the four CD62L and CD38 T cell subsets in any of the patients groups (*Supplementary Figure S1a*). As such, the frequency of mucosally-imprinted CD62L^{neg}CD38$^+$ T cells was not increased in CD patients with active small-intestinal inflammation (data not shown). We found no differences in the percentage of CD45RA$^+$ cells within the CD4$^+$ T cell population between patients and controls (*Supplementary Figure S1b*). Neither did we observe differences in the expression of CCR9 (*Supplementary Figure S1c, d*). As seen in pediatric patients, no difference was found in the percentage of CCR9$^+$ cells within the mucosally-imprinted CD62L^{neg}CD38$^+$ Foxp3$^+$ T cell subset from adult RCD patients (20.6% ± SD 14.1), treated CD patients (14.3% ± SD 6.9) when compared to controls (15.2% ± SD 5.4).

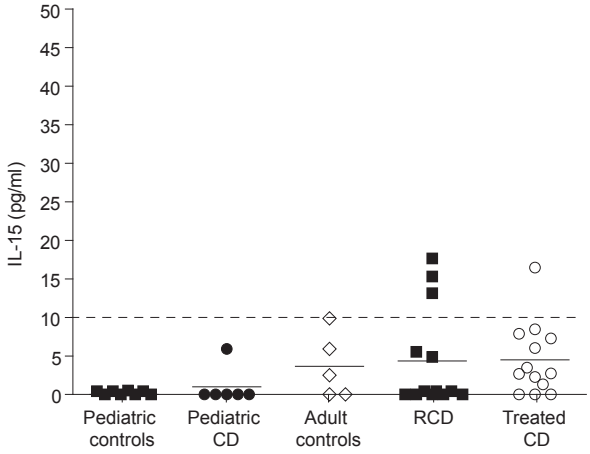
Increased numbers of Foxp3$^+$ cells in the lamina propria of pediatric and adult CD patients

Maintenance of intestinal homeostasis requires that Foxp3$^+$ T_{REG} cells are recruited to the small intestinal tissue. To examine a possible defect in Foxp3$^+$ T cell recruitment we performed immunohistochemical stainings to detect Foxp3$^+$ cells in duodenal biopsies from pediatric CD patients, adult CD patients, RCD patients and controls. Correlation with disease score revealed an increase in numbers of Foxp3$^+$ cells which was already detectable in patients with lower Marsh scores 1-2 (*Supplementary Figure S2*). Similar to the pediatric CD patient group, we found increased numbers of Foxp3$^+$ cells in the lamina propria of adult CD patients compared to controls (*Supplementary Figure S3*). In the treated CD group the numbers of Foxp3$^+$ cells were variable between patients. However, in the small number of RCD patients that was evaluated no increased Foxp3 positivity was seen. These data show that in our cohorts of pediatric and adult CD patients increased numbers of Foxp3$^+$ cells are detected locally in the inflamed intestinal mucosa and infer that recruitment of Foxp3$^+$ cells to the intestine is intact.

No increase of IL-15 plasma levels in CD patients

The cytokine interleukin (IL)-15 has been reported to stimulate Foxp3$^+$ T_{REG} cells.[31] In CD patients, IL-15 mRNA is over expressed in the small intestine and increased IL-15 concentrations are detected in serum.[32-34] Therefore, we investigated whether IL-15 plasma levels were related to increased frequencies of Foxp3$^+$ T_{REG} cells in peripheral blood of, adult CD patients. We were able to detect low levels IL-15 in plasma of some CD patients and controls. However, no relation between IL-15 plasma levels and increased frequencies of Foxp3$^+$ T_{REG} cells was detected (*Figure 4*).

Figure 4 ***No increase of IL-15 plasma levels in CD patients.*** *The IL-15 concentration in plasma was determined in pediatric controls (n=8), pediatric CD (n=6), adult controls (n=5), adult treated CD (n=13) and RCD patients (n=13) by enzyme-linked immunosorbent assay. Dashed line represents the detection limit. CD, celiac disease. Data are representative for three independent measurements.*



Discussion

Here we show that the frequency of natural and mucosally-imprinted Foxp3$^+$ T_{REG} cells is unaltered in the circulation of pediatric CD patients. In contrast, in treated adult CD and RCD patients increased frequencies of natural Foxp3$^+$ T_{REG} cells were detected whereas the mucosally-imprinted CD62L^{neg}CD38$^+$ subset remained unchanged. As such, systemic changes in natural CD4$^+$Foxp3$^+$ frequency are not inherent to all CD patients and appear related to a particular phase of disease. This is in contrast to the increased frequency of Foxp3$^+$ cells within the lamina propria on histology, which was observed in biopsies from both pediatric as well as adult CD patients.

In murine models for oral tolerance to dietary antigen inducible Foxp3$^+$ T cells have been shown to differentiate from naive T cells in the mesenteric lymph node under the control of CD103$^+$ DC that secrete TGF-β and RA.[11] Recently, we have shown that, in both human and mouse; mucosal imprinting in the mesenteric lymph node leads to a particular T cell phenotype characterized by low levels of CD62L expression and increased levels of CD38. In agreement, DQ2 tetramer staining revealed that gluten-specific T cells appearing in blood of treated celiac disease patients after oral gluten challenge were predominantly CD4$^+$CD62L^{neg}CD38$^+$. As this CD62L^{neg}CD38$^+$ T cell phenotype is maintained upon entering the circulation [24] it was used in the current study to distinguish mucosally-imprinted T cells within the total CD4$^+$ T cell pool. Here we report that no detectable changes are found in the frequency of circulating mucosally-imprinted CD4$^+$CD62L^{neg}CD38$^+$ Foxp3$^+$ cells in pediatric or adult CD patients. These data establish that there are no gross defects in mucosal T_{REG}-cell induction in non-treated CD. However, it is not excluded that transient alterations in gluten-specific Foxp3$^+$ T_{REG}-differentiation occur during the disease process.

Alternatively, the loss of tolerance to gluten in CD could be due to a defect in the effector phase of T_{REG}-cell function within the lamina propria rather than at the level of Foxp3 T-cell differentiation within the mesenteric lymph node. Indeed, multiple studies have provided evidence for such a localized loss of Foxp3 T-cell function. In particular, presence of IL-15 has been reported to abrogate suppression of isolated lamina propria effector T cells by intestinal Foxp3$^+$ cells in co-culture.[20] This defective suppression may in part be explained by an IL-15 induced resistance of effector T cells to suppression.[33] Similarly, IL-21 has been suggested to abrogate Foxp3$^+$$T_{REG}$ cell-function.[35] Loss of sensitivity to T_{REG} cell mediated suppression may in consequence allow inflammatory T cells to respond to gluten and to self-antigen that is exposed upon tissue damage.

T_{REG} cell mediated suppression requires sufficient T_{REG} cell numbers to be present in the inflamed area to counteract the effector T cells. As has been reported in several other studies we observed increased frequencies of Foxp3$^+$ cells in tissue biopsies of both pediatric and adult CD patients suggesting that recruitment of Foxp3$^+$ T cells to the inflamed tissue is intact or even increased.[16, 18-20, 33] Unfortunately, immunohistochemistry cannot distinguish between natural T_{REG} cells and mucosally imprinted T_{REG} cells. However, a large proportion of mucosally-imprinted CD4$^+$CD62L^{neg}CD38$^+$ Foxp3$^+$ cells from CD patients expressed the chemokine receptor CCR9 which should enable them to respond to CCL25, the chemokine required for small intestinal homing. Also in treated CD patients, numbers of lamina propria Foxp3$^+$ cells were increased. We speculate that this may be due to residual inflammation despite a GFD.[36] Contrary to our expectations, in the small number of RCD patients (n=5) no increased Foxp3 positivity was observed. Further investigation in a larger cohort of patients with defined immunosuppressive treatment is required to establish whether the absence of increased Foxp3$^+$ T cell numbers in lamina propria is a general phenomenon associated with RCD.

Changes in circulating Foxp3$^+$ T cells in CD patients have been observed in previous studies. However, until now it had not been investigated whether these cells were natural or induced T_{REG} cells. Here we demonstrate that the frequency of CD62L$^+$ natural Foxp3$^+$ T cells is increased in a subset of adult CD and RCD patients that are treated with a GFD but not in pediatric CD. Interestingly, these Foxp3$^+$ cells virtually all expressed Helios. These data infer that changes in the frequency of circulating natural Foxp3$^+$ T cells are not inherent to CD but are restricted to a subgroup of CD patients. In first instance, when combining all studies, increased frequencies of circulating Foxp3$^+$ cells seem restricted to adult patients.[15, 21, 23] However, this is not observed in all adult cohorts.[33] The variability of these data in the different adult patient cohorts shows that the changes in circulating Foxp3$^+$ T cells may be transient and possibly related to a particular stage of inflammation in CD patients. As increases in peripheral Foxp3$^+$ T_{REG} cells have also been reported for patients with cancer[37, 38], primary Sjögren's Syndrome and rheumatoid arthritis[39], psoriasis[40] and systemic sclerosis[41], we hypothesize that a non-specific chronic inflammatory mediator can cause this effect. To assess whether IL-15 levels in the circulation were related to increased frequencies of circulating Foxp3$^+$ cells we determined IL-15 concentrations in patient plasma. Overall the IL-15 levels did not reveal a possible role of IL-15 in Foxp3 expansion in CD. From this we conclude that circulating CD4$^+$Foxp3$^+$ T cell numbers are increased in adult patients with RCD and treated CD and hypothesize that this phenomenon may be related to a particular pattern of inflammation involving systemic immune activation.

Overall we conclude that the population of mucosally-imprinted CD62L^{neg}CD38$^+$ Foxp3$^+$ T_{REG} cells has a normal frequency in blood of CD patients suggesting that there are no severe general defects in induction of mucosal T_{REG} cells. Specific defects in gluten-reactive mucosal T_{REG} cells can not be excluded but are currently technically impossible to determine. Increased frequencies of Foxp3$^+$ natural cells are found in a subgroup of adult CD patients and may be related to systemic inflammation. In the lamina propria of all CD patients Foxp3$^+$ T cells are present in the inflammatory lesions but may be inactivated by the local inflammatory milieu. Whether loss of tolerance to gluten in CD patients is caused by such defective Foxp3$^+$ cells remains to be established.

Acknowledgments

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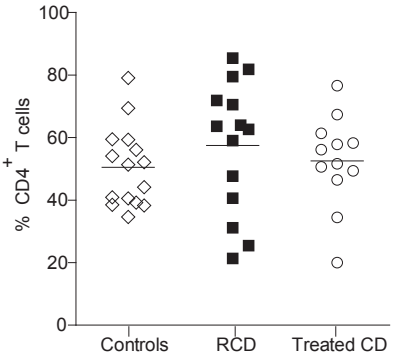
Supplementary figures

Supplementary Figure S1 ***Analysis of CD4⁺ CD62L/CD38 T-cell subsets in peripheral blood of adult CD patients and controls.*** *Peripheral blood was obtained from adult patients with RCD (n=14), CD patients responding to a GFD (treated CD, n=15) and healthy controls (n=14).* ***(a)*** *The percentages of total CD4⁺ T cells (within CD3⁺ T cell gate)* ***(b)*** *The percentage of CD45RA⁺ naive T cells (within the CD3⁺CD4⁺ T cell gate)* ***(c)*** *De percentage of total CCR9⁺ cells within the total (CD3⁺CD4⁺ T cell gate) or* ***(d)*** *within the different CD62L/CD38 T cells.*
CD, celiac disease; RCD, refractory celiac disease.

S1a



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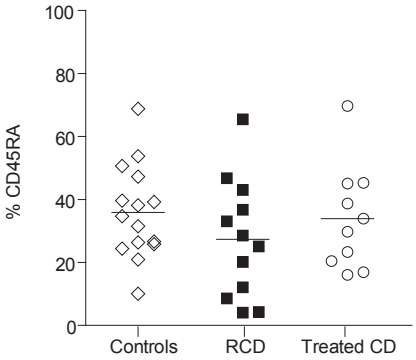

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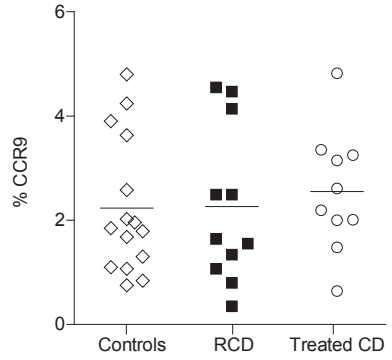

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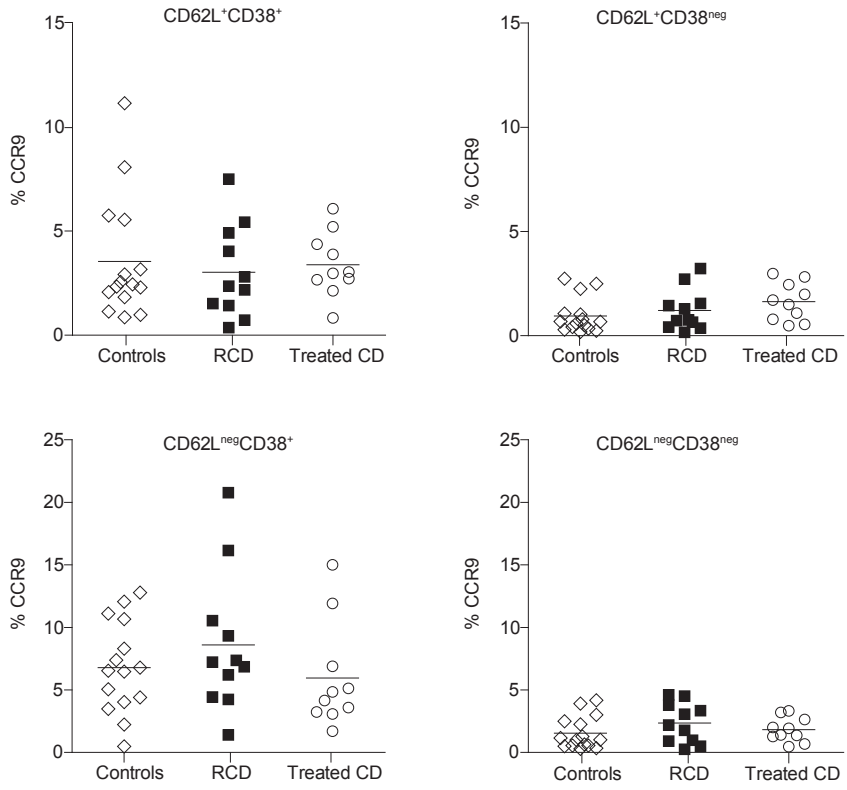
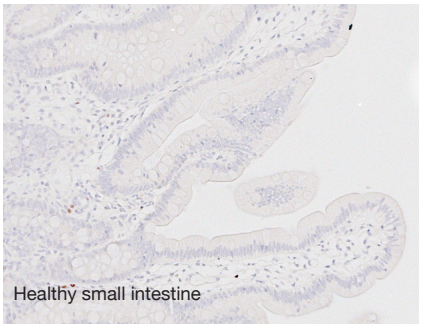

CD62L+CD38+
CD62L+CD38neg
CD62LnegCD38+
CD62LnegCD38neg
% CCR9
0
5
10
15
20
25
Controls
RCD
Treated CD

Supplementary Figure S2 ***Increased numbers of Foxp3⁺ cells in the lamina propria of pediatric CD patients.*** *Immunohistochemical detection of Foxp3 on paraffin embedded duodenal biopsies from pediatric controls and CD patients* ***(a-d)***. ***(e)*** *Isotype control antibody, mouse IgG_1, staining. Original magnification: x 20. Figures are representative for 16 different CD patients and controls. CD, celiac disease.*

S2a



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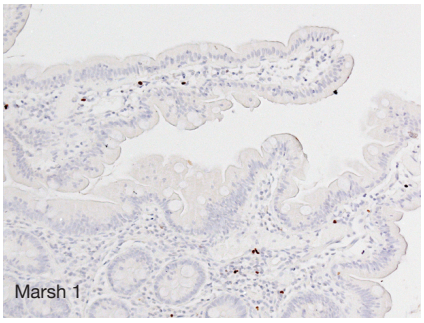

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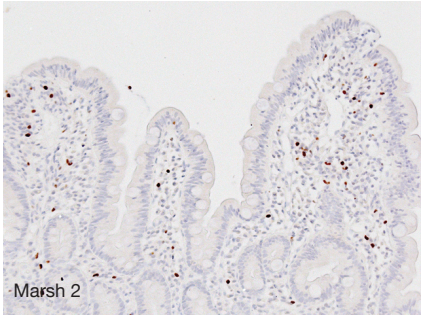

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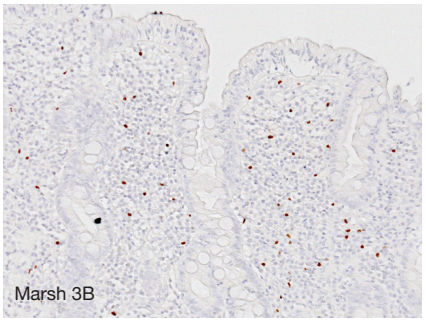

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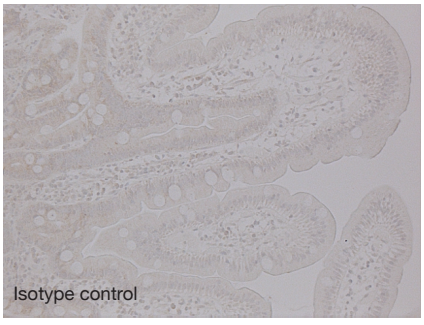
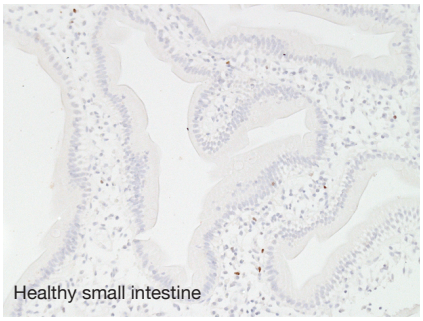


Supplementary Figure S3 ***Increased numbers of Foxp3⁺ cells in the lamina propria of adult CD.*** *Immunohistochemical detection of Foxp3 on paraffin embedded duodenal biopsies from healthy controls, treated CD and RCD patients* ***(a-e)****. Original magnification: ×20. Figures are representative for 12 different patients and controls. CD, celiac disease; RCD, refractory celiac disease.*

S3a



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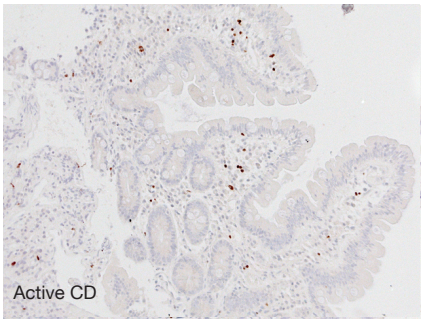

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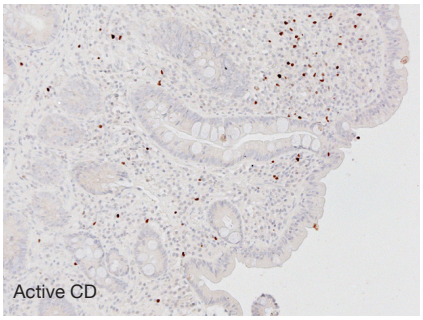

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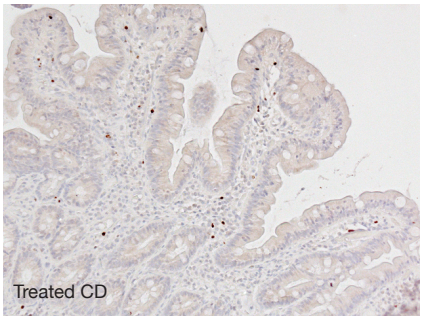

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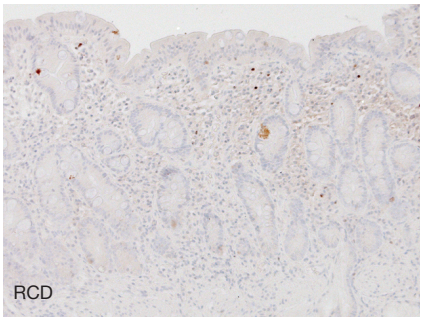

3

Functional consequences of a novel IL-10 receptor-α mutation on innate and adaptive immunity in inflammatory bowel disease

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Submitted

ABSTRACT

Inflammatory bowel disease (IBD) is driven by uncontrolled T-cell responses. Interleukin-10 (IL-10) receptor deficiency causes infantile-onset IBD. Currently, it is unclear how immune responses are affected by IL-10 receptor deficiency. We have characterized immunological consequences of IL-10 receptor deficiency in a now 11-year-old girl who presented with infantile-onset IBD and was diagnosed with an *IL10RA* mutation at 9 years of age. During our investigations, the patient had not received a hematopoietic stem cell transplantation and was in clinical remission of intestinal disease while receiving thalidomide and intravenous immunoglobulins.

The IL10RA-deficient immune system developed normally with respect to numbers and phenotype of circulating cells. Despite normal co-stimulatory molecule expression, LPS-stimulated monocyte-derived dendritic cells (DC) released higher levels of TNF-α and IL-6 *in vitro*. IL-10 was required to control DC cytokine production and anti-CD3-driven IFN-γ and IL-17 release by PBMCs. This agreed with high numbers of IL-17$^+$ and T-bet$^+$ cells in intestinal biopsies at disease onset. *In vitro*, intravenous immunoglobulins suppressed anti-CD3-driven IL-17 and IFN-γ release, while thalidomide inhibited LPS-mediated TNF-α production by PBMCs.

In sum, the immune system can develop in absence of IL-10 signaling but shows aberrant inflammatory cytokine release by DC and uncontrolled IFN-γ and IL-17 responses.

Introduction

Interleukin-10 (IL-10), a potent anti-inflammatory cytokine, plays a key role in limiting the extent of inflammatory immune responses against pathogens or pathogenic antigens. Additionally, in the intestine, IL-10 is crucial for mounting tolerance to harmless exogenous microbial antigens.[1] IL10- or IL-10 receptor (IL10R)-deficient mice develop spontaneous intestinal inflammation, which is dependent on bacterial colonization.[2, 3] In humans, the IL-10 signaling pathway plays a pivotal role in intestinal homeostasis, as infants with *IL10* or *IL10R* mutations develop severe, early-onset inflammatory bowel disease (IBD).[4-8] Furthermore, genome-wide association studies have associated polymorphisms in the IL-10 pathway with IBD; therefore, defective IL-10 signaling is strongly suspected to be involved in the pathogenesis of IBD.[8-11]

Currently, it is unclear how IL-10 deficiency leads to chronic intestinal inflammation. In particular, knowledge of IL-10-driven regulation of T helper 1 (T_H1) and T_H17 cells is lacking, especially as these cells drive chronic intestinal inflammation in IBD.[12] Moreover, it is unknown whether deficient IL-10 signaling leads to abnormal immune system development.

Here, we describe a currently 11-year-old female patient with a novel IL-10R alpha chain (*IL10RA*) mutation who developed severe early-onset colitis and perianal fistulizing disease in the first months of life. Treatment of patients with *IL10R* mutations has proven extremely difficult; allogeneic hematopoietic stem-cell transplantation (HSCT) is currently the optimal treatment in terms of survival and disease remission.[13, 14] Uniquely, prior to the detection of the IL10RA deficiency clinical remission of intestinal disease was achieved in this patient using thalidomide, colchicine, and intravenous immunoglobulin (IVIG). This allowed us to study the consequences of IL10RA deficiency on immune system development, and identify the mechanisms by which IL-10 regulates immune responses and intestinal immune homeostasis in humans.

Patients and methods

Patients

Several specific characteristics of disease phenotype and monocyte-derived macrophage function of this IL10RA-deficient patient have been included into prior cohort studies.[7, 15] Besides the patient, cohorts of pediatric patients with IBD and age-matched control orthopedic patients without underlying inflammatory or intestinal disease were also recruited for this study. All IBD patients were diagnosed

by endoscopy, histopathological, and clinical characteristics according to the Porto criteria (see *supplementary Table S1* for details).[16] PBMCs from 8 different adult healthy volunteers were used as controls for *in vitro* experiments. All participants or their parents gave written informed consent for the study. The studies were approved by the Erasmus MC ethical committee.

Homozygosity mapping and mutation analysis

Because of suspicion of a genetic defect in the IL-10/IL-10R pathway, genetic analysis was performed when the patient was 9 years old. Leukocyte DNA was hybridized to an Affymetrix GeneChip 260k Nsp1 SNP array. Regions of homozygosity were identified in the patient and her unaffected sister using CNAG software (version 3) using standard settings.[17] Regions of homozygosity larger than 1 Mb not overlapping with those of the healthy sister were studied for candidate genes.

After PCR amplification, the exons and flanking intron regions of *IL10RA* were sequenced.[6] The mutation was confirmed in an accredited diagnostic laboratory using an ABI 3730 DNA Analyser and SeqPatient genetic analysis software (JSI Medical Systems). NM_001558.3 was used as a reference sequence.

Cell isolation and Culture

PBMC were isolated using a Ficoll-Hypaque gradient. PBMC were stimulated with 100ng/ml lipopolysaccharide (LPS; Sigma-Aldrich) or anti-CD3 (500 ng/ml; Sanquin, the Netherlands) with or without 25 ng/ml IL-10 (R&D Systems) in Iscove's modified Dulbecco's medium (Lifetechnologies, Grand Island, NY, USA) supplemented with heat inactivated fetal calf serum, Glutamax (Lifetechnologies), 2-mercaptoethanol, penicillin and streptomycin. $CD4^+$ T cells were isolated by negative depletion using magnetically labeled antibodies (Dynabeads; Lifetechnologies) according to the manufacturer's instructions. $CD14^+$ monocytes were isolated by positive selection using anti-CD14 MACS beads (Miltenyi Biotec, Bergisch Gladbach, Germany) according to the manufacturer's instructions. The resulting >90% pure monocyte population was differentiated into monocyte-derived DC (moDC) during a 6-day culture with 800 U/ml GM-CSF (Novartis, Basel, Switzerland) and 400 U/ml IL-4 (R&D). Supernatants of stimulated PBMC or moDC cell cultures were collected and cytokine production were measured using enzyme-linked immunosorbent assays (ELISAs) for TNF-α (BD biosciences, Breda, the Netherlands), IFN-γ (eBiosciences) or IL-17A (Duoset; R&D Systems). Concentrations IL-8, IL-1β, IL-6, IL-10, TNF-α, IL-12p70 were detected by application of the standard protocol of the human inflammation Cytometric Bead Array (CBA) (BD biosciences).

Flow cytometry
For all functional assays, peripheral blood from the IL10RA-deficient patient was drawn at age 9 or 10 years while disease was in remission. STAT3 phosphorylation was induced in whole blood by incubation with IL-10 (25 ng/ml) or IL-6 (100 ng/ml; both R&D Systems) for 15 or 30 min, respectively. Staining for phosphorylated Tyr^{705} in STAT3 was performed according to the manufacturer's protocol (BD Phosflow).

For phenotype analysis whole blood samples were lysed and stained for flowcytometry. PBMCs were isolated, reactivated and subjected to intracellular cytokine staining as described below.

Antibodies for phenotypic analysis of whole blood samples
CD3 (HIT3), CD4 (RPA-T4), CD8 (SK-1), CD38 (HIT2), CD62L (DREG-56, all BD biosciences), CD45RA (MEM-56, Lifetechnologies), CCR9 (R&D Systems).

Intracellular cytokine staining
After reactivation for 4 h with 0.05 μg/ml phorbol 12-myristate 13-acetate (PMA; Sigma-Aldrich) and 0.5 μg/ml ionomycin (Sigma-Aldrich) in the presence of Brefeldin A (3 μg/ml eBiosciences) for the last 3h, the cells were stained for flow-cytometric analysis using monoclonal antibodies against CD3 (BD biosciences), fixed with 2% formalin solution and intracellular staining was performed using saponin (Sigma-Aldrich), followed by anti-IL-21 (clone eBio3A3-N2; eBiosciences), anti-IL-17A (clone eBio64DEC17; eBiosciences), anti-interferon (IFN)-γ (clone 4SB3, BD biosciences), or the appropriate isotype controls (all eBiosciences). Intracellular staining for Foxp3 (clone PCH101, eBiosciences) or the appropriate isotype control was performed with the Foxp3 staining buffer kit, according to manufacturer's protocol (eBiosciences). Flow-cytometric analysis was performed using the FACSCanto™ II (BD biosciences). Data were analyzed using FlowJo software (BD biosciences).

Immunohistochemistry on paraffin sections
For immunohistochemistry, paraformaldehyde fixed, paraffin embedded biopsies were sectioned and deparaffinized. Endogenous peroxidase activity was quenched with 3% H_2O_2 in PBS for 20 min. Antigen retrieval was performed by microwave treatment in citrate buffer (10 mM, pH 6.0) or enzymatic treatment with pepsin (0.1%). The sections were blocked for 1 h in 10% normal human serum plus 10% normal goat, rabbit or horse serum diluted in 10 mM Tris, 5 mM EDTA, 0.15 M NaCl, 0.25% gelatin, 0.05% Tween-20, pH 8. Antibody incubation was performed overnight at 4°C using anti-IL-10RA (polyclonal rabbit, raised against the cytoplasmic domain of human IL-10RA (residues 257-278), Millipore, Temecula, CA, USA), anti-CD3 (polyclonal rabbit, DakoCytomation, Glostrup, Denmark), anti-IL-17A (polyclonal goat IgG; R&D Systems, Abingdon, UK), anti-IL-21 (polyclonal rabbit IgG, Lifespan Biosciences, Seattle, WA, USA), anti-Foxp3 (mouse IgG_1 clone 236A/

E7, eBiosciences, San Diego, CA, USA) or anti-T-bet (mouse IgG clone 4Bio, eBiosciences). Immunoreactive sites were detected with biotinylated secondary rabbit anti-goat, goat anti-rabbit serum or horse-anti-mouse using the Vectastain ABC Elite Kit (Vector Laboratories, Burlingame, CA, USA) and 3,3'-diaminobenzidine tetrahydrochloride (Sigma-Aldrich, Zwijndrecht, The Netherlands), and tissues were counterstained with hematoxylin (Vector Laboratories). An isotype-matched negative control was performed for each immunostaining.

Images were acquired and analyzed using a Leica DM5500B upright microscope and LAS image acquisition software (Leica Microsystems, Rijswijk, The Netherlands).

Thalidomide and IVIG

Thalidomide (Celgene Corp.) was dissolved in dimethylsulfoxide (DMSO; Sigma-Aldrich). Cell cultures were treated with thalidomide, DMSO, IVIG (Nanogam; Sanquin), or human serum albumin (CeAlb; Sanquin).

Statistics

Prism (Graphpad Software Inc.) was used to perform paired sample *t*-tests or the Mann-Whitney *U* test as indicated; $P<0.05$ was considered significant.

Results

Patient

A currently 11-year-old female, with consanguineous parents of Turkish origin, presented with abscesses and fissures in the perianal region in the first months of life. Her older brother died at the age of 1 year due to complications of severe inflammatory enterocolitis. At the age of 3 months, the patient was diagnosed with aphthous colitis, proctitis, and perianal lesions. Histologically, at the age of 3 months the descending colon showed normal crypt differentiation and mild focal inflammation with inflammatory cell infiltration in the crypt epithelium. At the age of 9 months, colonic inflammation was more severe with tissue ulceration and abundant mononuclear and polymorphonuclear cell infiltration in the epithelium and lamina propria (*Supplementary Figure S1*). Based on clinical presentation, colonic disease predominated. As with most other IL10R-deficient patients described, the small intestine was not endoscopically investigated at the time of diagnosis.

The patient failed to achieve full clinical remission during treatment with exclusive elemental nutrition, antibiotics, sulfasalazine, prednisone, infliximab (anti-tumor necrosis factor-alpha antibody [anti-TNF-α]), and methotrexate. A provisional diagnosis of possible Behçet's disease was made which was the incentive to start immunosuppressive therapy with thalidomide and colchicine at the age of two.

From birth onwards, the patient suffered severe recurrent bacterial and viral respiratory tract infections with respiratory failure requiring ventilatory support on several occasions. In addition, the patient suffered otitis media shortly after birth and developed cutaneous folliculitis. The episodes of pneumonia were accompanied by weight loss (at 6 and 8 years respectively) as illustrated by the patients' growth curve (*Supplementary Figure S2*).

Diagnostic immunological analyses confirmed normal IgG, IgA, and IgM levels, and specific tetanus toxoid antibody titers in the lower normal range. Additional vaccination studies were not performed due to adverse reactions to previous vaccinations. As low numbers of memory B cells were detected and the patient suffered frequent respiratory tract infections, monthly IVIG was initiated, resulting in a reduced frequency of respiratory tract infections.

Currently, without claiming that the patient is in long-term remission, we report that she has not had gastrointestinal symptoms since the age of 3 while receiving combined treatment with thalidomide and colchicine and IVIG. During the last two years, no severe systemic infections have occurred while growth and development is normal (*Supplementary Figure S2*).

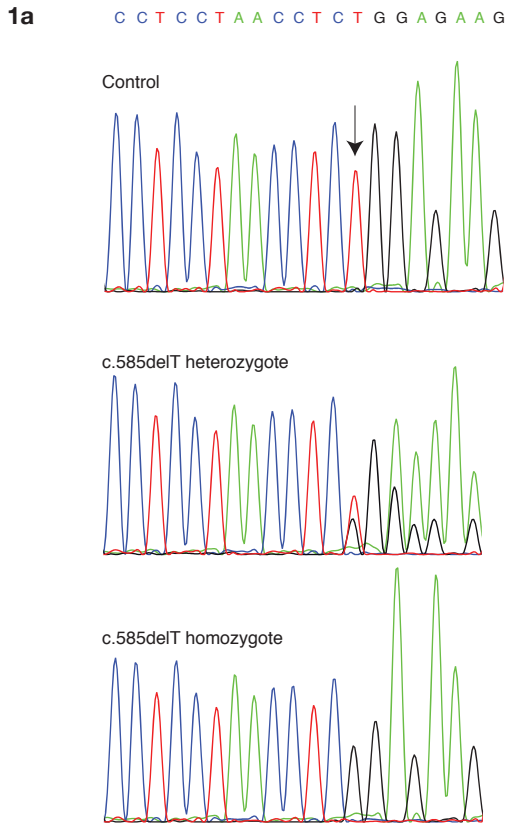
***IL10RA* mutation resulting in defective IL-10 signaling**

IL10RA was identified as the single candidate gene in different extended regions of homozygosity; Sanger sequencing identified a single nucleotide deletion resulting in a frame-shift and premature stop codon (c.585delT, p.Gly196Glufs*10; *Figure 1a*). Homozygosity was confirmed in the patient (fresh material) and her deceased brother (confirmation on DNA isolated from paraffin embedded intestinal biopsy); both parents are heterozygous carriers. The premature stop codon predicts a truncated receptor lacking the transmembrane and intracellular regions (*Figure 1b*). Absence of the intracellular domain of IL-10RA was demonstrated using immunohistochemistry in colonic biopsy specimens previously taken from the patient at time of diagnosis (*Figure 1c*).

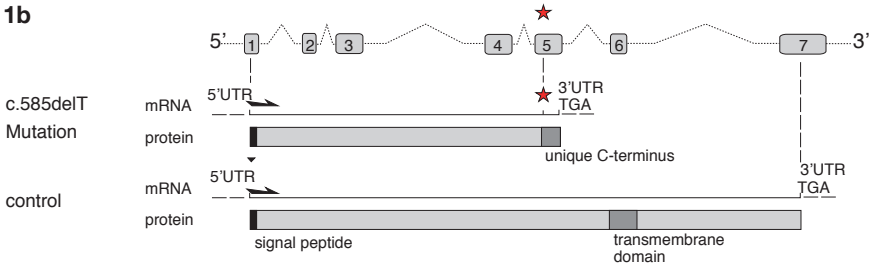
Compared to healthy control leukocytes, IL-10-mediated STAT3 phosphorylation was defective in leukocytes from the patient; however, normal IL-6-stimulated STAT3 phosphorylation was observed (*Figure 1d*). IL-10 inhibited TNF-α production in healthy control LPS-stimulated peripheral blood mononuclear cells (PBMCs), but not in LPS-stimulated PBMCs from the patient (*Figure 1e*). These data unequivocally demonstrate that the novel *IL10RA* mutation identified in our patient with early-onset IBD causes defective IL-10 signaling.

Figure 1 ***Functional analysis of a novel IL10RA mutation.***

(a) *Sequencing of the IL10RA region revealed a homozygous single nucleotide deletion (c.585delT, p.Gly196Glufs*10) in exon 5 compared to a normal control. The mutation of this patient has been briefly mentioned in two previous studies.*[7, 15] ***(b)*** *Intron-exon organization of the full-length and mutated IL10RA gene and the effect of the mutation at the protein level. Exons are shown in boxes, introns as lines, and mRNA as thick lines.* ***(c)*** *Confirmation of the absence of functional IL-10RA in the patient. Immunohistochemical detection of IL-10R using an antibody raised against the cytoplasmic domain of human IL-10RA (residues 257-278) in paraffin-embedded colon descendens sections from the IL10RA-deficient patient (left) and a pediatric IBD patient (right).* ***(d)*** *Control or patient whole blood was stimulated with IL-10 (25 ng/ml) for 15 min or IL-6 (100 ng/ml) for 30 min, and STAT3 phosphorylation was quantified by flow cytometry. Gated on monocytes for IL-10 stimulation (top) or the whole cell population for IL-6 stimulation (bottom).* ***(e)*** *Control and patient PBMCs were stimulated with LPS (100 ng/ml) in the absence or presence of IL-10 (25 ng/ml). After 6 h, supernatants were collected and TNF-α secretion was measured by an ELISA. *P<0.05, Mann-Whitney U test. IBD, inflammatory bowel disease; LPS, lipopolysaccharide.*



1b


5'
1
2
3
4
5
6
7
3'
c.585delT
Mutation
mRNA
5'UTR
3'UTR
TGA
protein
unique C-terminus
control
mRNA
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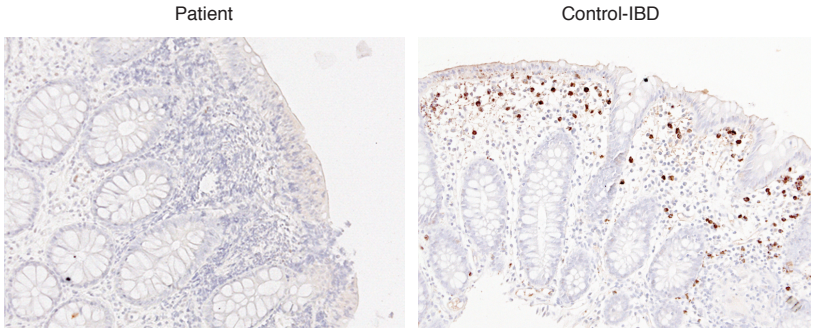
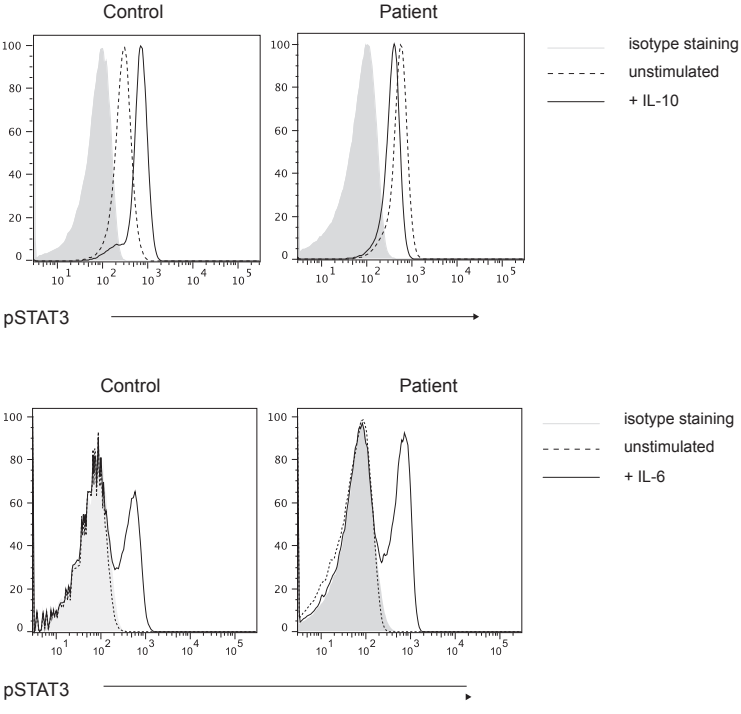
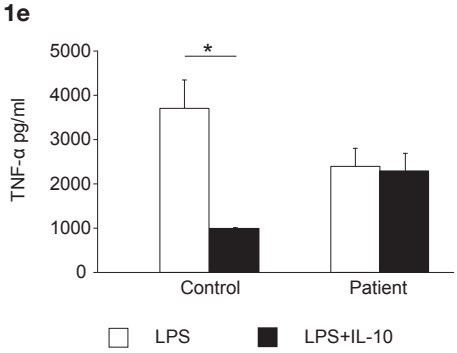
3'UTR
TGA
protein
signal peptide
transmembrane
domain

1c


Patient
Control-IBD

1d


Control
Patient
isotype staining
unstimulated
+ IL-10
pSTAT3
Control
Patient
isotype staining
unstimulated
+ IL-6
pSTAT3



Normal distribution of circulating dendritic cells and T cell subsets in the IL10RA-deficient patient during treatment

Primary immunodeficiency often leads to abnormal immune cell development, cellular dysfunction, or both. Therefore, before functional analysis, numbers and relative frequencies of immune cells were determined. The percentage of circulating $CD11c^{+}HLA\text{-}DR^{+}$ dendritic cells (DCs) in the patient was within the normal range for pediatric IBD patients (in remission or with active disease), and pediatric controls (*Figure 2a*). Co-stimulatory molecule expression on $CD11c^{+}HLA\text{-}DR^{+}$ cells was similar between the patient and healthy controls (data not shown). During remission the patients' plasma concentrations of TNF-α, IL-6, and IL-8 (data not shown), the percentages of $CD4^{+}$ and $CD8^{+}$ cells within $CD3^{+}$ T cells and $Foxp3^{+}$ cells within $CD4^{+}$ T cells (*Figure 2b*) were within the range of pediatric IBD patients in remission and pediatric controls.

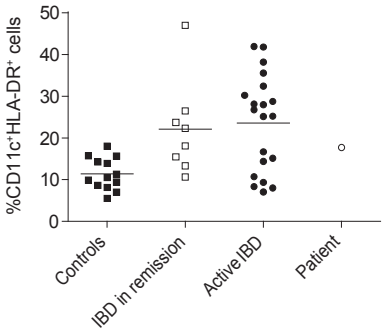
The $CD4^{+}CD62L^{neg}CD38^{+}$ T cell population in peripheral blood is enriched in effector memory cells with reactivity to mucosal antigens and contains the majority of $CCR9^{+}$ cells.[18, 19] In the patient, during remission, both the percentage of circulating mucosally-imprinted $CD62L^{neg}CD38^{+}$ cells (3.6%) and gut-homing $CCR9^{+}$ cells (2.9%) within the total $CD4^{+}$ population were not different to pediatric controls or pediatric IBD patients (*Figure 2c*), and the percentages of $CD3^{+}$ cells secreting IFN-γ, IL-21, and IL-17A were comparable to pediatric IBD patients in remission (*Figure 2d; Supplementary Figure S3*).

Therefore, in the absence of a functional IL-10R, during treatment with thalidomide, colchicine, and IVIG the immune system has developed with normal frequencies of $CD11c^{+}HLA\text{-}DR^{+}$ DCs and circulating effector memory T cell subsets.

Figure 2 ***Similar numbers of circulating CD11c⁺HLADR⁺ DCs and T cell populations in the IL10RA-deficient patient and pediatric controls.***

After erythrocyte lysis, peripheral blood from pediatric IBD patients, pediatric controls and the IL10RA-deficient patient was stained for CD11c, HLA-DR, CD4, CD8, CD62L, CD38, CCR9, and/or Foxp3, and analyzed by flow cytometry. ***(a)*** *Frequency of the DC population (CD11c⁺HLA-DR⁺).* ***(b)*** *Frequencies of CD4⁺, CD8⁺, and Foxp3⁺ cells gated on CD4⁺ lymphocytes.* ***(c)*** *Frequencies of CD62L^{neg}CD38⁺ and CCR9⁺ cells gated on CD4⁺ lymphocytes.* ***(d)*** *PBMCs were isolated from age-matched IBD patients in remission and the IL10RA-deficient patient, and reactivated for 4 h with PMA (0.05 µg/ml) and ionomycin (0.5 µg/ml) in the presence of Brefeldin A for the last 3 h. The cells were harvested, fixed, permeabilized, stained for IFN-γ, IL-21, and IL-17A and analyzed by flow cytometry. Representative cytokine dot plots for the IL10RA-deficient patient are shown; the numbers below the dot plots indicate the ranges of cytokine expression in five pediatric IBD patients in remission.*

2a



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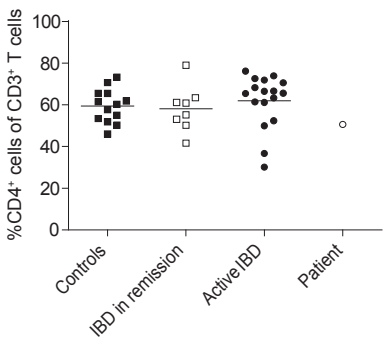
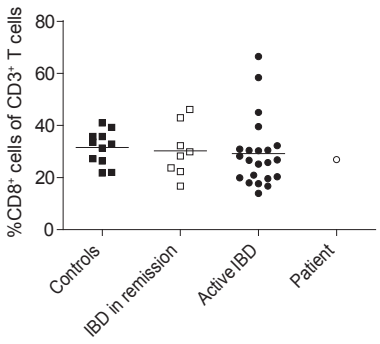
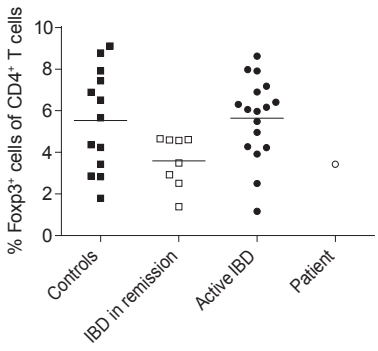
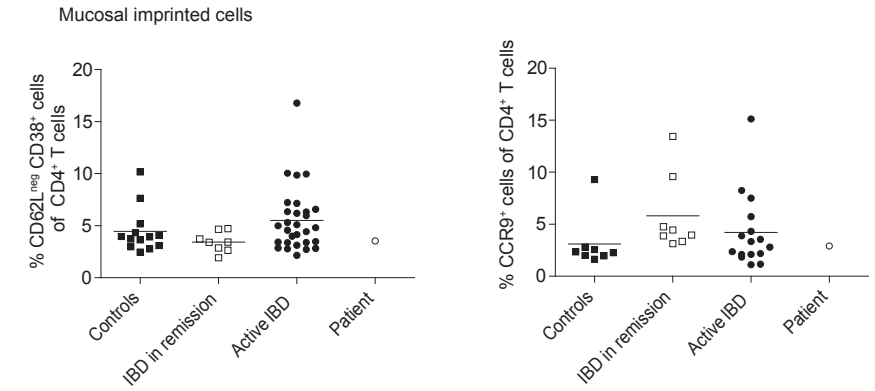


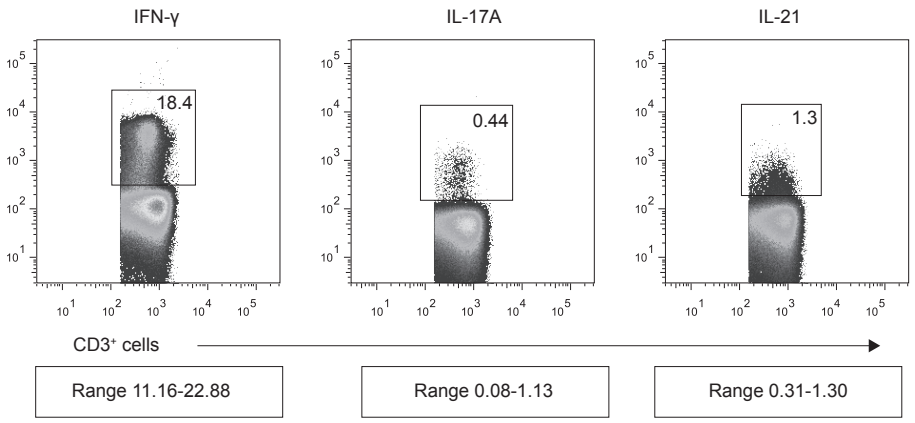




2c



2d

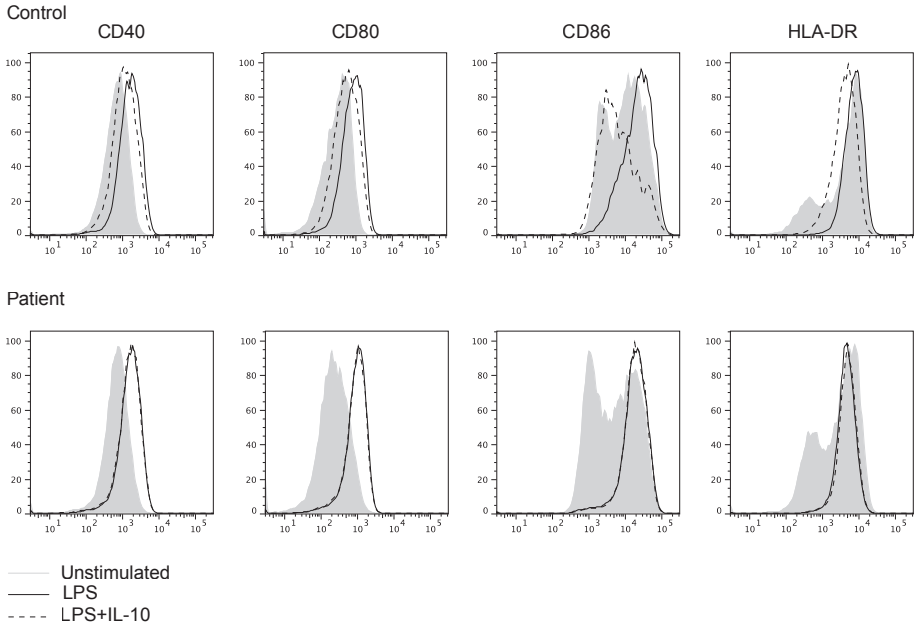


Functional consequences of defective IL-10 signaling on inflammatory DCs and T-cell responses *in vitro*

Next, we investigated the function of IL-10 in the differentiation of immature monocyte-derived DCs (MoDCs) into inflammatory DCs. MoDCs from the patient differentiated normally in response to GM-CSF and IL-4: after harvesting, the cells had an immature phenotype with low expression of HLA-DR, CD40, CD80, and CD86. Stimulation of moDCs with LPS increased HLA-DR, CD40, and CD80 expression as well as CD86 co-stimulatory molecule expression to a similar extent in DCs from the patient and healthy controls (*Figure 3a,b*), indicating that IL10RA-deficient DCs develop normally and do not hyper-express co-stimulatory molecules in response to LPS. As expected,[20] LPS-mediated co-stimulatory molecule expression was downregulated by exogenous IL-10 in healthy control DCs, but not in IL10RA-deficient DCs *(Figure 3a)*. In contrast, we observed hyper-responsive

Figure 3 ***Inflammatory DC and T-cell responses in the absence of functional IL-10R.*** ***(a)*** *Control and patient moDCs were matured with LPS (100 ng/ml) in the presence (dotted line) or absence (filled line) of IL-10 (25 ng/ml). After 20 h, moDCs were analyzed by flow cytometry for expression of CD40, CD80, CD86, and HLA-DR (black lines). Filled gray histograms represent staining with isotype-matched antibodies. Data shown is from one of three experiments with similar results.* ***(b)*** *Co-stimulatory molecule expression by moDC from healthy donors (filled line) and the IL10RA-deficient patient (dotted line).* ***(c)*** *Levels of TNF-α, IL-6, IL-12p40, IL-8, and IL-10 as determined by cytometric bead arrays. IL-10 was measured in the supernatants of moDC cultures from five adult controls and five separate IL10RA-deficient patient experimental supernatants* ***(d)*** *Control and patient PBMCs were activated in culture using anti-CD3 (500 ng/ml) in the absence or presence of IL-10 (25 ng/ml). After 48 h, IFN-γ and IL-17A mRNA expression were determined by PCR analysis and the supernatants were assayed for IFN-γ and IL-17A using an ELISA. Data are mean ± SEM of one of three representative experiments;*P<0.05, Mann-Whitney U test.*

3a



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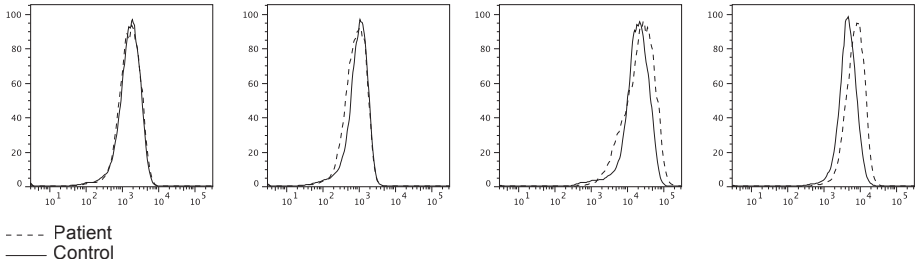
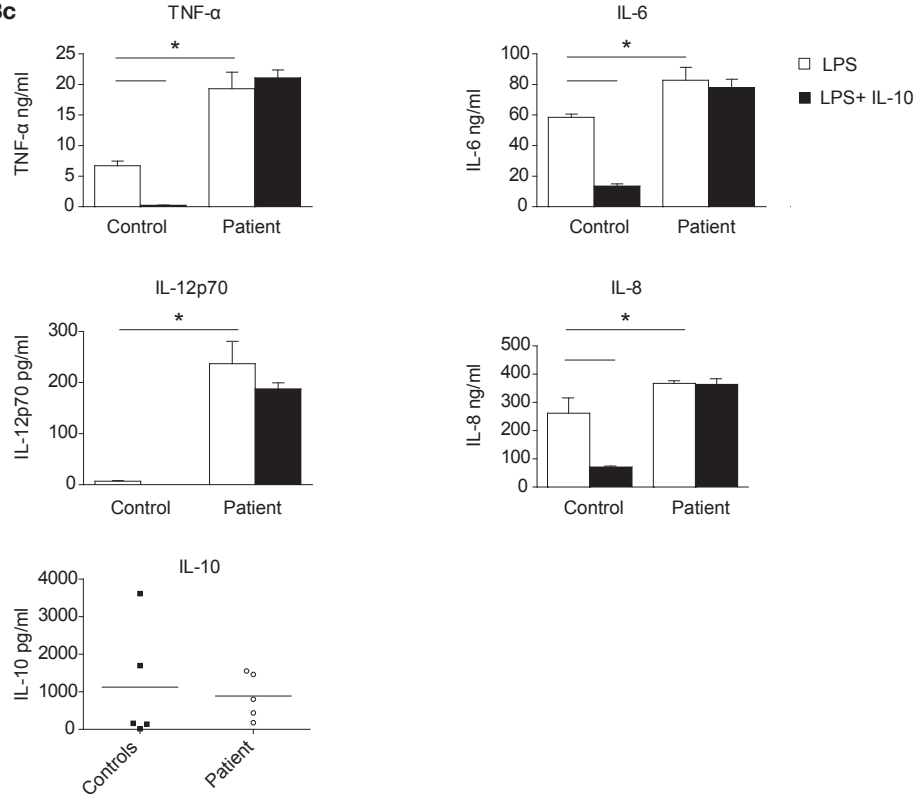


3c


TNF-α
TNF-α ng/ml
25
20
15
10
5
0
*
Control
Patient
IL-6
IL-6 ng/ml
100
80
60
40
20
0
*
Control
Patient
LPS
LPS+ IL-10
IL-12p70
IL-12p70 pg/ml
300
200
100
0
*
Control
Patient
IL-8
IL-8 ng/ml
500
400
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Control
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4000
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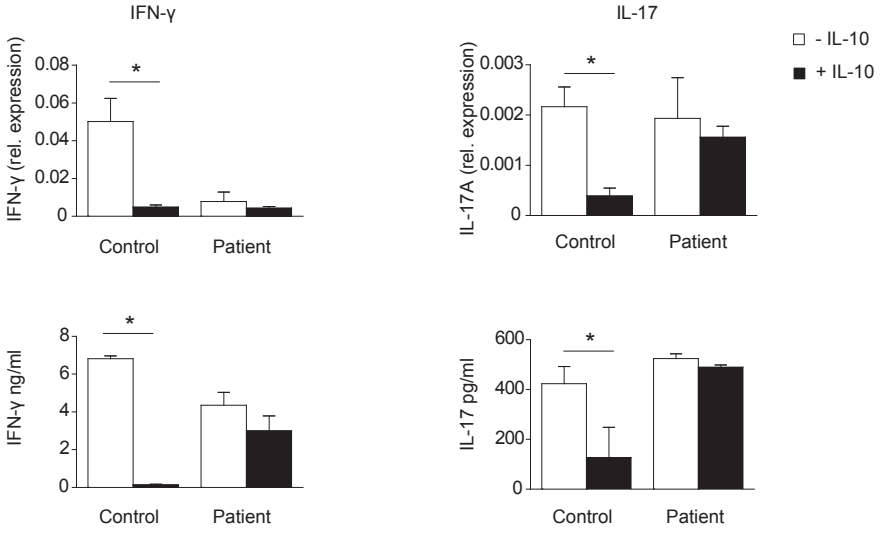
1000
0
Controls
Patient

3d


IFN-γ
IFN-γ (rel. expression)
0.08
0.06
0.04
0.02
0
*
Control
Patient
IL-17
IL-17A (rel. expression)
0.003
0.002
0.001
0
*
Control
Patient
- IL-10
+ IL-10
IFN-γ ng/ml
8
6
4
2
0
*
Control
Patient
IL-17 pg/ml
600
400
200
0
*
Control
Patient

cytokine secretion by IL10RA-deficient moDCs (*Figure 3c*), demonstrating a role for IL-10-mediated autocrine regulation of DC-derived cytokines. In particular, higher levels of TNF-α, IL-6, IL-8, and IL-12p70 were produced by IL10RA-deficient LPS-stimulated moDCs. IL-1β was not detectable in healthy control or patient moDCs, and IL10RA-deficient and healthy control moDCs secreted similar levels of IL-10 (*Figure 3c*). In contrast to healthy control moDCs, exogenous IL-10 did not inhibit cytokine production by IL10RA-deficient moDCs (*Figure 3c*). Hyper-responsive cytokine release peaked upon stimulation with 100 ng/ml LPS whereas lower concentrations (10 ng/ml) had less dramatic effects (*Supplementary Figure S4*), suggesting that deregulation of DC activation and loss of tolerance is only achieved after reaching threshold levels of stimulation, and that tolerance remains operational at lower levels of cellular activation.

To assess the role of IL-10R signaling in T-cell activation, PBMCs were stimulated with anti-CD3. Activated PBMCs from the patient and healthy controls secreted comparable amounts of IFN-γ and IL-17 (*Figure 3d*). IL-21 secretion could not be detected (data not shown). These data indicate that during remission, IL10RA-deficient T cells were not hyper-activated by only T-cell receptor ligation. However, IL-10 inhibited IFN-γ and IL-17 mRNA expression and secretion in healthy control PBMCs, but not in IL10RA-deficient PBMCs (*Figure 3d*).

In summary, endogenous IL-10 signaling is primarily required to restrain inflammatory TNF-α, IL-6, and IL-8 release by LPS-stimulated DCs, but does not affect co-stimulatory molecule expression. In the presence of functional IL-10 signaling, exogenous IL-10 regulates cytokine release by both T cells and DC, and reduces DC co-stimulatory molecule expression.

Intestinal lesions contain CD3$^+$ cells with T_H1 and T_H17 cell characteristics

To identify whether a particular T_H cell subtype response promoted intestinal disease in our patient, we performed immunohistochemical analysis of paraffin-embedded biopsies taken at the age of 3 and 9 months. Descending colon tissue showed an influx of CD3$^+$ cells in the lamina propria and intra-epithelial CD3$^+$ cells (*Figure 4a-c*). Co-staining of serial sections revealed that CD3$^+$ cells co-localized with IL-21$^+$, IL-17A$^+$, and T-bet$^+$ cells in the inflamed mucosa. Clusters of CD3$^+$IL-21$^+$ and IL-17^{neg} cells were present, indicating non-simultaneous IL-17 and IL-21 release. High numbers of intra-epithelial CD3$^+$ cells, mostly IL-21$^+$, were present at the age of 9 months (*Figure 4c*). Foci of Foxp3$^+$ cells were detected in the intestinal mucosa lesions, suggesting normal Foxp3$^+$ T cell recruitment (Isotype stainings are shown in *Supplementary Figure S5*). Therefore, in the absence of functional IL-10RA, inflammatory lesions contain both infiltrating T_H1–like and T_H17-like cells.

Figure 4 ***Presence of CD3⁺, IL-21⁺, IL-17⁺, T-bet⁺ and Foxp3⁺ cells in the inflamed intestinal mucosa of an IL10RA-deficient patient with infant IBD.***

Representative immunohistochemical staining for CD3, IL-21, IL-17A, T-bet, and Foxp3 in serial sections of paraffin-embedded biopsies of the colon descendens from the IL10RA-deficient patient taken at the age of ***(a)*** *3 months and* ***(b,c)*** *9 months. Original magnification: ×20.*

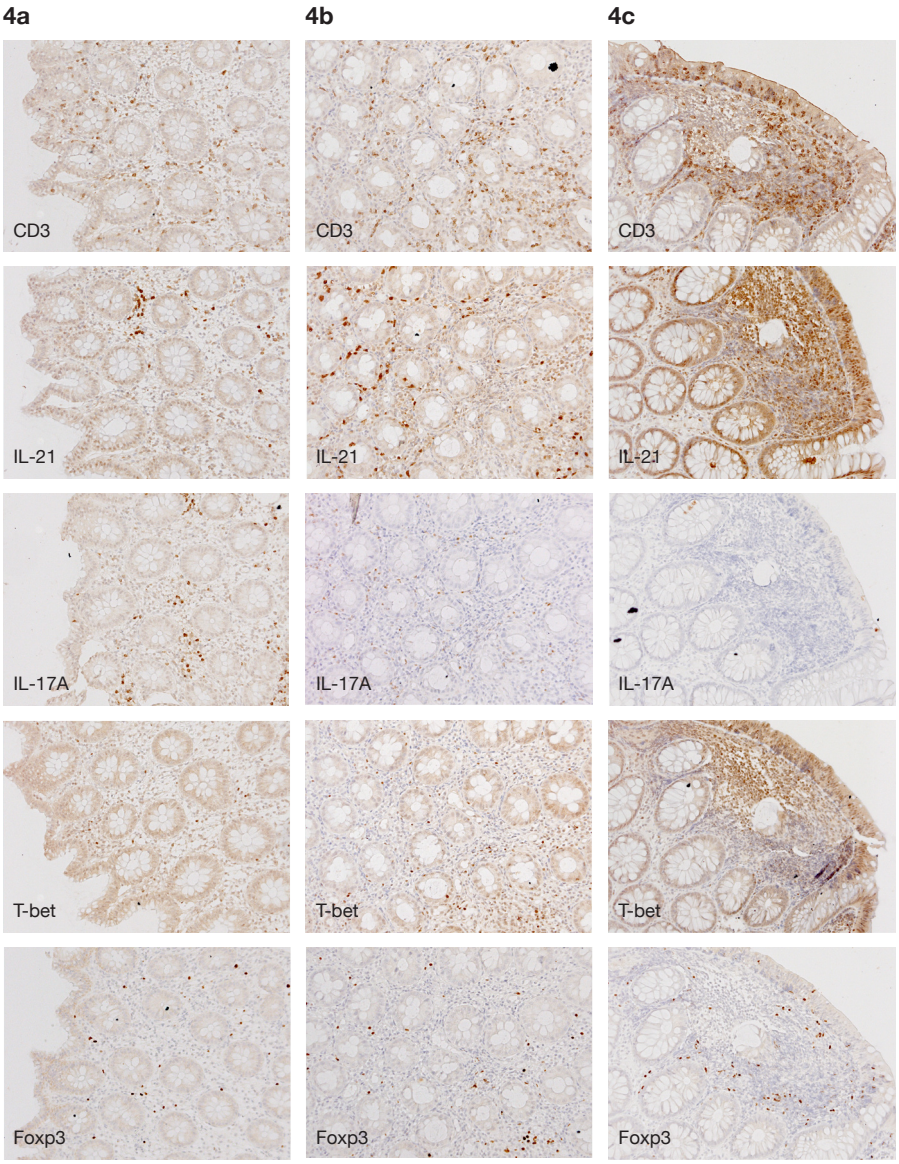
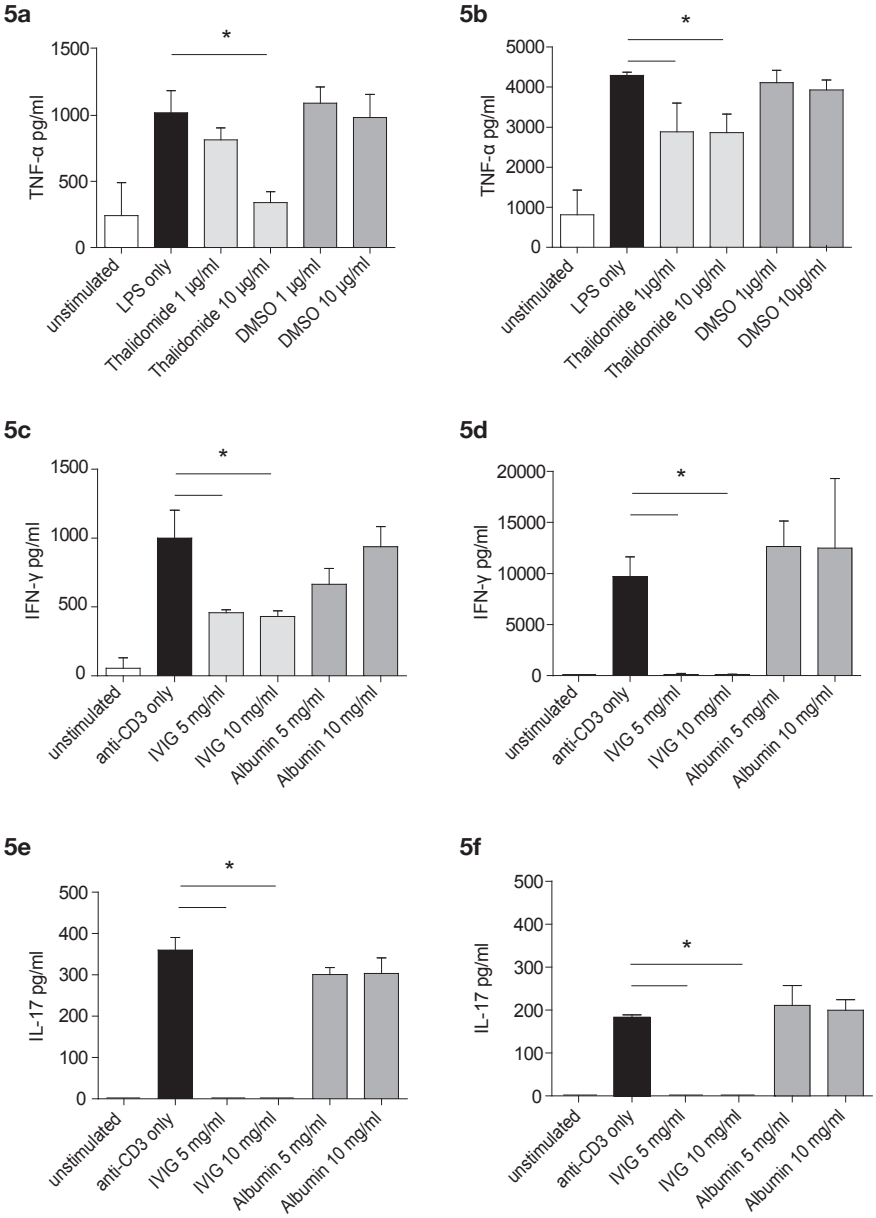


Figure 5 ***Thalidomide inhibits LPS-mediated TNF-α release and IVIG inhibits anti-CD3-driven IFN-γ and IL-17 release.***

(a, b) *PBMCs were isolated and stimulated with LPS (100 ng/ml) in the presence of thalidomide (1 or 10 µg/ml) or DMSO (1 or 10 µg/ml). After 20 h, cytokine levels in the supernatant were measured by an ELISA. TNF-α response for* ***(a)*** *three different healthy adult donors and* ***(b)*** *the IL10RA-deficient patient.* ***(c, d)*** *PBMCs were stimulated with anti-CD3 in presence of IVIG (5 or 10 mg/ml) or albumin (5 or 10 mg/ml). After 48 h, cytokine levels were measured in the supernatant. IL-17 and IFN-γ response for* ***(c)*** *three different healthy adult donors and* ***(d)*** *the IL10RA-deficient patient.*



Thalidomide inhibits LPS-mediated TNF-a production and IVIG inhibits anti-CD3-driven IFN-γ and IL-17 production by PBMCs

We addressed the effect of the patient's current therapy on IL10RA-deficient DC and T-cell responses in vitro. Thalidomide significantly decreased TNF-α production by LPS-stimulated PBMCs from both healthy controls and the patient. Thalidomide had no significant effect on anti-CD3-mediated cytokine production (data not shown). However, IVIG significantly reduced IL-17 and IFN-γ secretion by PBMCs from both healthy controls and the patient (*Figure 5*). Thus, combined thalidomide and IVIG may regulate cytokine release *in vitro* independently of sufficient IL-10 signaling.

Discussion

We report an 11-year-old patient with an *IL10RA* loss-of-function mutation who developed severe, early-onset infant IBD and frequent lower respiratory tract infections. As the patient had not received HSCT[6, 13] and was receiving immune suppressants without having clinical symptoms of intestinal disease, this clinical situation allowed an in-depth analysis of the mechanisms by which IL-10 regulates immune responses and intestinal immune homeostasis.

Deficient IL-10 signaling did not lead to detectable abnormal immune cell development. During treatment, the number of circulating $CD11c^{+}HLA\text{-}DR^{+}$ DCs and co-stimulatory molecule expression were normal, indicating sufficient bone marrow output and myeloid precursor differentiation. Whether local differentiation of various subpopulations of antigen-presenting cells in mucosal tissues is affected by defective IL-10 signaling could not be studied. Functionally, mature IL10RA-deficient moDCs displayed hyper-reactive LPS-induced cytokine secretion, but not MHCII or co-stimulatory molecule expression; this differential regulation by endogenous IL-10 is notable, as previous neutralization studies proposed a role for IL-10 in both autocrine co-stimulation and cytokine release.[21,22] However, our data agree with observations in $CD11c^{+}$ DC-specific IL10R-deficient mice, in which only DC cytokine release, and not co-stimulatory molecule expression, was affected.[23] Disparate regulation of co-stimulatory molecules and cytokines by endogenous IL-10 may possibly be explained by the use of different inhibitory signaling cascades, as IL-10-mediated regulation involves direct and indirect pathways which may act sequentially or are activated depending on the phase of cellular maturation.

As IL-10 was originally identified as an enhancer of thymocyte proliferation,[24] IL-10 deficiency could affect development of the T cell population. The composition of peripheral blood T cell subsets did not differ between the IL10RA-deficient patient

and pediatric IBD patients and controls. Moreover, Foxp3$^+$ cells were detected in the inflamed intestinal tissue of the patient, indicating normal Foxp3$^+$ T_{REG}-cell migration. Exogenous IL-10 regulated both IFN-γ and IL-17 production in healthy activated T cells *in vitro*. In agreement, the inhibitory effects of exogenous IL-10 on IFN-γ and IL-17 production were clearly defective in the IL10RA-deficient patient PBMCs. Moreover, the patients' intestinal lesions at diagnosis contained CD3$^+$ T cells with T_H1 and T_H17 cell cytokine profiles, demonstrating the importance of IL-10 signaling in limiting mucosal inflammatory T_H1- and T_H17-cell responses. Whether IL-10 regulates T_H1 and T_H17 cells directly or indirectly via antigen presenting cells or by favoring T_{REG} cell-mediated suppression remains unknown.[25,26] In mice, T_H17 cells, but not T_H1 cells, express IL-10R[27]; therefore, treatment of IL-10R deficiency may require both DC inhibition and T-cell suppression to fully inhibit T-cell driven inflammation.

The primary aim of our manuscript was to reveal how IL10R deficiency alters the function of the immune system. We do not advocate that immune suppression is a first treatment option for this genetic deficiency as HSCT clearly is desirable in view of possible increased risk of malignancy.[28] The treating physician discussed HSCT, but until now the family has refused to consider this option. It is striking that the patient has survived and until now has been in clinical remission of her intestinal symptoms. A possible explanation may be that the patient has modifying genes that have kept the intestinal disease mild. Based on our findings, we anticipate that inhibition of both innate and adaptive immune responses are required to achieve remission. The observed inhibitory effect of thalidomide on TNF-α production by LPS-stimulated IL10RA-deficient PBMCs suggests that TNF-α inhibition contributes to suppression of innate responses. However, as the patient failed to respond to infliximab, other mechanisms may also play a role. Other immuno-modulatory effects of thalidomide include inhibition of NF-κB activity and increased degradation of TNF-α mRNA.[29,30] Moreover, thalidomide binds cereblon, of which interferon regulatory factor (IRF-4) is a downstream target.[31,32] IRF-4 is an important regulator of T_H17 cells, and IRF-4-deficient mice display reduced *IL-17* gene expression during chronic intestinal inflammation.[33] Although thalidomide had no effect on T_H1 and T_H17 cell cytokine production in vitro, we cannot exclude an effect of thalidomide on T_H1 and T_H17 cells *in vivo*.

Although IVIG is primarily recognized to inhibit innate immune cells, we demonstrated that IVIG inhibited adaptive T_H cell cytokines, i.e. IFN-γ and IL-17. IVIG consists of pooled serum IgG and is used as replacement therapy in immunodeficient individuals or to suppress pathological immune responses.[34] IVIG exerts pleiotropic immune-modulatory actions, which can be indirectly mediated by DCs *in vitro*[35] or mediated by direct interaction with T cells[36,37]. It is unclear whether treatment with colchicine, a microtubule formation inhibitor more often used to treat Behçet's disease, promoted

the induction of remission in our patient. Currently, the dose of colchicine has been drastically reduced without clinical deterioration.

Our data provides fundamental information on the functioning of the immune system in an IL-10R-deficient patient who appears responsive to immunosuppressive therapy. As our patient did not respond to classical treatments, including infliximab and sulfasalazine, we envisage that IBD patients with less severe defects in IL-10 signaling may be similarly unresponsive to immunosuppressive regimens. Therefore, we aim to investigate whether patients who are resistant to classical IBD therapy are more likely to have suboptimal IL-10 signaling. Overall, our findings promote our knowledge of intestinal immune regulation, which is urgently required to advance our ability to classify the varying clinical pathologies of IBD.

Acknowledgements

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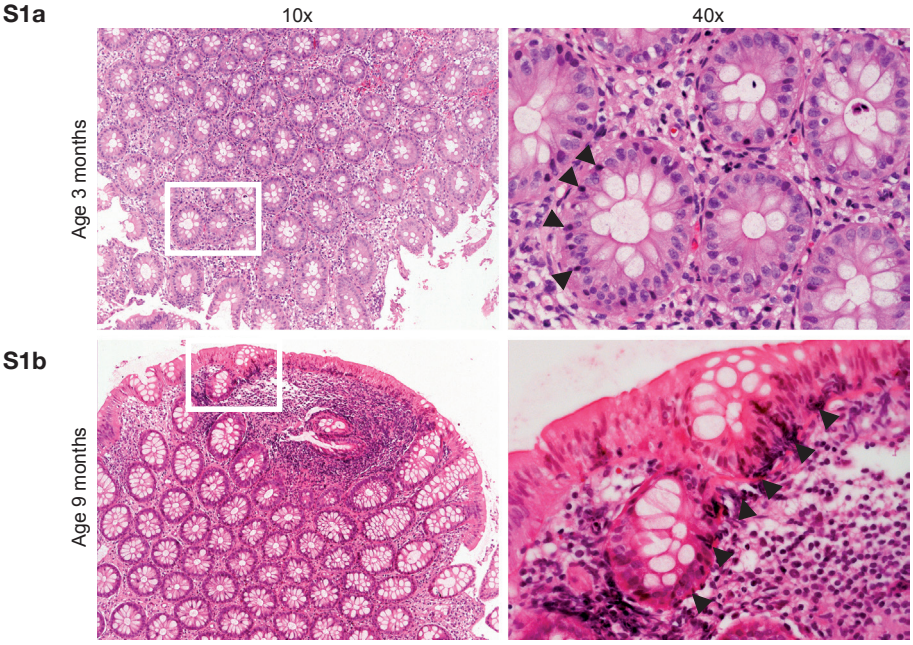
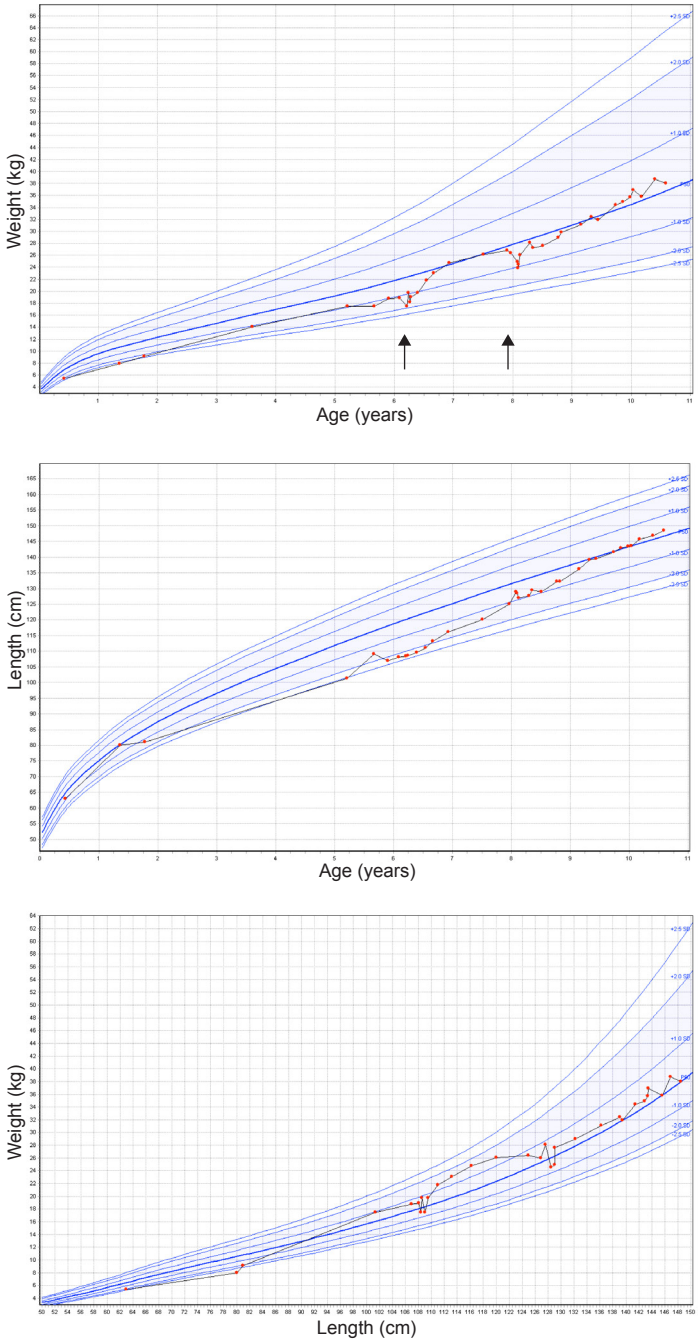
Supplementary figures and table

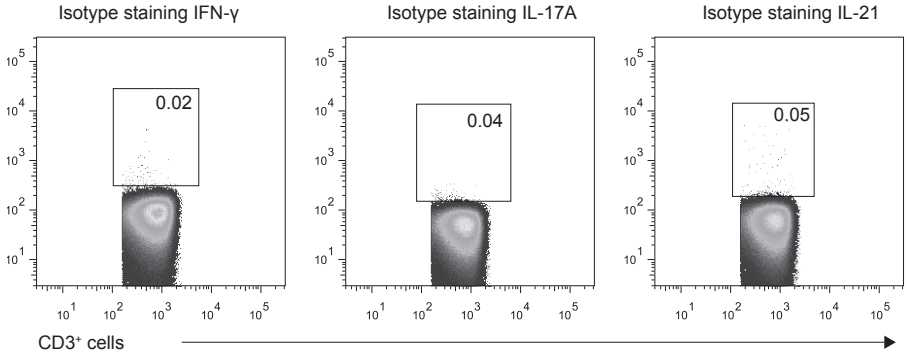
Supplementary Figure S1 *Intestinal inflammation in an IL10RA-deficient patient with inflammatory bowel disease in infancy. Histological analysis of paraffin-embedded specimens obtained from sites of inflammation in the colon descendens* ***(a)*** *at the age of 3 months and* ***(b)*** *9 months. Sections were stained with hematoxylin-eosin. Arrowheads indicate immune cells which have infiltrated into the epithelial layer. Original magnifications: ×10 (left) and ×40 (right).*



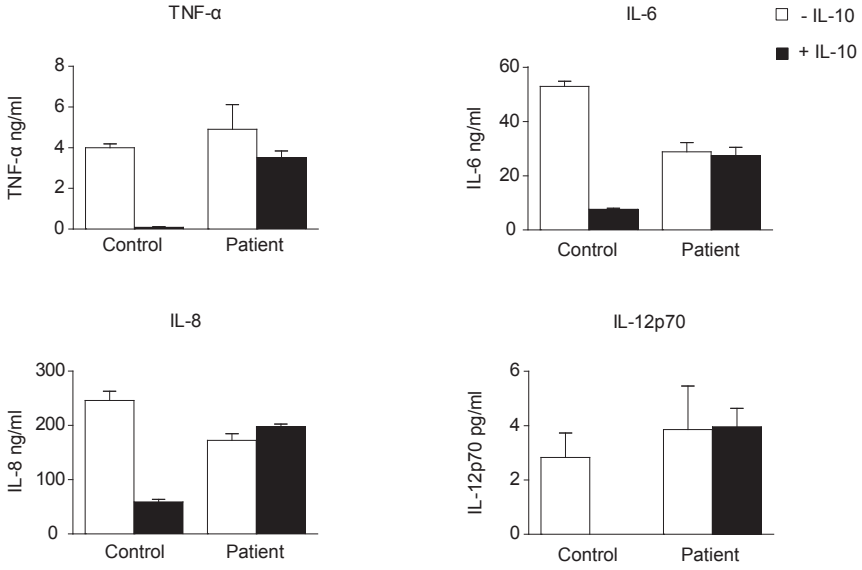
Supplementary Figure S2 *Growth curves for the IL10RA-deficient patient. Arrows indicate weightloss associated with respiratory symptoms.*



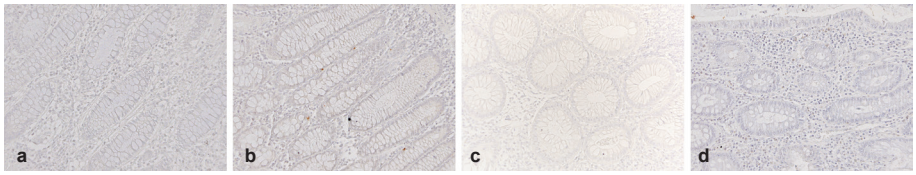
Supplementary Figure S3 *Representative flowcytometric dot plots for isotype control staining of the activated PBMCs from age-matched IBD patients in remission and the IL-10R-deficient patient shown in Figure 2D.*



Supplementary Figure S4 *Control and patient moDCs were matured with LPS (10ng/ml) in the presence or absence of IL-10 (25 ng/ml). After 20 h, the levels of TNF-α, IL-6, IL-8, and IL-12p40 in the culture supernatants were determined using a Cytometric Bead Array. Data are mean ± SD.*



Supplementary Figure S5 *Representative isotype control staining of paraffin embedded specimens obtained from the colon descendens of pediatric patients with IBD.* ***(a)*** *Polyclonal rabbit anti-goat Ig isotype control staining for IL-21 and CD3.* ***(b)*** *Goat IgG1 isotype control staining for IL-17A.* ***(c)*** *Mouse IgG1 isotype control staining for Foxp3.* ***(d)*** *Mouse IgG1 isotype control staining for T-bet. Sections were counterstained with hematoxylin. Original magnification: × 20.*



Supplementary Table S1 *Patient characteristics*

	Orthopedic controls	IBD in remission	Active IBD
Number	13	8	30
Age in years, mean (SD)	13.9 (2.4)	16.8 (0.6)	13.4 (3.2)
Male (n)	8	1	18
Female (n)	5	7	1
Diagnosis			
Colitis Ulcerosa		3	9
Crohn's disease		5	20
Intermediate disease			1
Treatment			
no			8
azathioprine		3	15
mesazaline		2	4
infliximab		2	4
methotrexaat		1	2
prednisolone			1
mercaptopurine			1

4

Increased production of interleukin-21, but not interleukin-17A, in the small intestine characterizes pediatric celiac disease

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ABSTRACT

Celiac disease (CD) is caused by inflammatory $CD4^+$ T-cell responses to dietary gluten. It is unclear whether interleukin (IL)-21 and IL-17A contribute to CD onset and lesion severity; therefore, we investigated IL-21 and IL-17A expression in biopsies from pediatric CD patients with different histopathological scores. High numbers of IL-21-producing cells were observed in pediatric CD lesions, even Marsh 1-2 lesions while increased numbers of IL-17-secreting cells were not observed. Intraepithelial lymphocytes, $CD3^+$ T cells but also neutrophils secreted IL-21. Flow cytometry of lamina propria cells revealed a large population of IL-21- and IFN-γ-secreting $CD4^+$ T cells that did not secrete IL-17A. Adult CD patient biopsies also contained high numbers of IL-21-positive cells; however, enhanced numbers of IL-17-positive cells were observed in a small subgroup of patients with severe lesions. As duodenal tissue damage increases contact with microbe-associated molecular patterns, we hypothesized that microbial sensing by Toll-like receptors (TLR) modulates T cell-derived cytokine secretion. Co-stimulation with TLR3 ligands during polyclonal T-cell activation significantly increased IL-21 secretion, while TLR2 ligands selectively enhanced IL-17A. These results demonstrate that an IL-17A-independent increase in IL-21 production by $CD4^+$ T cells is characteristic of pediatric CD. We hypothesize that incidental IL-17 secretion is caused by tissue damage rather than gluten-specific responses.

Introduction

Celiac disease (CD) is characterized by small intestinal enteropathy with villous atrophy, crypt hyperplasia and increased numbers of infiltrating lymphocytes in the epithelium and lamina propria. $CD4^+$ T lymphocytes with specificity for dietary gluten drive the mucosal inflammation in CD via the secretion of proinflammatory cytokines. In particular, production of the cytokine interferon gamma (IFN-γ) by gluten-specific $CD4^+$ T cells is thought to play a role in intestinal epithelial damage.[1,2] However, other $CD4^+$ T cell-derived cytokines, in particular interleukin-21 (IL-21) have recently been suggested to contribute to the pathogenesis of CD.[3]

IL-21 has received increasing attention, as genome-wide association studies for CD identified risk variants in the *IL-2/IL-21* gene region.[4] Detection of increased IL-21 mRNA expression in duodenal biopsies of untreated adult CD patients has supported a possible role for IL-21 in disease pathogenesis.[3] $CD4^+$ T cells,[5] natural killer T cells,[6] and neutrophils[7] have the capacity to secrete IL-21. Isolated lamina propria lymphocytes (LPL) and intra-epithelial lymphocytes (IEL) from CD patients were shown to produce IL-21.[8] Additionally, IL-21 can be produced by gluten-reactive $CD4^+$ T cells derived from biopsies of CD patients.[9] IL-21 may contribute to intestinal damage via enhancing the production of IFN-γ, as neutralization of IL-21 in gliadin-stimulated biopsy cultures results in reduced IFN-γ production.[3] However, IL-21 may be involved in multiple aspects of CD pathogenesis as it can also regulate the growth, survival and function of B, T and natural killer cells and limit the differentiation and suppressive capacity of regulatory T cells.[10,11] Currently, the relative frequency, localization and phenotype of the IL-21-producing cells in CD lesions remain largely unknown. Moreover, the relationship between IL-21 secretion and the severity of intestinal lesions, based on the Marsh score, has not determined. IL-21 may be derived from different $CD4^+$ T cells. For example, T helper 17 cells (Th17) produce IL-21, in addition to IL-17A, IL-17F, and IL-22 [10,12-14] , and T follicular helper cells[15] and T regulatory-1 cells can also produce IL-21.[16] As two studies have demonstrated increased expression of IL-17 mRNA in mucosal biopsies from CD patients, compared to biopsies from patients on a gluten-free diet or healthy tissue[17,18], it is conceivable that Th17 cells are the source of IL-21 in CD. However, this hypothesis is contradicted by the finding that gliadin-specific T cell lines secrete IL-21 but not IL-17.[9]

To dissect the role of IL-21 in the pathogenesis of CD, we investigated the expression of IL-21 *in situ* by immunohistochemical staining of small intestinal biopsies from a well-defined cohort of new-onset pediatric CD patients with different histopathological scores.

Furthermore, we phenotyped the IL-21-producing cells by flow cytometric analyses of polyclonally restimulated lamina propria lymphocytes (LPL) and by immunohistochemical staining for IL-17 in small intestinal biopsies from CD

patients. Our analysis focused on pediatric CD patients and was aimed at new-onset disease. For comparison, we also analyzed biopsies from adult patients with CD. To obtain insight into the possible causes of the varied patterns of IL-17 release observed in pediatric and adult CD patients, we determined whether particular microbe-associated molecular patterns (MAMPs) were involved in enhancing the secretion of IL-17 or IL-21 by $CD4^+$ T cells.

Our results demonstrate that increased IL-21 production is observed in pediatric CD patients, even in lesions with low Marsh scores. The IL-21-secreting cells were mostly $CD4^+$ and secreted IFN-γ and low levels of IL-10, but not IL-17. In a small subgroup of adult patients, IL-17 secretion was more intense. Such incidental increases in IL-17 secretion may be associated with the encounter of Toll-like receptor 2 (TLR2) ligands in lesional tissue, as TLR2 ligation of activated T cells selectively enhanced IL-17 release, while TLR3 ligation favored IL-21 release.

Methods

Patients and controls

Pediatric patients (age 1-11 years, mean 4.9) with a suspected diagnosis of CD who underwent an esophagogastroduodenoscopy (EGD) at Erasmus MC – Sophia Children's Hospital, Rotterdam, The Netherlands were approached for participation in this study. After diagnosis, the patients with biopsy-proven CD were included in the patient group, whereas children with a normal intestinal histology and negative auto-antibodies were included in the control group. Patients diagnosed with other diseases were excluded from the study. The histopathological grade of the duodenal biopsies was based on the Marsh score[19] (Marsh 0, n = 10; Marsh 1, n = 9; Marsh 2, n = 2, Marsh 3A, n = 8; Marsh 3B, n = 11, Marsh 3C, n = 12). All CD patients had positive serum anti-endomysium and/or anti-transglutaminase-2 antibodies. For comparison, intestinal biopsies from children with inflammatory bowel disease (IBD) diagnosed with colitis ulcerosa (n = 10) or Crohn's disease (n = 9) were also obtained (age 7-18 years, mean 13.9). The study received ethical approval from the local Ethics Committee (Erasmus MC, Rotterdam, The Netherlands) and the parents or guardians of all patients provided signed informed consent.

Immunohistochemistry on paraffin sections

For immunohistochemistry, the sections were deparaffinized and endogenous peroxidases were quenched with 3% H_2O_2 in methanol for 20 min. Antigen retrieval was performed by microwave treatment in citrate buffer (10 mM, pH 6.0). The sections were blocked for 1 h in 10% normal human serum diluted in 10 mM Tris, 5 mM EDTA, 0.15 M NaCl, 0.25% gelatine, 0.05% Tween-20, pH 8. Antibody incubation was performed overnight at 4°C using anti-IL-17A (1:50; R&D Systems, Abingdon,

UK) or anti-IL-21 (1:100; Lifespan Biosciences, Seattle, WA, USA). Immunoreactive sites were detected with biotinylated secondary rabbit anti-goat or goat anti-rabbit serum using the Vectastain ABC Elite Kit (Vector Laboratories, Burlingame, CA, USA) and 3,3'-diaminobenzidine tetrahydrochloride (Sigma-Aldrich, Zwijndrecht, the Netherlands), and the nuclei were counterstained with hematoxylin (Vector Laboratories).

For double immunofluorescent staining, anti-IL-21 and anti-CD4 Ab-8 (Thermo Scientific, Fremont, CA, USA) or anti-CD15 (BD-Biosciences, Breda, the Netherlands) were used followed by goat anti-rabbit secondary antibody fluorescently labeled with DyLight-488 or goat anti-mouse secondary antibody labeled with DyLight-594 (Thermo Scientific). The sections were mounted in medium for fluorescence containing DAPI (Vector Laboratories). An isotype-matched negative control was performed for each immunostaining. The positive cells were counted by two independent investigators in a field of 6000 μm^2.

Images were acquired and analyzed using a Leica DM5500B upright microscope and LAS-AF image acquisition software (Leica Microsystems, Rijswijk, The Netherlands).

Lymphocyte isolation

Peripheral blood mononuclear cells (PBMC) were isolated using a Ficoll-Hypaque gradient. $CD4^+$ T cells from 6 CD patients (Marsh score > 3A) were purified by negative depletion using magnetically labeled antibodies (Dynabeads; Invitrogen, Carlsbad, CA, USA) according to the manufacturer's instructions. Lamina propria lymphocytes (LPL) or intra-epithelial cells (IEL) were isolated from the duodenal samples of 5 CD patients, as previously described.[20] The average yield of LPLs was approximately 1-1.5 x 10^6 cells per patient.

TLR expression on purified T cells

$CD4^+$ T cells, $CD4^+CD45RA^+$ T cells and $CD4^+CD45RO^+$ T cells were purified from PBMC obtained from a healthy donor using flow cytometric cell sorting; the purity of the populations was 99%, 99% and 90%, respectively. Total RNA was purified from the sorted T cells and non-sorted PBMC (control) using the NucleoSpin RNA XS kit (Machery-Nagel, Düren, Germany). Up to 1 µg RNA was reverse transcribed to single-stranded cDNA using a mix of random hexamers (2.5 µM) and oligo(dT) primers (20 nM). The reverse transcription reaction was performed in a total volume of 25 µl containing 0.2 mM of each dNTP (Amersham Pharmacia BioTech, Piscataway, NJ, USA), 200 U Moloney murine leukemia virus reverse transcriptase (M-MLV RT; Promega, Madison, WI, USA) and 25 U RNAsin (Promega). The conditions for the reverse transcription reaction were 37°C for 30 min, 42°C for 15 min and 94°C for 5 min. The cDNA was diluted five-fold and stored at –20°C. Conventional polymerase chain reaction (PCR) or real-time quantitative PCR using the AbiPrismR

7900 Sequence Detection System (PE Applied Biosystems, Foster City, CA, USA) using general fluorescence-based detection with SYBR green were performed using the specific primers listed below. The PCR products were purified and visualized by agarose gel electrophoresis.

TLR primers

Specific primers were designed across different constant region exons resulting in the following primers: *GAPDH*: forward 5'-GTCGGAGTCAACGGATT-3', reverse 5'-AAGCTTCCCGTTCTCAG-3'; *TLR2*: forward 5'-GCTGGTG GCAATAACTTC-3', reverse 5'-AGGCCCACATCATTTTC-3'; *TLR3* forward 5'-GCACGAATTTGACTGAACT-3', reverse 5'-TCCAATTGCGTGAAAAC-3'; *TLR4* forward 5'-CCTGGACCTGAGCTTTAAT-3', reverse 5'-CCACCA GCTTCTGTAAACTT-3'; *TLR7* forward 5'- CTGCCC-TGTGATGTCACT-3', reverse 5'- CGCTGGGGAGATGTCT-3'.

Cell culture and cytokine analysis

Isolated $CD4^+$ T cells were activated in culture using anti-CD3 anti-CD28 beads (Dynabeads, Invitrogen) in the absence (control) or presence of 1 μg/ml Pam3Cys (EMC Microcollections, Tübingen), 25 μg/ml polyinosinic-polycytidylic acid (Poly I:C; Sigma-Aldrich), 5 μg/ml lipopolysaccharide (LPS; Sigma-Aldrich) or 5 μg/ml of the imidazoquinoline-derivative CL097 (Invivogen, San Diego, USA). After 48 h and 120 h, the concentrations of IL-21 and IL-17A in the cell supernatants were measured using the Ready-SET-go! Kit (eBiosciences, San Diego, CA, USA) and Duoset (R&D Systems) enzyme-linked immunosorbent assays (ELISAs), respectively.

Flow cytometry

PBMC and LPL were reactivated for 5 h with 0.05 μg/ml phorbol 12-myristate 13-acetate (PMA; Sigma-Aldrich) and 0.5 μg/ml ionomycin (Sigma-Aldrich) in the presence of Brefeldin A for the last 4 h (3 μg/ml eBiosciences). After reactivation, the cells were stained for flow cytometry using monoclonal antibodies against CD3, CD4, CD8, αβ TCR and γδ TCR (all BD-Biosciences, Breda, the Netherlands), fixed with 2% formalin solution and intracellular staining was performed using saponin (Sigma-Aldrich), followed by IL-21 (eBiosciences), IL-17A (eBiosciences), interferon-gamma (IFN-γ, BD-Biosciences), IL-10 (BD-Biosciences) or the appropriate isotype controls (all eBiosciences). Flow cytometric analysis was performed using the FACSCanto™ II (BD-Biosciences).

Statistics

Prism (Graphpad Software Inc., La Jolla, CA, USA) was used to perform statistical analysis using paired sample *t*-tests or the Mann-Whitney *U* test, as indicated in the figure legends. *P* values < 0.05 were considered statistically significant.

Results

Biopsies from pediatric CD patients contain increased numbers of IL-21-producing cells

To investigate the presence of IL-21-secreting cells *in situ* in biopsies from pediatric CD patients, an IL-21 immunohistochemical detection method was developed for paraffin-embedded tissue. In control tissue biopsies with a Marsh score of 0, only a few IL-21-positive cells were observed in the lamina propria, while the isotype control staining was negative (*Figure 1a, f*). Relative to control tissue with a Marsh score of 0, duodenal mucosa with a Marsh score of 1-3 contained higher numbers of IL-21-secreting cells (*Figure 1a-e*). The majority of the IL-21-positive cells were randomly distributed within the lamina propria (*Figure 1d, e*). Quantification of the numbers of IL-21 positive cells demonstrated a significant difference between CD patients with a Marsh score of 1-3 and control patients ($P < 0.05$; *Figure 2a*). It is of note that even the biopsies from patients with low Marsh scores (Marsh 1-2) contained significantly higher numbers of IL-21-positive cells than the control tissues (Marsh 0). In tissue with Marsh 3B or 3C lesions, IL-21-positive cells were also detected between the epithelial cells. These results are consistent with recent data showing that IL-21 is also produced by IELs in the lesional tissue of CD patients.[8] Quantification of these cells on histology revealed increased numbers of IL-21-secreting IELs in tissue with higher Marsh scores (*Figure 2b*). It should be noted that the number of IL-21 positive IELs varied between patients. Moreover, mildly positive cells were difficult to detect due to background staining in the inflamed tissue. Therefore, flow cytometric analysis was used to confirm that IELs isolated from lesional tissue secreted IL-21 (*Figure 2c*).

The majority of IL-21-producing cells co-express CD4 and IFN-γ

As IL-21 can be produced by $CD4^+$ T cells[5], we performed immunohistochemical double staining for IL-21 and CD4 on paraffin-embedded duodenal biopsies from pediatric CD patients. Double staining for IL-21 and CD4 demonstrated the presence of IL-21 in $CD4^+$ cells (*Figure 3a,b*), although $CD4^{neg}$ $IL\text{-}21^+$ cells were also detected. Analysis of the $CD4^{neg}$ revealed that polymorphonuclear cells within the lamina propria stained positive for IL-21. $CD15^+$ neutrophils have been shown to have to capacity to secrete IL-21.[7] Double staining for CD15 and IL-21 revealed the presence of $IL\text{-}21^+CD15^+$ cells in CD lesions (*Figure 3c*). Next, we isolated LPL from the duodenal biopsies of 5 pediatric CD patients. IL-21-producing cells were further phenotyped by flow cytometric analysis of freshly isolated phorbol ester (PMA) and calcium ionophore-restimulated peripheral blood mononuclear cells (PBMC) and LPL from the same 5 pediatric CD patients. $IL\text{-}21^+$ LPL revealed no IL-21 staining in $CD3^+CD8^+$ cells, suggesting that the IL-21-producing cells may be $CD3^+CD4^+$. Further analysis of the $CD3^+$ population revealed that 92.91% ± 5.16% of the IL-21 producing cells were αβ T cell receptor (TCR) positive, whereas 1.95% ± 0.79% of the

Figure 1 ***Increased numbers of IL-21-producing cells are observed in biopsies from pediatric CD patients. (a-e)*** *Immunohistochemical detection of IL-21 in paraffin-embedded duodenal biopsies from* ***(a)*** *a pediatric control patient (Marsh score 0) and CD patients with a* ***(b)*** *Marsh score of 1,* ***(c)*** *Marsh score of 3A,* ***(d)*** *Marsh score of 3B and* ***(e)*** *Marsh score of 3C.* ***(f)*** *Isotype control antibody staining of a biopsy from a CD patient with a Marsh score of 3B. Original magnification: ×20. The images are representative of all 57 CD patients and controls.*

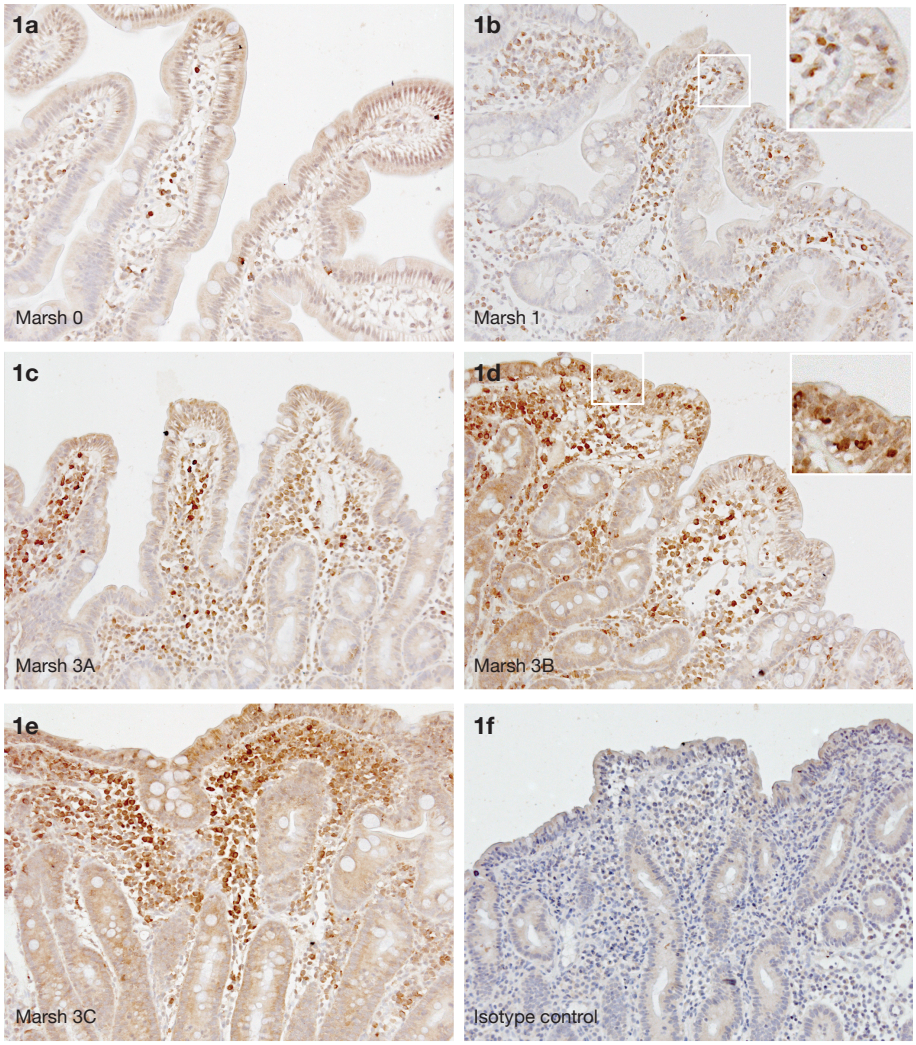


Figure 2 ***Increased numbers of IL-21-positive cells are observed in pediatric CD mucosa compared to control mucosa. (a)*** *Quantification of the number of IL-21-positive cells in the lamina propria in the different patient groups. Positive cells were counted at an original magnification of ×63 in a field of 6000 µm² **(b)** Quantification of the number of intra-epithelial IL-21-positive cells in the different patient groups. Positive cells were counted at an original magnification of ×63 per 500µm length. The slides were analyzed in blinded fashion by two independent investigators. Each point represents a single subject; the horizontal bars represent the mean values. * P<0.05 compared to Marsh score 0, Mann-Whitney U tests. IELs were isolated from CD patients, the cells were stimulated for 5 h with PMA/ionomycin and Brefeldin, and the expression of intracellular cytokines was detected by flow cytometry. **(c)** Representative dotplots of isotype staining and IL-21 staining in CD3⁺ IELs of a Marsh 2 patient.*

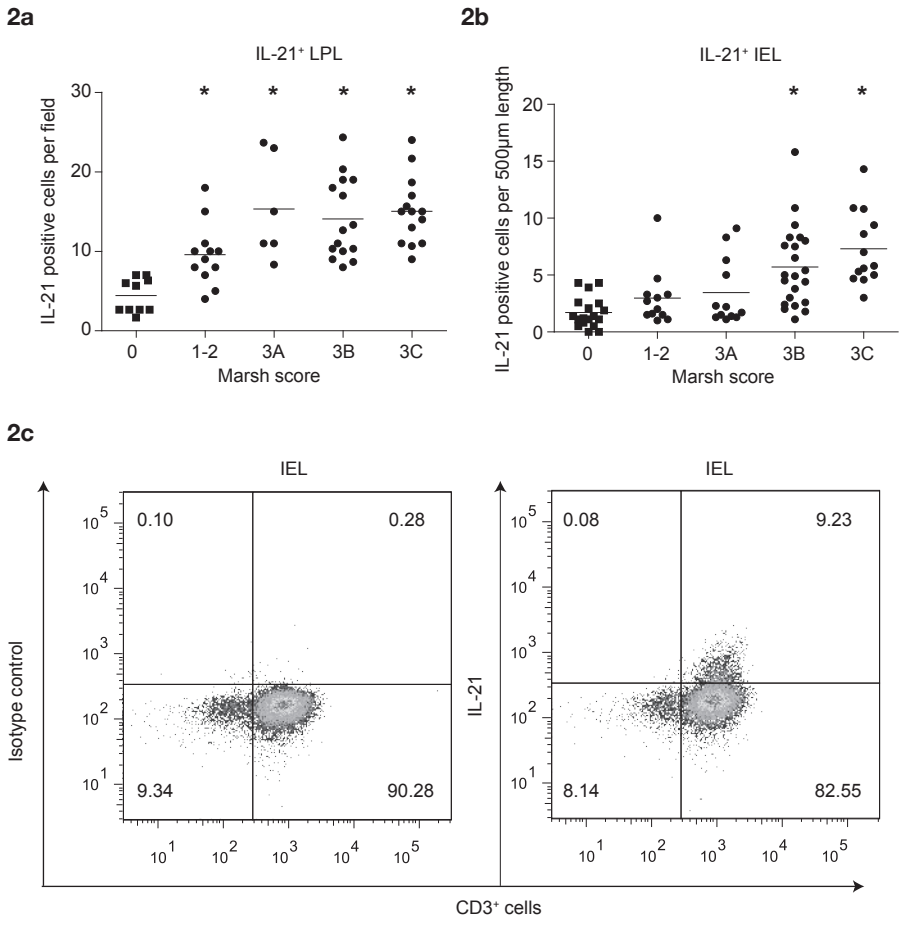
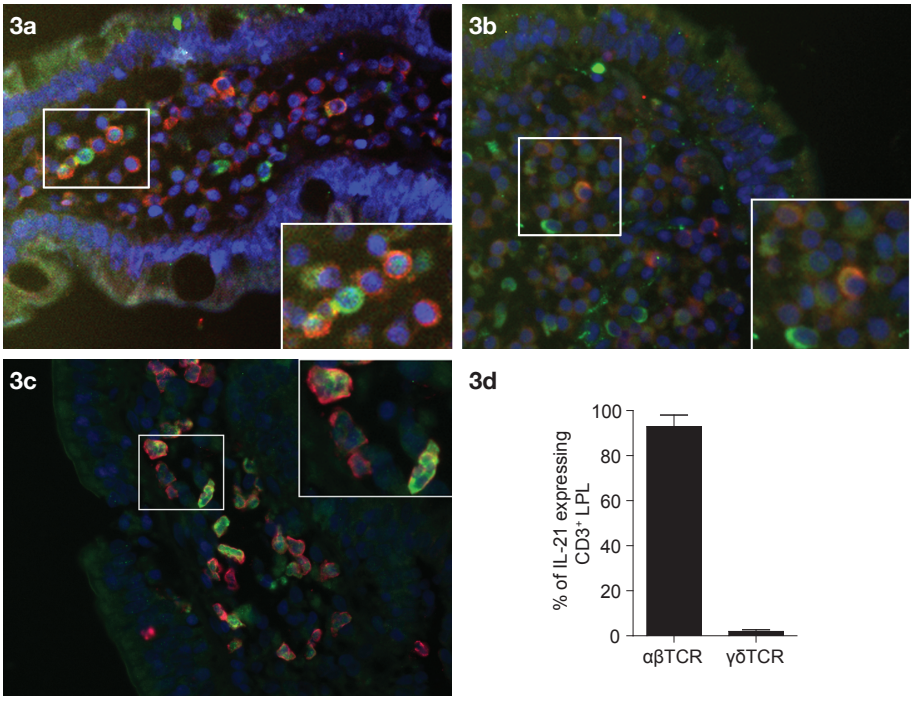
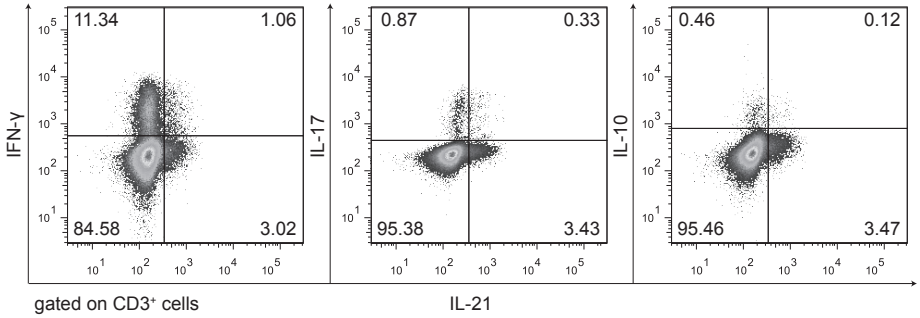


Figure 3 ***The majority of IL-21-producing cells in CD biopsies co-express CD4 and IFN-γ. (a)*** *Representative immunofluorescent double staining of paraffin-embedded duodenal biopsies from a control patient (Marsh score 0) and a CD patient with a Marsh score of 3C* ***(b)****. Green indicates IL-21 staining, red indicates CD4 staining; blue indicates DAPI nuclear counterstaining. Original magnification: ×20.* ***(c)*** *Representative immunofluorescent double staining of paraffin-embedded duodenal biopsies from a control patient CD patient with a Marsh score of 3A. Green indicates IL-21 staining, red indicates CD15 staining; blue indicates DAPI nuclear counterstaining. Original magnification: x20. Peripheral and lamina propria lymphocytes were isolated from 5 different CD patients. The cells were stimulated for 5 h with PMA/ionomycin and Brefeldin, and the expression of αβ TCR and γδ TCR and intracellular cytokines were detected by flow cytometry.* ***(d)*** *Percentage of $CD3^+IL\text{-}21^+$ LPLs expressing αβ TCR and γδ TCR in 5 CD patients. Data indicate mean ± SD.* ***(e,f)*** *Representative dotplots of double stained cells showing the co-expression of IL-21 and IFN-γ (left panel), IL-21 and IL-17A (middle panel) or IL-21 and IL-10 (right panel) gated using the appropriate isotype control antibodies. Quantitative analysis of the percentage of cytokine-producing cells within the $CD3^+$ population of* ***(g)*** *peripheral blood lymphocytes and* ***(h)*** *lamina propria cells isolated from five 5 CD patients; box and whiskers plot; the horizontal bars represent the mean values.*



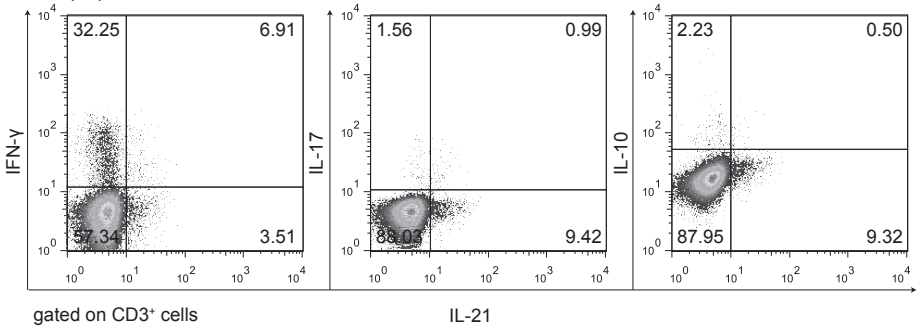
3e

Peripheral blood



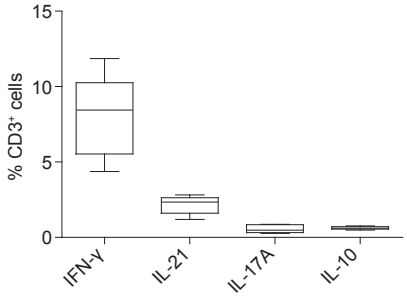
3f

Lamina propria



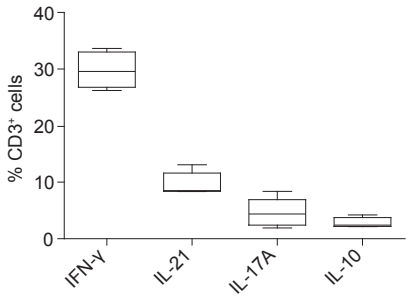
3g

Peripheral blood



3h

Lamina propria



IL-21$^+$ cells were γδ TCR positive (*Figure 3d*). Moreover, the analysis did not reveal strong expression of the T follicular helper cell marker C-X-C chemokine receptor type 5 (CXCR5) in IL-21 secreting cells (data not shown). These data demonstrate that the majority of IL-21-producing T cells in the lamina propria of pediatric CD patients consist of CD3$^+$ αβ TCR positive T cells.

Next, the expression of T helper cell-related cytokines by T cells was determined (*Figure 3e-h*). In all of the LPL isolates, a clear CD3$^+$IL-21$^+$ population was detected, while the population of IL-17A-producing cells was small. Nearly two-thirds of the IL-21-positive cells co-expressed IFN-γ, whereas less than 12% of the IL-21-positive cells were IL-17A-positive. Interestingly, IL-10-producing cells were virtually absent in restimulated LPL (*Figure 3f,h* and *Supplementary Figure S1* for isotype stainings). All of the cytokines tested were detectable in PBMCs, including IL-17A, yet similarly to LPL, only a small percentage of IL-21$^+$IL-17A$^+$ cells could be detected (*Figure 3e,g* and *Supplementary Figure S1* for isotype stainings). In conclusion, the majority of IL-21-secreting LPL co-express IFN-γ but are negative for IL-17 and IL-10.

Increased numbers of IL-17A-producing cells are not observed in biopsies from pediatric CD patients

To establish whether a small number of Th17 cells could be detected in IL-21-positive CD biopsies, immunohistochemical staining for IL-17A was performed in serial sections of the duodenal tissues from the pediatric CD patients. As shown in *Figure 4*, IL-17A-positive cells were detected in the lamina propria of CD patients and controls. However, in contrast to the increased numbers of IL-21-secreting cells observed with increasing Marsh score, the numbers of IL-17A-positive cells did not vary with the Marsh score (*Figure 4b-e*). Consequently, quantification of the numbers of IL-17A-positive cells in all biopsies demonstrated no significant difference between CD patients and control tissues (*Figure 4f*). These data indicate that IL-21 is not produced by Th17 cells in the inflamed tissue of untreated, new-onset pediatric CD patients.

Presence of IL-21- and IL-17A-secreting cells in the mucosa of pediatric patients with inflammatory bowel disease

IL-17A-secreting cells are involved in the pathogenesis of inflammatory bowel disease (IBD).[21, 22] To detect co-localizing IL-17A- and IL-21-producing cells, intestinal biopsies from pediatric IBD patients diagnosed with Crohn's disease or ulcerative colitis were immunohistochemically stained for IL-21 and IL-17A. Although most of the biopsies from active IBD patients contained high numbers of IL-21-secreting cells, the localization of IL-21-secreting cells in IBD was different to CD. As previously stated, IL-21-secreting cells were randomly distributed within the lamina propria in CD (*Figure 1*). In IBD, IL-21-secreting cells were located in the lamina propria, with the appearance of increased numbers of cells in inflamed regions, such as in damaged crypts or ulcerative lesions (*Figure 5 a,b,e,f*). By co-staining serial sections of the same

Figure 4 ***The number of IL-17A-producing cells is not increased in biopsies from pediatric CD patients. (a-d)*** *Representative IL-17A staining of paraffin-embedded duodenal biopsies from* ***(a)*** *a pediatric control patient (Marsh score 0) and CD patients with a* ***(b)*** *Marsh score of 3A,* ***(c)*** *Marsh score of 3B and* ***(d)*** *Marsh score of 3C.* ***(e)*** *IL-17A isotype control antibody staining of a biopsy from a CD patient with a Marsh score of 2. Original magnification: ×20. The images are representative of all 41 CD patients and controls.* ***(f)*** *Quantification of the number of IL-17A-positive cells in the different pediatric patient groups. Positive cells were counted at an original magnification of ×63 in a field of 6000 μm². All slides were analyzed by two independent investigators in blinded fashion. Each point represents a single subject; the horizontal bars represent the mean values. No significant differences were observed between the groups, Mann-Whitney U test.*

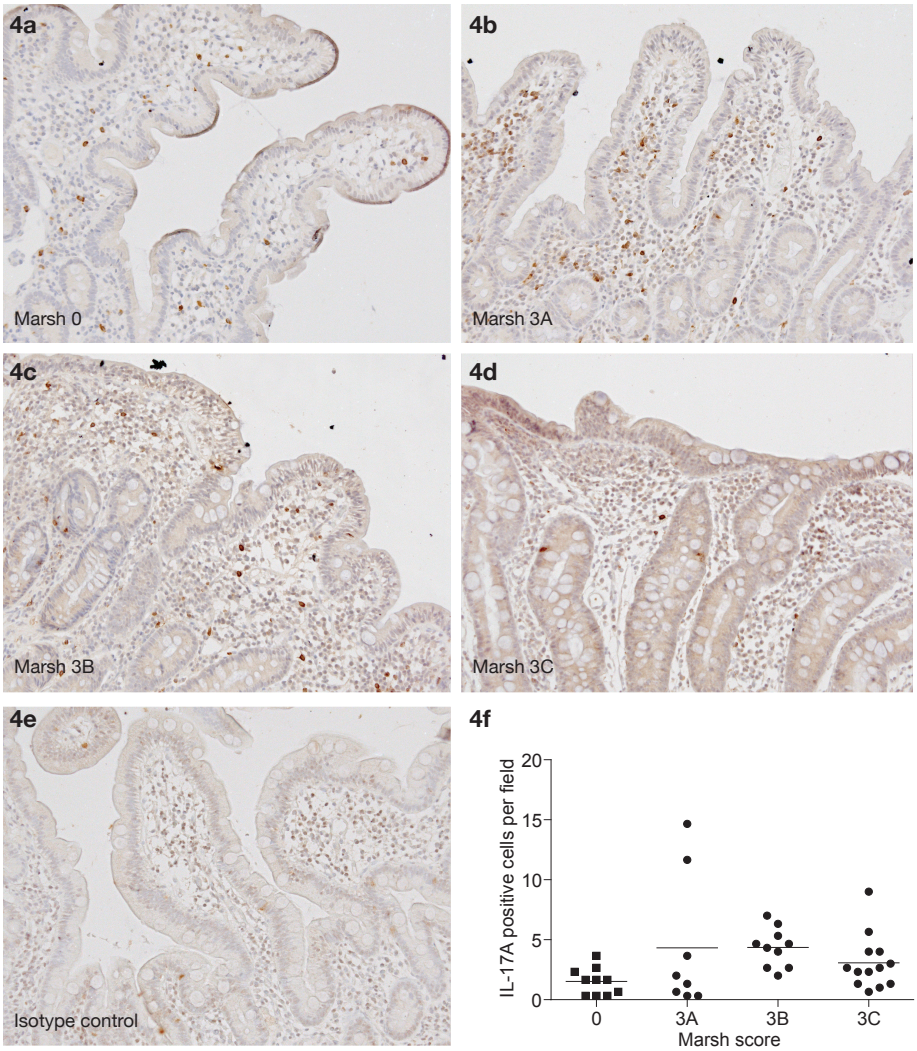
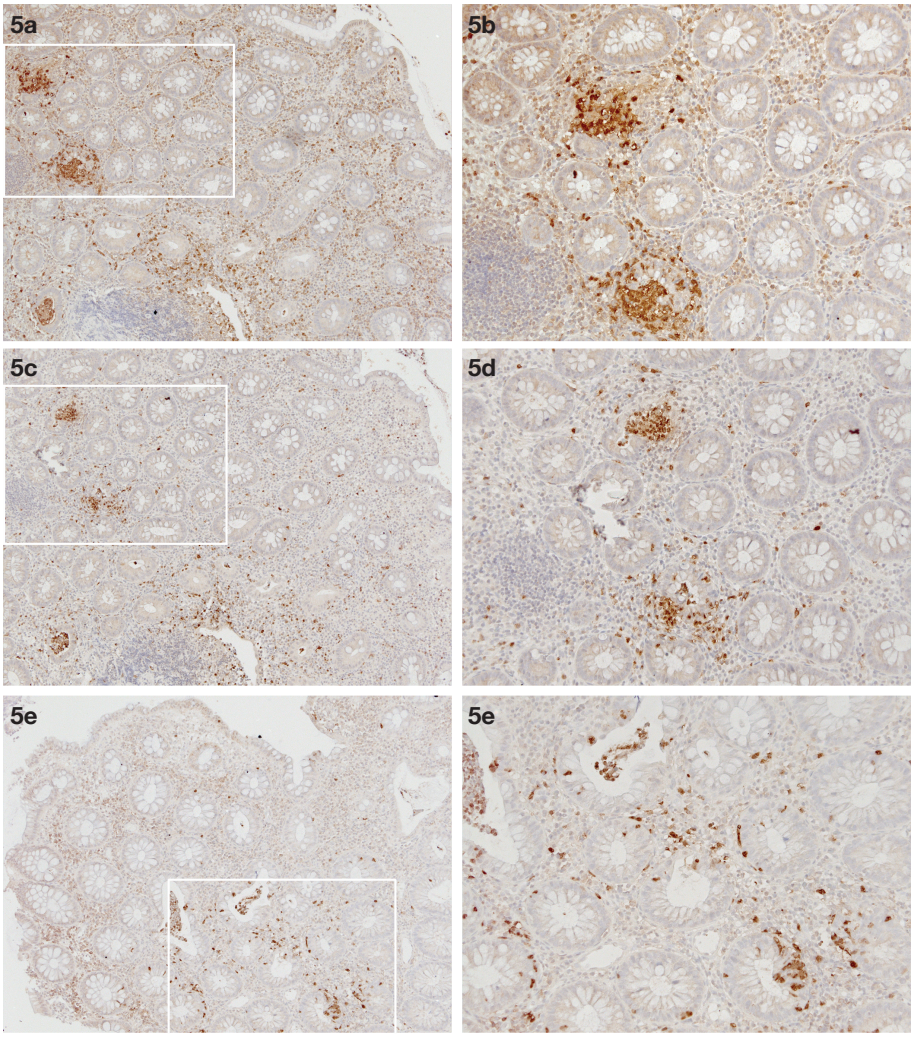
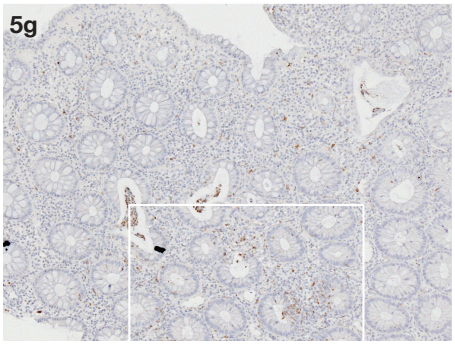
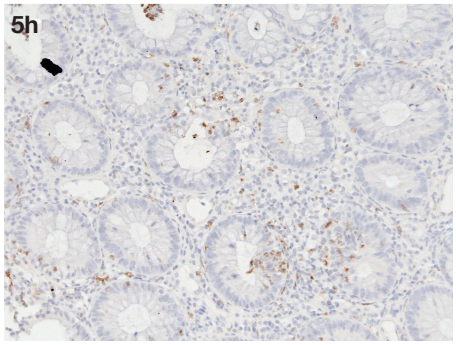
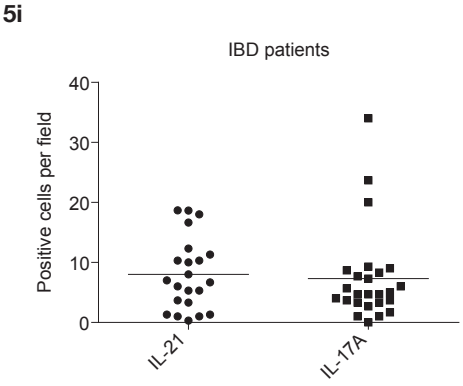


Figure 5 ***Presence of IL-21- and IL-17A-secreting cells in the mucosa of pediatric patients with inflammatory bowel disease (IBD).*** *Representative IL-21 and IL-17A staining of serially-sectioned paraffin-embedded biopsies from pediatric IBD patients.* ***(a,b)*** *IL-21 staining and* ***(c,d)*** *IL-17A staining of a terminal ileum biopsy from a patient with Crohn's disease.* ***(e,f)*** *IL-21 staining and* ***(g,h)*** *IL-17A staining of a colon descendens biopsy from a Colitis ulcerosa patient. Original magnification: ×20 or 40×.* ***(i)*** *Quantification of the number of IL-21- and IL-17A-positive cells in the IBD patient group. Data from colonic biopsies (n=19) and small intestinal (terminal ileum) biopsies (n=7) of the IBD patients group were combined. Positive cells were counted at an original magnification of ×63 in a field of 6000 μm^2. The slides were analyzed by two independent investigators in blinded fashion. Each point represents a single subject; the horizontal bars represent the mean values.*









intestinal biopsies for IL-21 and IL-17A, we detected that IL-21-secreting cells co-localized to the same areas of inflamed mucosa as the IL-17A-secreting cells (*Figure 5c,d,g,h*). Quantification of IL-21- and IL-17-secreting cells is depicted in *Figure 5i*. These findings demonstrate that enhanced, concomitant IL-21 and IL-17A release may be in detected in the inflammatory infiltrates of biopsies from pediatric IBD patients.

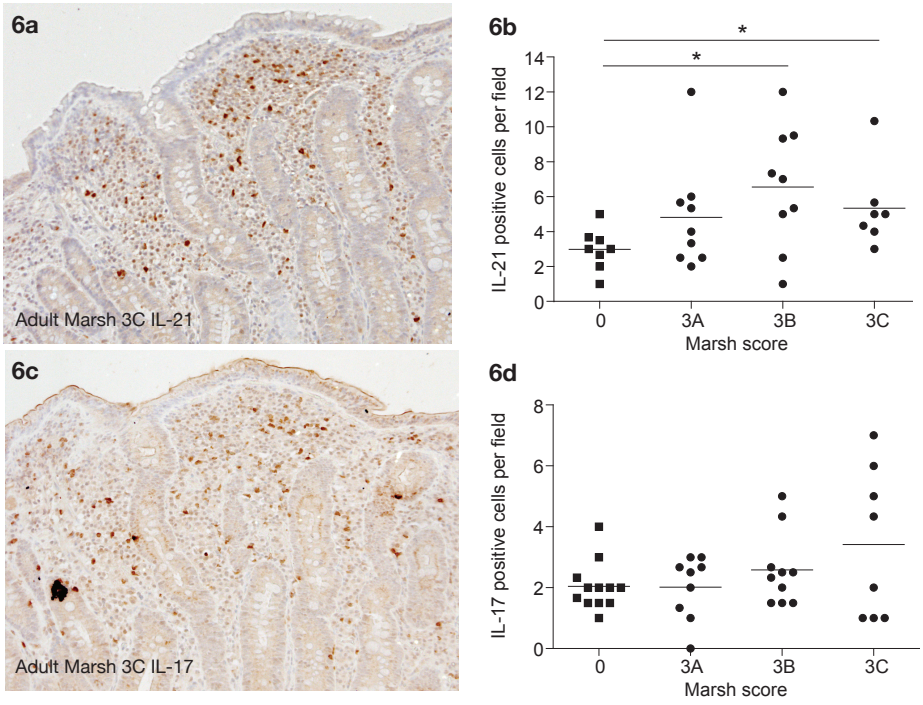
A small subgroup of adult CD patients exhibits simultaneous expression of IL-21 and IL-17A

To assess whether the enhanced secretion of IL-21 in the absence of increased numbers of IL-17-producing cells was specifically attributed to early onset CD in children, IL-21 and IL-17A were analyzed using immunohistochemistry in biopsies from a limited number of adult CD patients. Overall, as in pediatric CD, higher numbers of IL-21-secreting cells were observed in the lamina propria of adult CD patients, while the numbers of IL-17A-positive cells did not significantly increase with lesion severity. However, a small subgroup of Marsh 3C biopsies contained slightly increased numbers of IL-17A-positive cells (*Figure 6a-d*). As shown in *Figure 6a,c*, immunohistochemical staining of IL-21 and IL-17 in serial sections of these few Marsh 3C biopsies with

high numbers of IL-17-secreting cells revealed both increased numbers of IL-21- and IL-17A-secreting cells within the same region.

In conclusion, overall, increased numbers of IL-21-producing cells, but not IL-17A-producing cells, were present in biopsies from adult CD patients, as well as pediatric CD patients. Incidentally, concomitant expression of IL-21 and IL-17 was observed in severe Marsh 3C lesions in biopsies from adult CD patients.

Figure 6 ***A small subgroup of adult CD patients exhibit simultaneous expression of IL-21 and IL-17A. (a)*** *IL-21 staining of a biopsy from an adult CD patient with a Marsh score of 3C.* ***(b)*** *Quantification of the number of IL-21-positive cells in the different adult patient groups.* ***(c)*** *IL-17A staining of a serial section of the biopsy shown in* ***b****. This adult CD patient biopsy with a Marsh score of 3C was representative of the subgroup which showed increased numbers of IL-17⁺ cells.* ***(d)*** *Quantification of the number of IL-17A positive cells in the different patient groups. Positive cells were counted at* ***an*** *original magnification of ×63 in a field of 6000 μm². All slides were analyzed by two independent investigators. Each point represents a single subject; the horizontal bars represent the mean values. * P < 0.05 compared to Marsh score 0, Mann-Whitney U test*

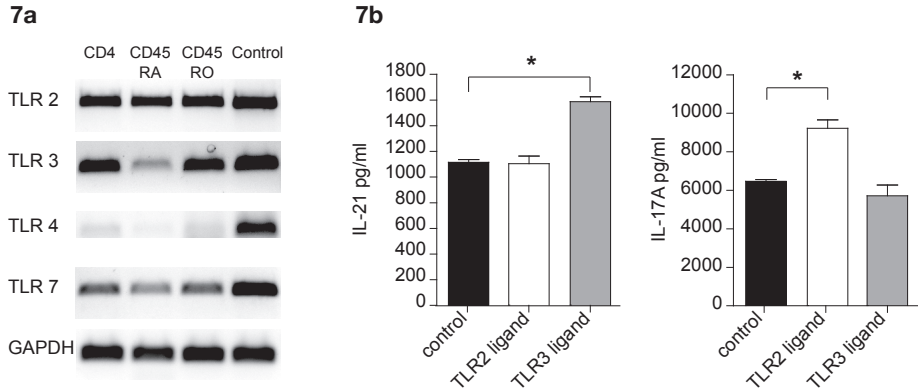


TLR2- and TLR3- ligation on CD4⁺ T cells differentially enhance the release of IL-17A and IL-21 respectively

As an incidental, concomitant increase in the production of IL-21 and IL-17 was observed in severe CD lesions in biopsies from adult patients, we hypothesized that a bystander stimulus associated with tissue destruction and barrier dysfunction may be involved in the stimulation of IL-17 release. Microbe-associated molecular patterns, e.g. TLR ligands, are released in response to damage and may modulate cytokine release by CD4⁺ T cells. Previous studies have shown that CD4⁺ T cells are responsive to microbial stimuli resulting in the modulation of cytokine secretion.[23] Therefore, we firstly investigated which TLR are expressed by CD4⁺ T cells. The mRNA expression profile of highly purified CD4⁺ T cells obtained from healthy donors was determined. Comparison of naive (CD4⁺CD45RA) and memory (CD4⁺CD45RO) or total CD4⁺ T cells to PBMC revealed that TLR2, TLR3, and TLR7 mRNA were expressed by purified healthy donor-derived naive and memory CD4⁺ T cells, whereas the expression of TLR4 was very weak or absent (*Figure 7a*). Next, we investigated whether the production of IL-21 and IL-17 by CD4⁺ T cells were differentially affected by stimulation with particular TLR ligands. When healthy donor-derived CD4⁺ T cells were activated for 48 h or 120 h in the presence of the TLR3 ligand polyinosinic-polycytidylic acid (poly I:C), a significant increase in IL-21 release, but not IL-17A secretion, was induced (*Figure 7b,c* and *Supplementary Figure S2a*). In contrast, stimulation with the TLR2 ligand Pam3Cys enhanced IL-17A release, but did not alter IL-21 secretion (*Figure 7b* and *Supplementary Figure S2a*). The patterns of cytokine release in response to TLR ligation varied between donors and was more consistent for TLR3 activation (*Figure 7c*). Activation of CD4⁺ T cells in the presence of a TLR7 ligand CL097 did not affect IL-21 or IL-17 release (data not shown). Flow cytometric analysis showed no increase in the frequency of IL-21-producing cells after poly I:C stimulation or Pam3Cys stimulation at 48 h (data not shown). However, after 120 h of co-culture with TLR3 ligand the percentage of IL-21-producing cells, but not IL-17-producing cells, was increased (*Figure 7d*). Moreover, stimulation for 120 h with TLR2 ligand enhanced the percentage of IL-17-, but not IL-21-producing cells (*Figure 7d*). It should be noted that the increase in the percentage of cytokine producing cells was dependent on the strength of the T-cell activation with a ratio of 1 anti-CD3 anti-CD28 coated bead to 4 T cells giving maximal effect for TLR2 mediated IL-17 responses (*Figure 7d* and *Supplementary Figure S2b*). Furthermore, TLR3 stimulation of highly purified memory (CD45RO) and naive (CD45RA) CD4⁺ T cells during 48h of anti-CD3 anti-CD28 activation revealed that memory T cells were the main population of IL-21 secreting cells whereas no IL-21 secretion by naive T cells was detected (*Supplementary Figure S2c*). Further study with different conditions is required to establish whether TLR3 activation affects IL-21 secretion during naive T-cell differentiation. These results demonstrate that ligation of TLR3 during CD4⁺ T-cell activation acts as a co-stimulus which enhances IL-21 release

but not IL-17 release, while ligation of TLR2 enhances IL-17 release but not IL-21 release. As such, the local encounter of TLR ligands within the intestinal lamina propria could modulate cytokine secretion during T-cell activation and elicit cytokine release as a bystander effect to the gliadin-induced cytokine response.

Figure 7 ***TLR3 ligation enhances the release of IL-21, but not IL-17A, by CD4+ T cells.*** ***(a)*** *Expression of TLR2, TLR3, TLR4 and TLR7 and GAPDH mRNA was determined in highly-purified healthy donor-derived cell-sorted CD4 cells, CD4⁺CD45RA cells, CD4⁺CD45RO cells and peripheral blood mononuclear cells (control) using PCR. Data from one of two representative experiments is shown.* ***(b,c)*** *CD4⁺ T cells were purified from 6 healthy donors by depletion of non-CD4⁺ T cells using magnetically labeled antibodies (Dynal). The isolated CD4⁺ T cells were activated in culture using anti-CD3 anti-CD28 beads (Dynabeads) at a ratio of 1 bead per 2 T cells in the absence (control) or presence of poly I:C (25 µg/ml) and/or Pam3Cys (1 µg/ml). After 48 h, the supernatants were collected and the secretion of IL-21 and IL-17A protein were measured using ELISAs.* ***(b)*** *IL-21 and IL-17A response in the activated CD4⁺ T cells of a single representative individual.* ***(c)*** *Cytokine release by activated CD4⁺ T cells in response to TLR stimulation, expressed as the percentage increase compared to control anti-CD3 anti-CD28 bead stimulation. Each dot represents a single individual; the horizontal bars represent the mean values.* ***(d)*** *After 120 h of culture, the CD4⁺ T cells were reactivated for 5 h with PMA (0.05 µg/ml) and ionomycin (0.5 µg/ml) in the presence of Brefeldin A for the last 4 h. The cells were harvested, fixed, permeabilized, stained for IL-21 and IL-17A and analyzed by flow cytometric analysis. The percentage of cytokine secreting cells in response to TLR stimulation is expressed as the percentage increase compared to control anti-CD3 anti-CD28 bead stimulation. Each dot represents a single individual; the horizontal bars represent the mean values. Data are representative of two independent experiments using cells from three different donors; * P < 0.05 versus control (n = 3), paired t-tests. TLR, toll-like receptor.*



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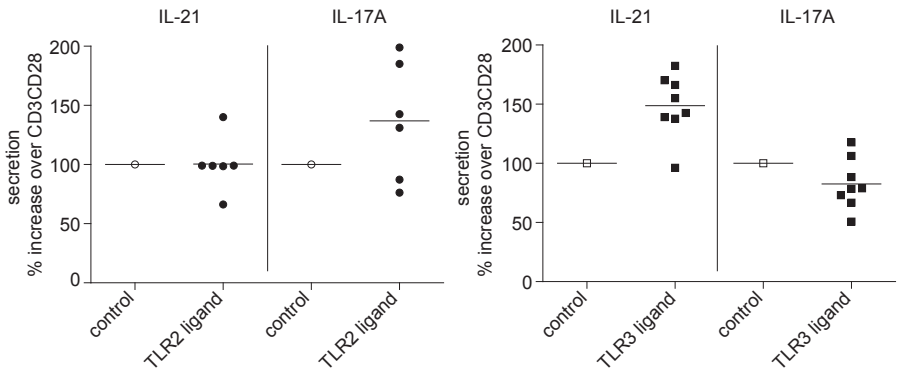

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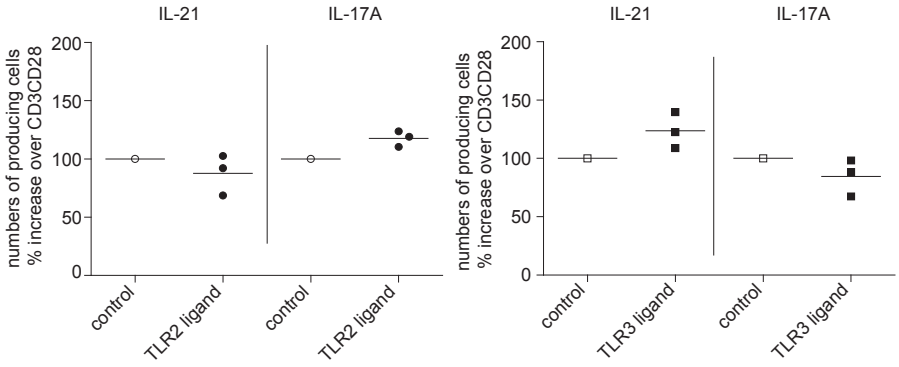

Discussion

In this study we investigated the role of IL-21-producing cells in pediatric CD using two complementary methods, i.e. direct immunohistochemical analysis of biopsies and intracellular flow cytometric analysis of freshly isolated LPLs. This study demonstrates that increased numbers of IL-21-producing cells were present in the lamina propria of the small intestine in pediatric CD patients ranging from 1-11 years old (mean, 4.9), compared to non-CD control patients. Phenotypically, the IL-21$^+$ cells were predominantly CD4$^+$ αβ$^+$ T cells, of which the majority co-produced IFN-γ, but not IL-17 or IL-10.

Previously, it has been shown that the expression of IL-21 is upregulated in the small intestinal mucosa of adult CD patients, compared to healthy controls. In particular, in adult CD patients IELs and LPLs were shown to produce IL-21.[3,8] Here, we show for

the first time that increased numbers of IL-21-producing cells can readily be detected *in situ* in the lamina propria of early-diagnosed CD patients. Extensive analysis of this cohort of pediatric patients demonstrated that high numbers of IL-21-positive cells were already present in biopsies with mild lesions with a Marsh score of 1-2. Not only $CD3^+\alpha\beta$ TCR^+ lymphocytes and IEL secreted IL-21, as a clear population of neutrophilic IL-21-secreting cells was also detected on histology. Neutrophils have recently been shown to have IL-21 secreting capacity.[7] These B cell-helper neutrophils were reported to stimulate diversification and production of immunoglobulin in the marginal zone of the spleen. In CD, neutrophilic influx has been reported previously.[24] Whether IL-21-secreting neutrophils in the celiac lesion have a specific function in B-cell stimulation will need to be investigated. Using serial tissue sections of our pediatric CD patient cohort we established that the number of IL-17-secreting cells did not increase with disease score and remained comparable to non-diseased control tissues. Previous studies which used different cell culture techniques to study the role of IL-21 and IL-17 in CD have led to discrepancies regarding the role of IL-17-producing cells in the pathogenesis of CD.[9,18] In one study, biopsy-derived purified T cells from CD patients contained an increased percentage of IL-17-producing cells, and stimulation of these cells with gliadin for 5 days lead to increased IL-17 mRNA expression.[25] In a second study, a combination of intracellular cytokine staining with DQ2-α-II-gliadin peptide tetramer staining revealed that polyclonal T cell lines derived from CD biopsies contain IL-17-secreting cells; however, the gluten-reactive T cells which produced IL-21 and IFN-γ were IL-17 negative.[9] These data infer that differences in the technical approaches yield different interpretations of the role of Th17 cells in CD pathogenesis. The advantage of immunohistochemistry is that it circumvents the possible pitfall of selective survival of T cell subsets in culture. Therefore, our data establishes that IL-21 secretion occurs independently of IL-17 release in early onset CD. It should be noted that increased numbers of IL-17A-positive cells were detected in the inflamed mucosa of pediatric IBD patients, and IL-17A-positive cells co-localized with IL-21-positive cells in the infiltrates of IBD lesions, in line with the role of Th17 cells in the pathogenesis of IBD.[21,26] To further confirm our findings we performed polyclonal restimulation of freshly isolated lamina propria cells from newly diagnosed pediatric patients with phorbol ester and calcium ionophore, and demonstrated that a large majority of lamina propria IL-21-secreting $CD4^+$ T cells produced IFN-γ; however, few IL-17 producing cells were detected. Our data shows that no increase in the number of IL-17-producing cells is detected in pediatric CD, irrespective of T cell receptor (TCR) specificity. This data agrees with a recent study demonstrating that human leukocyte antigen (HLA)-DQ2-restricted gluten-reactive $CD4^+$ T cell lines derived from adult CD patients produce IFN-γ and IL-21, but not IL-17.[9]

Having established that IL-21-producing cells predominate in Marsh 1-2 CD lesions, and the numbers of IL-17-producing cells did not increase with Marsh score

in young, newly diagnosed CD patients, it was questioned whether the pattern of IL-21-and IL-17-producing cells changed with age at diagnosis. Indeed, increased numbers of IL-21-secreting cells and low numbers of IL-17-positive cells were also detected in biopsies from adult CD patients. However, the number of IL-17-secreting cells in adult CD biopsies was more variable. In particular, a subgroup of adult patient biopsies with Marsh scores of 3C had higher numbers of IL-17-producing cells. Mucosal IL-17A-producing cells contribute to protective anti-microbial responses and epithelial barrier integrity.[27] Therefore, a possible explanation for the incidental increase in IL-17 secretion observed in the biopsies from our adult CD cohort may be that IL-17 is secreted in response to bacterial triggers in damaged tissue, but is not directly related to the gluten-specific T-cell response. As $CD4^+$ T cells express TLRs (*Figure 7*) and have been shown to respond to TLR stimulation[23] we hypothesized that an incidental increase in IL-17 production by $CD4^+$ T cells may be stimulated by TLR ligation. Indeed, our data demonstrated that stimulation of $CD4^+$ T cells with the TLR2 ligand Pam3cys favoured the release of IL-17, not IL-21. A technical consequence of our finding is that TLR ligand contamination should be ruled out in digested gliadin restimulation cultures, to ensure gliadin-specific restimulation effects in T cells.

Further analysis of TLR stimulation revealed that stimulation with the TLR3 ligand during $CD4^+$ T cell activation enhanced IL-21 release, but not IL-17 release. TLR3 is activated by double stranded RNA from viruses[28] and mRNA released from necrotic cells[29] which has relevance for CD as an epidemiological study showed that repeated infections with rotavirus, a double-stranded RNA virus, may increase the risk of CD autoimmunity in childhood.[30] Moreover, poly (I:C) is a synthetic double stranded RNA TLR3 ligand which, when injected into mice, results in transient activation of tissue-transglutaminase-2[31] and villous atrophy[31,32] in the small intestine, both hallmarks of human CD enteropathy. The fact that enhanced IL-21 release is mediated by TLR3, but not TLR2 ligation, in $CD4^+$ T cells strengthens the hypothesis that the pattern of CD inflammation has the signature of a TLR3 mediated response. It should be noted that TLR3 ligation acts as a cofactor enhancing IL-21. A T cell receptor signal is a prerequisite for the production of IL-21 as culture of T cells with TLR3 alone did not yield detectable IL-21 release (data not shown). As activation of T cells in the presence of TLR3 ligand enhanced the percentage of IL-21 secreting cells *in vitro* we hypothesize that TLR3 ligation in response to tissue damage may play a possible role in specifically perpetuating the IL-21 driven inflammation in CD.

In summary, this study demonstrates that increased numbers of IL-21-secreting $CD4^+$ T cells in the lamina propria are characteristic for CD and are even detectable in mild lesions. Our data, together with existing knowledge, argues that IL-21 plays a causative role in the pathogenesis of CD. IL-21 is a pleiotropic cytokine which, in cooperation with cytokines such as IL-15, may be involved in multiple inflammatory pathways. IL-21 may be an important enhancer of IFN-γ production in $CD4^+$ T cells,

as blocking of IL-21 activity in CD mucosal biopsy cultures reduced IFN-γ secretion.[3] With respect to IELs, IL-21 may be involved in increasing the number of intra-epithelial $CD8^+$ T cells, as overexpression of IL-21 in mice selectively enhances the accumulation of memory $CD8^+$ T cells.[33] IL-21 may also contribute to CD-associated auto-antibody production, as IL-21 acts directly on B cells to regulate Bcl-6 expression and germinal center responses.[34] As IL-21 has been shown to increase the resistance of inflammatory T cells to regulatory T cells in an *in vitro* culture system[11], IL-21 may be involved in inhibition of the anti-inflammatory response in CD. Future studies are required to determine the precise role of IL-21 in the course of CD development.

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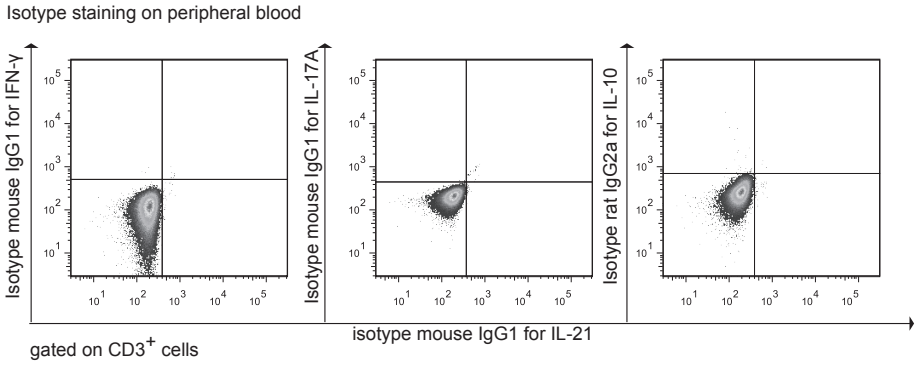
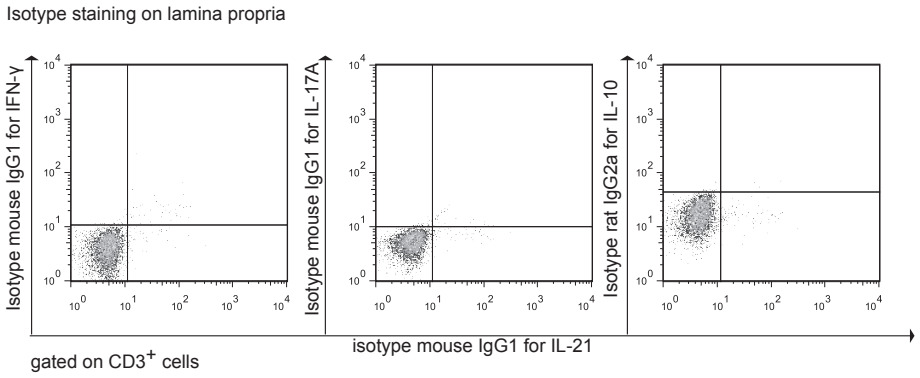
Supplementary figures

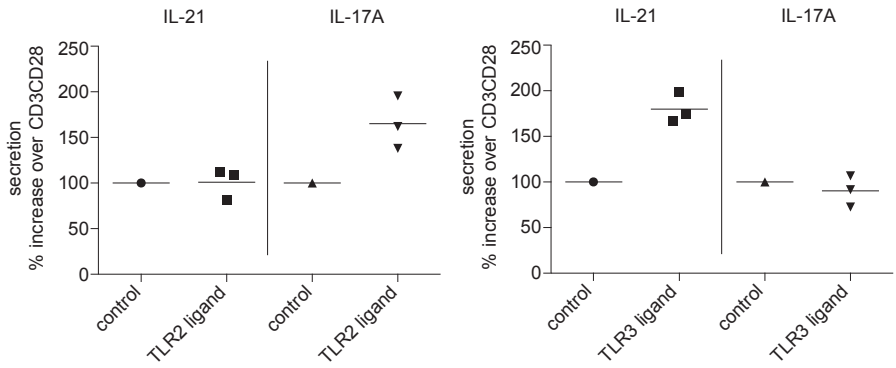
Supplementary Figure S1 *Representative dotplots of isotype control staining for double stained cells as shown in Figure 3. Isotype control antibodies on peripheral blood (top) or lamina propria lymphocytes (bottom).*



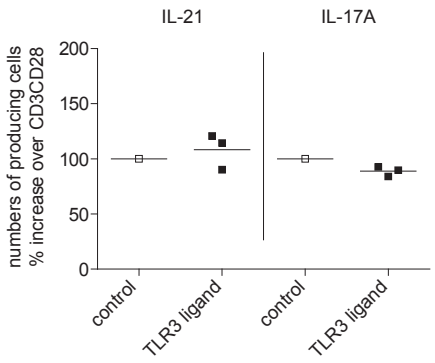


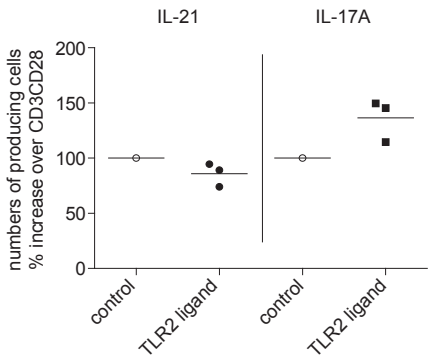
Supplementary Figure S2 *CD4⁺ T cells were purified from 6 healthy donors by depletion of non-CD4⁺ T cells using magnetically labeled antibodies (Dynal). The isolated CD4⁺ T cells were activated in culture using anti-CD3 anti-CD28 beads (Dynabeads) in the absence (control) or presence of poly I:C (25 µg/ml) and/or Pam3Cys (1 µg/ml). After 120 h the supernatants were collected and the secretion of IL-21 and IL-17A protein were measured using ELISAs.* ***(a)*** *Cytokine release by activated CD4⁺ T cells in response to TLR stimulation, expressed as the percentage increase compared to control anti-CD3 anti-CD28 bead stimulation (beads at a ratio of 1 bead per 2 T cells). Each dot represents a single individual; the horizontal bars represent the mean values.* ***(b)*** *After 120 h of culture, the CD4⁺ T cells were reactivated for 5 h with PMA (0.05 µg/ml) and ionomycin (0.5 µg/ml) in the presence of Brefeldin A for the last 4 h. The cells were harvested, fixed, permeabilized, stained for IL-21 and IL-17A and analyzed by flow cytometric analysis. The percentage of cytokine secreting cells in response to TLR stimulation is expressed as the percentage increase compared to control anti-CD3 anti-CD28 bead stimulation (beads at a ratio of 1 bead per 4 T cells).* ***(c)*** *Peripheral blood mononuclear cells were isolated from 3 different healthy donors. CD4⁺ T cells were highly purified by first depleting the non-CD4⁺ T cells using magnetically labeled antibodies (Dynal) followed by flow cytometric cell-sorting of the naive CD4⁺CD45RA⁺ and memory CD4⁺CD45RO⁺ populations. The isolated CD45RO⁺ and CD45RA⁺ T cells were activated in culture using anti-CD3 anti-CD28 beads at a ratio of 1 bead per 2 T cells (Dynabeads) in the absence (control) or presence of poly I:C (25 µg/ml). After 48 h, the supernatants were collected and the secretion of IL-21 and IL-17A protein were measured using ELISAs. The naive T cells did not secrete detectable amounts of IL-21 or IL-17. Each dot represents a single individual; the horizontal bars represent the mean values. TLR, toll-like receptor.*

S2a

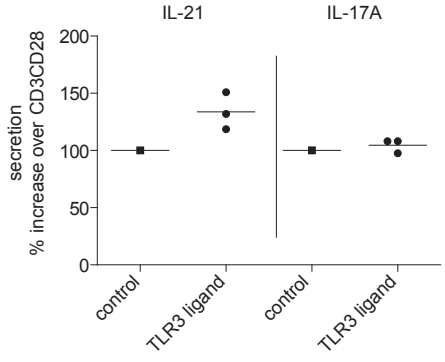


S2b t=120h T cell beads ratio 4:1





S2c t=48h $CD4^{+}CD45RO^{+}$



5

Characterization of human duodenal myofibroblasts and their interaction with T cells in celiac disease

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Manuscript in preparation

ABSTRACT

Celiac disease (CD) is a T cell-mediated chronic inflammation of the small intestine induced by the dietary protein gluten. It is unclear how memory gluten-specific T cells are viable in the lamina propria. We hypothesize that intestinal myofibroblasts may contribute to the chronic inflammation in CD by enhancing memory T-cell viability. Currently, phenotype and immune function of duodenal myofibroblasts have not been studied. Therefore, we isolated duodenal myofibroblasts of CD and non-CD patients, as confirmed by CD90 and α-smooth muscle actin expression. These human duodenal myofibroblasts expressed sensing, cell adhesion and regulatory molecules. TLR3 stimulation of duodenal myofibroblasts resulted in high levels of chemokine expression in comparison with other TLR ligands. Duodenal myofibroblasts expressed IL-1β and exogenous IL-1β altered the IFN-γ, IL-21 and IL-10 secretion by T cells. Furthermore co-culture with duodenal myofibroblasts reduced early T-cell apoptosis *in vitro*. Duodenal myofibroblasts from CD patients and non-CD patients responded variably. Therefore, larger numbers of cell-lines need to be analyzed to assess possible changes in myofibroblast function in CD patients. These data reveal that myofibroblasts can participate in intestinal immune responses. Further research is needed to establish the role of myofibroblasts in CD.

Introduction

Celiac disease (CD) is a T cell-mediated chronic inflammation of the small intestine induced by the dietary protein gluten, found in wheat, barley and rye.[1]

In CD, gluten peptides are enzymatically modified by the tissue repair enzyme tissue transglutaminase-2 (TG2) which is activated in lamina propria cells upon tissue injury.[2] The disease-predisposing HLA-DQ2 or DQ8 molecules expressed on the antigen-presenting cells can bind the TG2 modified gluten peptides and subsequently trigger gluten-specific T-cell responses.[3-5] Presumably, this antigen-presentation takes place in organized lymphoid tissue, from which primary gluten-specific effector T cells migrate to the lamina propria of the small intestine. These inflammatory gluten-specific T cells produce large amounts of interferon (IFN)-γ[6,7] and are expected to be key contributors to the development of the small intestinal lesions characterized by villous atrophy, crypt hyperplasia and increased numbers of lymphocytes.

The only available treatment for CD is a gluten-free diet, which leads to restoration of the intestinal architecture. However, despite strict long-term adherence to the gluten-free diet, intake of gluten will lead tot reactivation of intestinal memory T cells and results in inflammation. Currently, it is not known which mechanisms contribute to long-term survival of gluten-specific effector memory T cells in the lamina propria.

Growing evidence suggests that intestinal stromal cells participate in immune responses and inflammation.[8] Stromal cells are non-hematopoietic structural tissue cells which are long lived and resident in the lamina propria. Stromal cells can respond to microbial signals and have immunomodulatory properties.[9,10] It has been shown that in other chronic inflammatory diseases, such as rheumatoid artritis and chronic airway inflammation stromal cells are important enhancers of chronic inflammation by expressing cytokines.[11-13]

In the lamina propria of human intestine, multiple stromal cell populations can be identified. These include fibroblasts, myofibroblasts, pericytes, endothelial cells and smooth muscle cells.[14] Intestinal myofibroblasts are localized between the epithelium and the immune cells in the lamina propria and are characterized by expression of microfilament α-smooth muscle actin (α-SMA), type 3 intermediate filament vimentin and the surface protein CD90.[15] We hypothesize that intestinal myofibroblasts contribute to chronic inflammation in CD by sustaining memory T-cell responses.

In contrast to human colonic myofibroblasts,[14,16,17] duodenal myofibroblasts are not well characterized. Therefore, we aimed to identify how duodenal myofibroblasts are activated, determined their chemokine and cytokine profile and assessed their capacity to modulate T-cell activation and survival. Duodenal myofibroblast function was compared to tonsil myofibroblasts. To assess whether duodenal myofibroblast function is altered in CD, responses of duodenal myofibroblasts from CD patients and non-CD patients were compared in all assays.

Methods

Isolation and culture of human duodenal myofibroblasts

Human duodenal myofibroblasts were isolated from biopsy specimens of seven pediatric patients that were suspected to have CD. After diagnosis, those with biopsy-proven CD were included in the patient group (n=3) whereas biopsies from children with a normal intestinal histology and negative auto-antibodies were included in the control group (n=4). Duodenal myofibroblasts were isolated by selecting for adherent cells, as described by Mahida et al.[18] All patients were HLA-DQ2 positive. For comparison we used tonsil myofibroblasts isolated from 3 children who underwent tonsillectomy. The study received ethical approval from the local Ethics Committee (Erasmus MC, Rotterdam, the Netherlands).

Myofibroblasts were cultured in MesenPRO RS™ medium (Invitrogen™, Carlsbad, CA), containing 1% L-glutamin (Cambrex biosciences, Verviers, Belgium), 0.5% penicillin and streptomycin (Invitrogen, Paisley, UK), in a humidified incubator at a temperature of 37˚C in 5% CO_2 atmosphere. Culture flasks (Greiner Bio One cellstar, Alphen a/d Rijn) were coated with 1% gelatin (Sigma-Aldrich, St. Louis, USA) for 30 minutes at 37˚C. Cells were regularly passaged by disrupting the monolayers with 1x trypsin-ethylenediamine tetraacetate (Sigma-Aldrich) after washing the cells with phosphate-buffered saline (PBS) with Ethylene Diamine Tetracetic Acid (EDTA). In general, myofibroblasts were passaged at approximately 80% confluence and experiments were performed with cells at passages 6-20.

Flow cytometry

After isolation, myofibroblasts were stained for flow cytometry using monoclonal antibodies against CD90 (BD-Biosciences, Breda, the Netherlands). 7-Amino-Actinomycin D (7-AAD) was used to gate the viable cell population. Flow cytometric analysis was performed using the FACSCanto™ II (BD Biosciences).

Immunohistochemistry

For immunohistochemistry, myofibroblasts were cultured on microscopic slides and after 12 hours cells were fixed in 100% methanol for 5 minutes. To visualize morphology, cells were stained for 3 minutes in May-Grünwald eosin methylene blue solution (Merck KGaA, Darmstadt, Germany) and incubated for 15 minutes in Giemsa's azure eosin methylene blue solution (Merck). For anti-SMA and anti-TG2 staining, a solution of PBS, 0.1% sodium azide, and 0.33% hydrogen peroxide was used to block the cells for 15 minutes. Subsequently, cells were blocked for 30 minutes with 10% normal human serum in addition of 10% normal goat serum or normal horse serum, depending on the secondary antibody. Antibody incubation was performed overnight at 4˚C for anti-alpha-SMA (Mouse-anti-human α-SMA, Sigma) and anti-TG2 (Rabbit polyclonal antibody Transglutaminase II, Thermo Fisher Scientific, USA). Immunoreactions were detected using biotinylated secondary antibodies and

a complex of avidine and biotin (Vectastain ABC Elite Kit, Vector Laboratories, Burlingame, CA), with 3,3-diaminobenzidine tetrahydrochloride (DAB, Sigma-Aldrich, Zwijndrecht, The Netherlands) as chromogen. Cells were counterstained with hematoxylin. Control negative staining with the conjugate was performed for each immunostaining. Images were acquired and analyzed using a Leica DM5500B upright microscope and LAS image acquisition software (Leica Microsystems, Rijswijk, The Netherlands).

Cell culture and cytokine analysis

Stimulation with recombinant IFN-γ (100 U/mL; Immunotools, Friesoythe, Germany) was performed for 48 hours. Before Toll like receptor (TLR) ligand stimulation, medium was removed, cells were washed with PBS supplemented with EDTA and new culture medium was added. Next, myofibroblasts were further cultured for 24 hours in the absence (medium control) or presence of 10μg/ml Pam3Cys (EMC Microcollections, Tübingen, Germany), 25μg/ml polyinosinic–polycytidylic acid potassium salt (poly I:C) (Sigma-Aldrich), 5μg/ml lipopolysaccharide (LPS) (Sigma-Aldrich). After 24 h, cells were harvested in lysis buffer (Macherey-Nagel, Düren, Germany) and the concentrations of cytokine and chemokines in the cell supernatants were measured. Concentrations of CXCL-8, CXCL-9, CXCL-10, IL-10 and IFN-γ were determined by human enzyme-linked immunosorbent assay (ELISA) sets (BD Biosciences, San Diego, USA). The concentration of IL-21 in was measured using the Ready-SET-go! kit (eBiosciences, San Diego, CA). Concentrations of IL-1β and IL-6 were measured using matched antibody pairs for ELISA. (IL-1β, R&D systems, Minneapolis, US; IL-6, immunotools). For standards, recombinant human IL-1β (R&D) or recombinant human IL-6 (Human IL-6 CytoSet, invitrogen) were used.

Real time quantitative PCR

Total RNA was purified from the cultured myofibroblasts using the NucleoSpin RNA II kit (Machery-Nagel, Düren, Germany). Up to 1 μg RNA was reverse transcribed to single-stranded cDNA using a mix of random hexamers (2.5 μM) and oligo(dT) primers (20 nM). The reverse transcription reaction was performed in a total volume of 25 μl containing 0.2 mM of each dNTP (Amersham Pharmacia BioTech, Piscataway, NJ, USA), 200 U Moloney murine leukemia virus reverse transcriptase (M-MLV RT; Promega, Madison, WI, USA) and 25 U RNAsin (Promega). The conditions for the reverse transcription reaction were 37˚C for 30 min, 42˚C for 15 min and 94˚C for 5 min. The cDNA was diluted five-fold and stored at –20˚C. Conventional polymerase chain reaction (PCR) or real-time quantitative PCR using the AbiPrismR 7900 Sequence Detection System (PE Applied Biosystems, Foster City, CA, USA) using general fluorescence-based detection with SYBR green were performed using the specific primers listed below. Relative expression to glyceraldehyde-3-phosphate dehydrogenase (GAPDH) was calculated as relative expression=$2^{-\Delta Ct}$, where $\Delta Ct=Ct_{target}-Ct_{GAPDH}$.

Primers

Specific primers were designed across different constant region exons resulting in the following primers: *GAPDH*: forward 5'-GTCGGAGTCAACGGATT-3', reverse 5'-AAGCTTCCCGTTCTCAG-3'; *TLR2*: forward 5'-GCTGGTGGC AATAACTTC-3', reverse 5'-AGGCCCACATCATTTTC-3'; *TLR3* forward 5'-GCACGAATTTGACTGAACT-3',reverse5'-TCCAATTGCGTGAAAAC-3'; *TLR4* forward 5'-CCTGGACCTGAGCTTTAAT-3', reverse 5'-CCACCA GCTTCTGTAAACTT-3'; *TLR7* forward 5'-GCCCATCTCAAGCTGAT-3', reverse 5'- CTGGGGAGATGTCTGGTAT-3'; *CD40* forward 5'-TTGGGGTC AAGCAGATT-3' reverse 5'-CCTGGGGACCACAGAC-3'; *CD80* forward 5'-TGGGCCATTACCTTAATCT-3' reverse 5-'TCTGCGGACACTGTTAT ACA-3'; *CD86* forward 5'-TGGGGTCATTTCCAGATA-3' reverse 5'-GTG CGGCCCATATACTT-3'; *PD-L1* forward 5'-TTGGGAAATGGAGGATAAG-3' reverse 5'-GGGGCATTGACTTTCAC-3'; *PD-L2* forward 5'-TTGGCTGCT TCACATTTT-3' reverse 5'-CAGGCCACCGAATTCT-3'; *COX-2* forward 5'-CCACCCGCAGTACAGA-3' reverse 5'-GGCCGAGGCTTTTCTA-3'; *VCAM-1* forward 5'-TTGCCGAGCTAAATTACAC-3' reverse 5'-TCGCTGGA ACAGGTCA-3'; *ICAM-1* forward 5'-ATGGGCAGTCAACAGCTA-3' reverse 5'-GCAGCGTAGGGTAAGGTT-3'; CCL-2 forward 5'-TCCAGCATGAAA GTCTCTG-3' reverse 5'-CGAGCCTCTGCACTGA-3'; *IL-1β* forward 5'-CCGCGTCAGTTGTTGT-3' reverse 5'-GGAGCGTGCAGTTCAG-3'; *IL-6* forward 5'-CCCCCAGGAGAAGATTC-3' reverse 5'-GCTGCTTTCACA CATGTTACT-3'

Lymphocyte stimulation

Peripheral blood mononuclear cells (PBMC) were isolated from one healthy HLA-DQ2^{+} donor using a Ficoll-Hypaque gradient. Cells were activated using human T-activator anti-CD3 anti-CD28 beads (Dynabeads, Gibco by life technologies) and cultured for 72 hours in the presence or absence of 50 ng/ml or 100ng/ml recombinant human IL-1β (R&D).

Co-culture of stromal cells and PBMCs

Duodenal myofibroblasts were activated with IFN-γ for 48 hours and stimulated with or without poly I:C for 24 hours as described above. Co-cultures were initiated by introducing PBMC to stromal cell monolayers in Iscove's modified Dulbecco's medium (Lifetechnologies, Grand Island, NY, USA) supplemented with heat inactivated fetal calf serum, Glutamax (Lifetechnologies), 2-mercaptoethanol, penicillin and streptomycin. At 72 hours, non-adherent cells were isolated for apoptosis assay by gentle rinsing with PBS and centrifugation for 5 minutes at 1500 rpm.

In order to determine cellular apoptosis, PBMC were removed from the stromal cell monolayer by gentle re-suspension. Adherent cells were not used for further

analyses. Cells were blocked for 10 minutes with 10% normal human serum and stained with APC-H7 labeled mouse-anti-human CD4 antibody clone SK3 (BD Pharmingen) and FITC labelled mouse-anti-human CD45 antibody clone MEM-28 (Immunotools) for 30 minutes on ice. 20 seconds before analysis cells were stained with APC labelled Annexin V (1:1000, invitrogen) and propidium iodide (PI) (1:250, Nexins research) Flow-cytometric analysis was performed using the FACSCanto™ II (BD-Biosciences).

Statistics

Results are presented as mean values ± range or standard deviation (SD) as indicated in figure legends. Prism (Graphpad Software Inc., La Jolla, CA, USA) was used to perform statistical analysis using paired sample *t*-tests, as indicated in the figure legends. *P* values < 0.05 were considered statistically significant.

Results

Isolated human duodenal myofibroblasts are α-SMA positive and express CD90

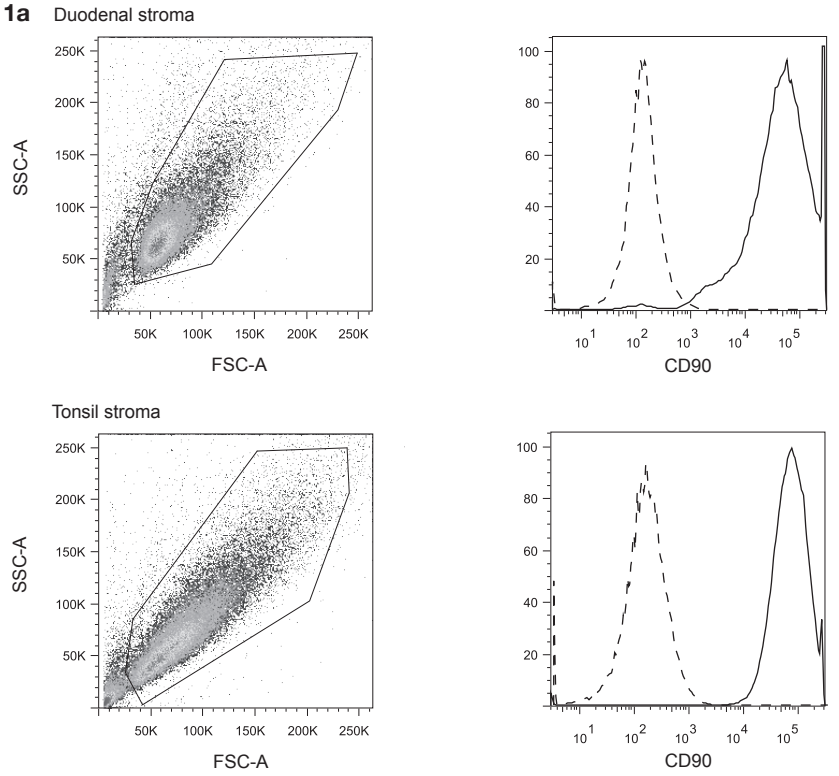
Duodenal myofibroblasts were isolated from CD and non-CD patients and compared to tonsillar myofibroblasts. After isolation, flow-cytometric analysis identified a homogenous $CD90^+$ fibroblast population (*Figure 1a*). Cells from duodenal tissue appeared to be smaller than cells from tonsil (*Figure 1b*). No differences in morphology were found between stromal cells from 3 CD patients and 4 non-CD patients (*Figure 1c*). Immunohistochemistry for α-SMA, a marker characteristic for myofibroblasts, revealed that >90% of the isolated cells from tonsil, CD duodenum and non-CD duodenum were myofibroblasts (*Figure 1c*). Therefore, the cells are hereafter denoted as 'myofibroblasts'. In addition, myofibroblasts from both tonsil and duodenum stained positive for cytoplasmic tissue transglutaminase (*Figure 1d*).

Taken together, CD90 expression and α-SMA staining indicates that myofibroblasts were successfully isolated from duodenal tissue. No differences in cellular morphology were detected between CD and non-CD myofibroblasts.

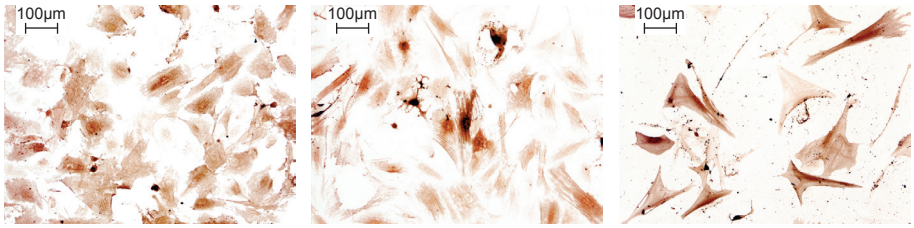
Human duodenal myofibroblasts express sensing, cell adhesion and regulatory molecules

To study the microbial sensing capacity of duodenal myofibroblasts, we determined the mRNA expression of TLR2, TLR3, TLR4 and TLR7 in cells stimulated with or without IFN-γ. TLR2 mRNA expression was detectable at low levels in tonsil and duodenal myofibroblasts and did not increase upon IFN-γ stimulation. TLR3 mRNA was detected in duodenal and tonsillar myofibroblasts and was upregulated after IFN-γ stimulation. TLR4 mRNA expression was detected in unstimulated and IFN-γ stimulated tonsillar myofibroblasts but was low or absent in duodenal

Figure 1 ***Adherent isolated human duodenal myofibroblasts express CD90, α-SMA and tissue transglutaminase 2. (a)*** *Representative flowcytrometric analysis of adhered duodenal myofibroblasts stained for CD90 (black line) compared to unstained cells (dotted line). Isolated cells from duodenal and tonsil biopsies were cultured on slides and immunohistochemical staining was performed using* ***(b)*** *Giemsa,* ***(c)*** *an antibody directed to α-SMA* ***(d)*** *or an antibody directed to tissue transglutaminase 2, followed by nuclear staining using haematoxylin.*



1c



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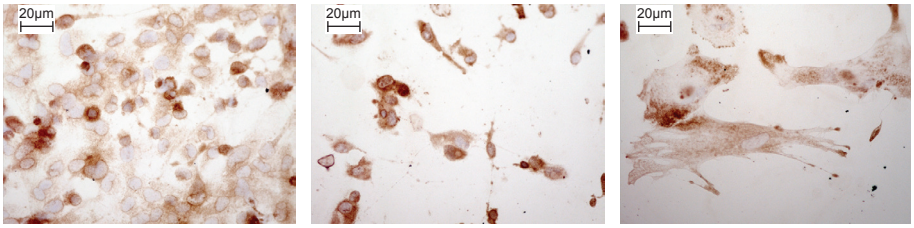
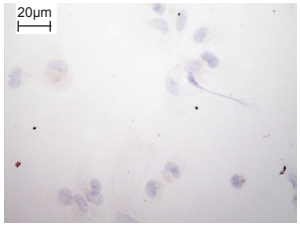


Negative Control

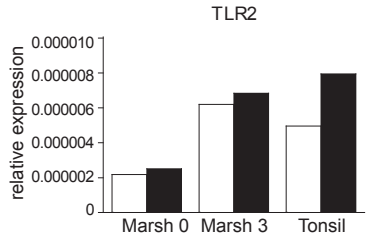


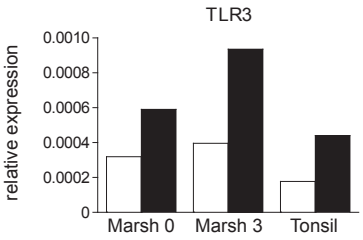
myofibroblasts (*Figure 2a*). TLR7 mRNA was not detected in duodenal or tonsillar myofibroblasts (data not shown). Myofibroblasts from CD patients expressed TLR in the same pattern and degree when compared to non-CD myofibroblasts (*Figure 2a*).

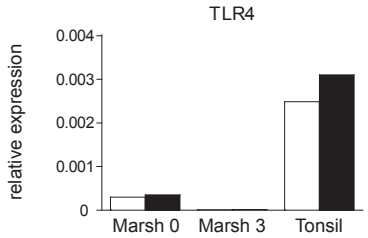
We further characterized myofibroblasts by determining the expression levels of activation markers, co-stimulatory molecules and immunomodulatory molecules. Myofibroblasts from both tonsil and duodenum expressed the activation marker Vascular Cellular Adhesion Molecule-1 (VCAM-1) mRNA. The activation marker Intercellular Adhesion Molecule-1 (ICAM-1) mRNA expression was low in tonsil myofibroblasts, and slightly higher in intestinal myofibroblasts (*Figure 2b*). Both activation markers increased after IFN-γ stimulation. Co-stimulatory molecules HLA-DQ, CD40, CD80, or CD86 molecules were not detected at baseline or in IFN-γ stimulated duodenal myofibroblasts (data not shown). The immune-regulatory co-stimulatory molecules Programmed cell death 1 ligand (PD-L1) and Programmed cell death ligand 2 (PD-L2) were detected in myofibroblasts from both duodenum

Figure 2 ***Human duodenal myofibroblasts express sensing TLR, activation markers and regulatory molecules. (a-d)*** *Myofibroblasts were isolated from a pediatric CD, non-CD patient and tonsil and cultured in the presence (black bars) or absence (white bars) of IFN-γ for 48 hours. Representative mRNA expression of* ***(a)*** *TLR2, TLR3, TLR4* ***(b)*** *VCAM-1, ICAM-1* ***(c)*** *PD-L1, PD-L2* ***(d)*** *and COX2 in myofibroblasts. Relative expression was calculated to GAPDH. Expression levels are representative for n=3 Marsh 0 or non-CD patients and n=2 Marsh 3 or CD patients.*

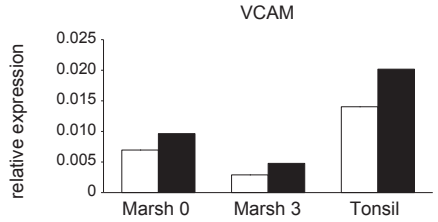
2a

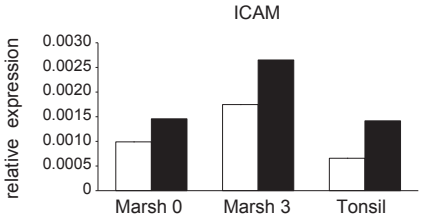






2b





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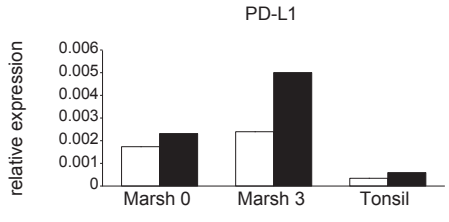
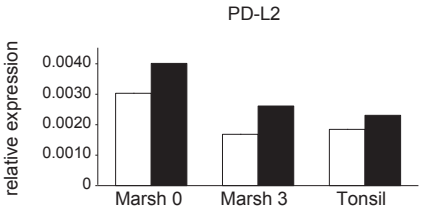
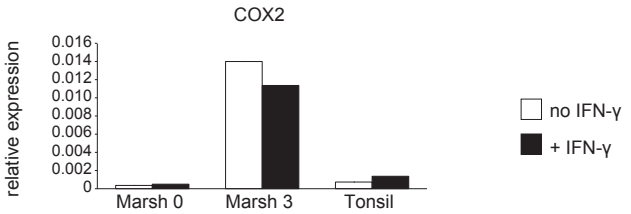




2d



and tonsil. PD-L1 expression was higher in duodenal myofibroblasts compared to tonsil (*Figure 2c*). For all these costimulatory and activational markers no difference in expression was seen between CD and non-CD derived myofibroblasts except for a slightly enhanced expression of PD-L1 in IFN-γ-stimulated myofibroblasts from CD patients when compared to stimulated non-CD myofibroblasts. Colon derived intestinal myofibroblasts are known to have the capacity to express cyclooxygenase 2 (COX-2).[18] Interestingly, COX-2 mRNA was detected at high levels only in duodenal myofibroblasts from CD patients but not in non-CD patients or tonsillar myofibroblasts (*Figure 2d*). In conclusion, duodenal myofibroblasts express TLR2, TLR3 but not TLR4. Upon activation the cells upregulate VCAM and ICAM. Duodenal myofibroblasts do not detectably express costimulatory molecules except PD-L1 and PD-L2. Myofibroblasts from CD and non-CD duodenum are similar in their expression of most molecules tested except for a clear difference in COX-2 in CD.

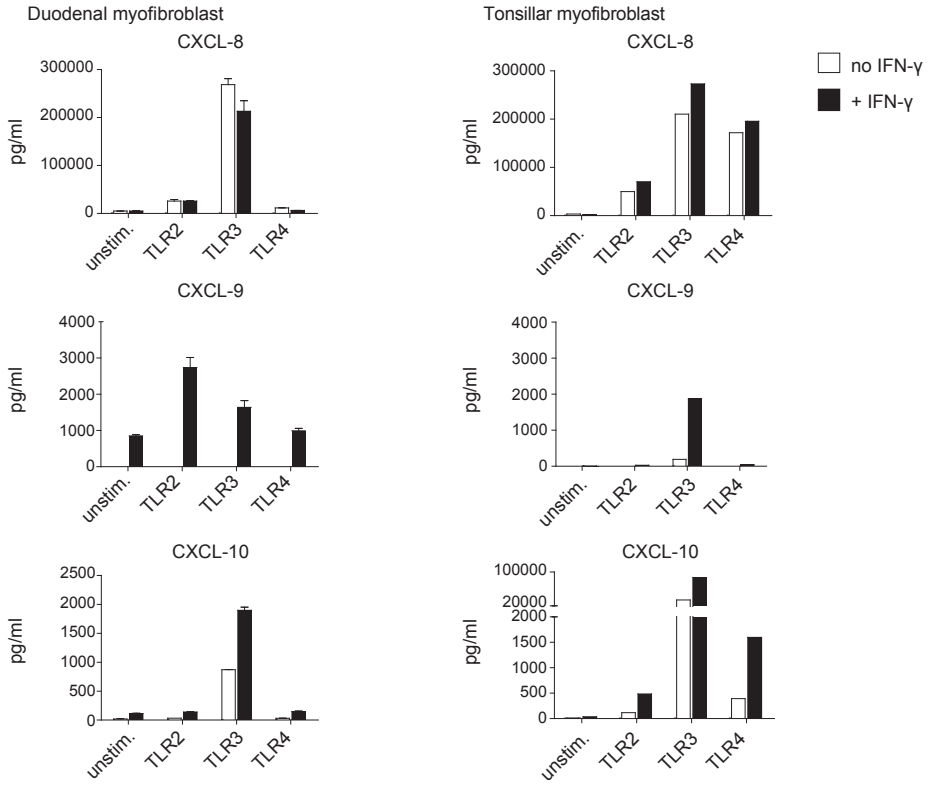
TLR-3 ligation of duodenal myofibroblasts results in up-regulation of chemokine expression

In secondary lymphoid tissue, stromal cells play an important role in cellular trafficking by secreting chemokines.[19, 20] Since myofibroblasts express microbe-associated molecular patterns (MAMPs) we investigated the production of chemokines after TLR ligation.

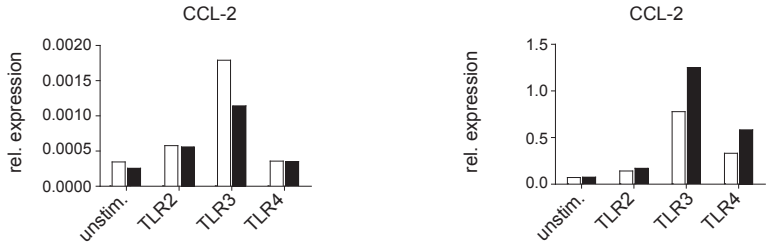
After 24 hours of TLR3 stimulation with poly I:C both tonsillar- and duodenal myofibroblasts secreted very large quantities of the neutrophil chemoattractant CXCL-8 and the T-cell chemoattractants CXCL-9 and CXCL-10 compared to unstimulated cells (*Figure 3a*). In addition, the myofibroblasts expressed the monocyte chemoattractant CCL-2 (*Figure 3b*). For all chemokines, duodenal cells responded most to TLR3 stimulation although TLR2 stimulation did lead to increased CXCL-9 production, but not CXCL-8 and CXCL-10 (*Figure 3a*). CXCL-9 production was dependent on IFN-γ presence. In agreement with the absence of TLR4 mRNA in duodenal myofibroblasts, stimulation through this TLR did not elicit CXCL-8 and CXCL-10 production whereas these chemokines were elicited through TLR4 on tonsillar myofibroblasts. Comparison of CD versus non-CD patient duodenal myofibroblasts showed similar response patterns (*Figure 3c*).

Figure 3 ***TLR3 ligation of duodenal myofibroblasts resulted in up-regulation of chemokine expression. (a-c)*** *Tonsillar stromal cells and duodenal stromal cells were activated with or without IFN-γ for 48 hours and thereafter stimulated with TLR ligands Pam3Cys (TLR2), poly I:C (TLR3) and LPS (TLR4) for 24 hours.* ***(a)*** *Protein concentrations for CXCL-8, CXCL-9 and CXCL-10 were measured in the cell culture supernatant* ***(b)*** *Cells were harvested and expression of CCL-2 mRNA relative to GAPDH was determined by q-PCR analysis.* ***(c)*** *Chemokine expression of CD n=3 versus non-CD patients n=3 after IFN-γ and TLR3 stimulation. Figure a,b are showing the expression of one CD patient myofibroblast cell-line (Marsh 3C) and one tonsillar-myofibroblast cell-line the expression levels are representative for n=3 CD patients- and n=2 tonsillar myofibroblast cell-lines. Results are represented as mean values ± range. Unstim. is unstimulated. An error bar represents SD.*

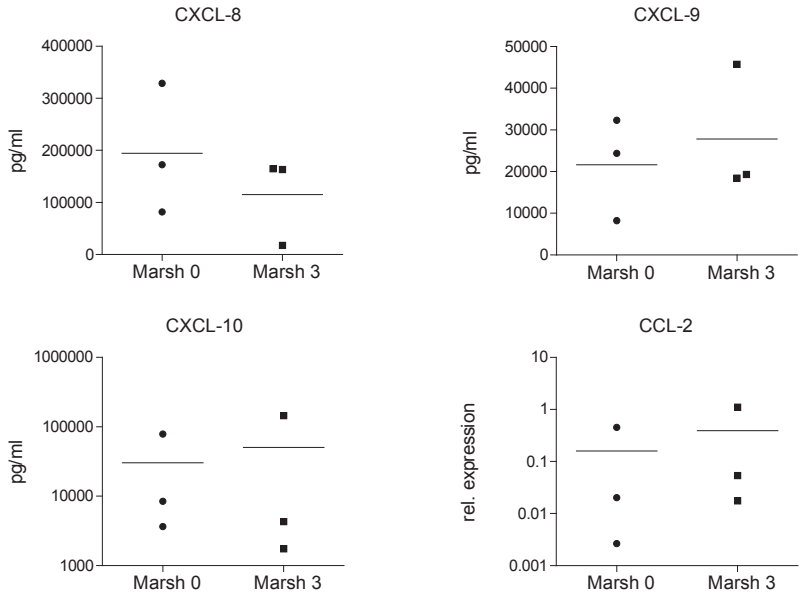
3a



3b



3c



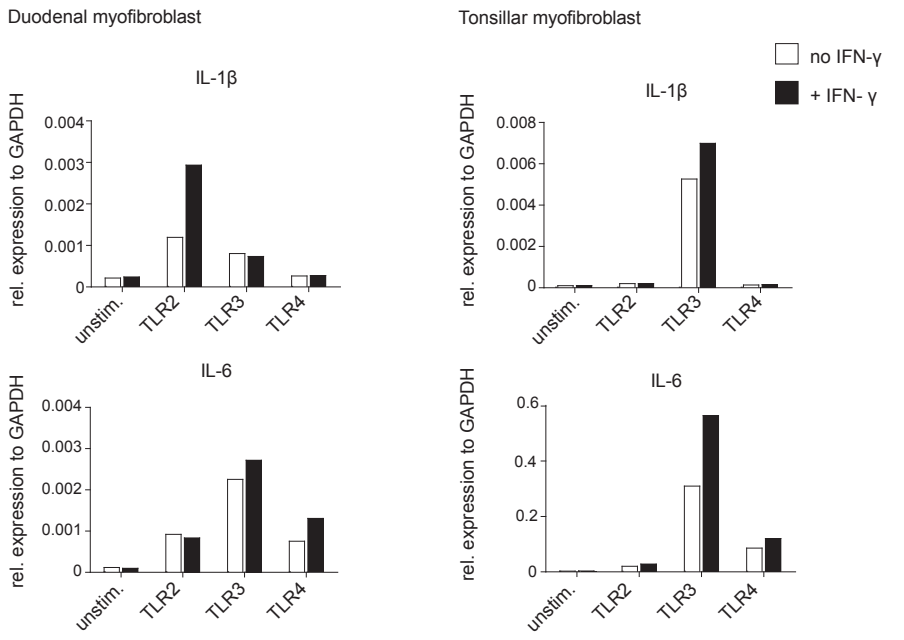
These results demonstrate that duodenal myofibroblasts are potent producers of chemokines upon TLR activation. In particular TLR3 triggering induced the highest response in duodenal myofibroblasts.

IL-1β produced by duodenal myofibroblasts could alter the IFN-γ, IL-21 and IL-10 secretion by T cells

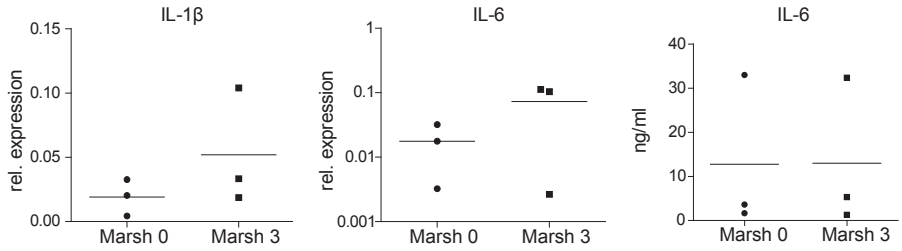
Next, we wished to investigate whether myofibroblasts secreted immune modulatory cytokines. After 24 hours of TLR stimulation, mRNA expression of IL-1β and IL-6 by both tonsillar- and duodenal myofibroblasts was upregulated (*Figure 4a*). While TLR3 ligation was the main trigger for IL-6 expression in duodenal myofibroblasts the cells expressed the most IL-1β after stimulation with TLR2 ligand Pam3Cys.

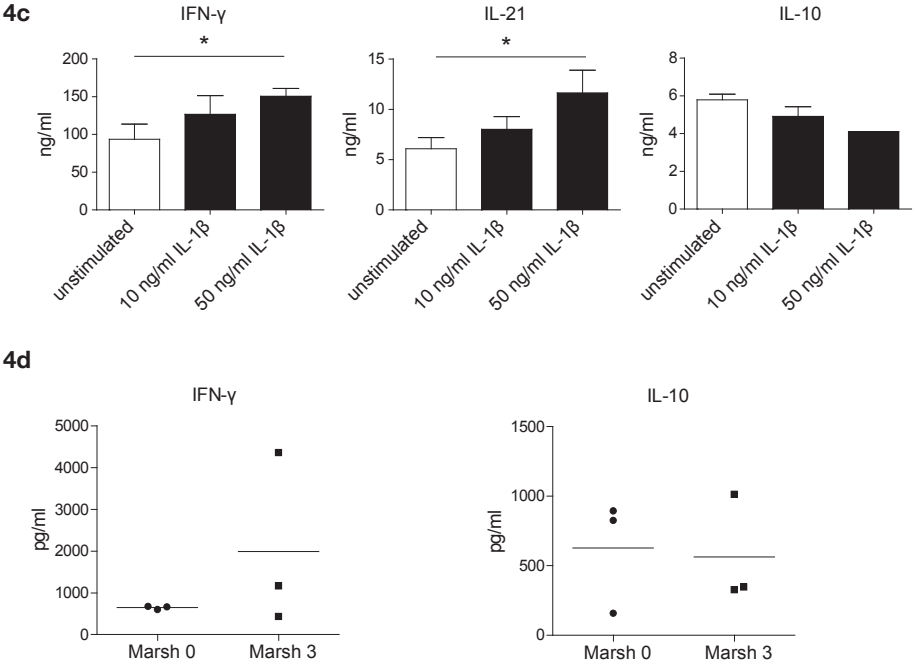
Figure 4 ***Duodenal myofibroblasts express proinflammatory cytokines upon TLR activation. (a,b)*** *Tonsillar and duodenal myofibroblasts were activated with or without IFN-γ for 48 hours and thereafter stimulated with or without TLR ligands Pam3Cys (TLR2), poly I:C (TLR3) and LPS (TLR4) for 24 hours.* ***(a)*** *Cells were harvested and expression of IL-6 and IL-1β was determined by qPCR analysis. Figures showing one representative CD duodenal myofibroblasts cell-line (Marsh 3C) of a total of n=3 and one representative tonsil myofibroblast cell-line of a total of n= 2.* ***(b)*** *Cytokine expression of duodenal myofibroblasts of CD versus non-CD patients after IFN-γ and TLR3 stimulation* ***(c)*** *Peripheral blood mononuclear cells were activated in culture using anti-CD3 anti-CD28 beads (Dynalbeads) in the absence (control) or presence of recombinant IL-1β. After 48 hours, the supernatants were collected and the secretion of IFN-γ, IL-21 and IL-10 proteins was measured using ELISAs (n=3).* ***(d)*** *PBMC of one donor were activated using anti-CD3 and TLR3 in the presence of duodenal stromal cells of CD patients (n=3) or non-CD patients (n=3). After 72 hours, the supernatants were collected and IFN-γ and IL-10 were measured.*

4a



4b

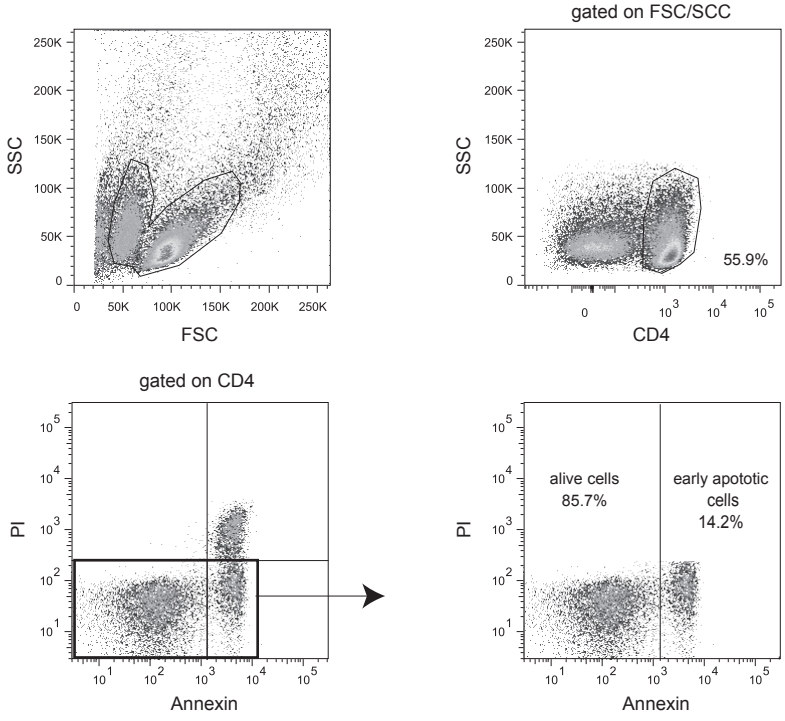




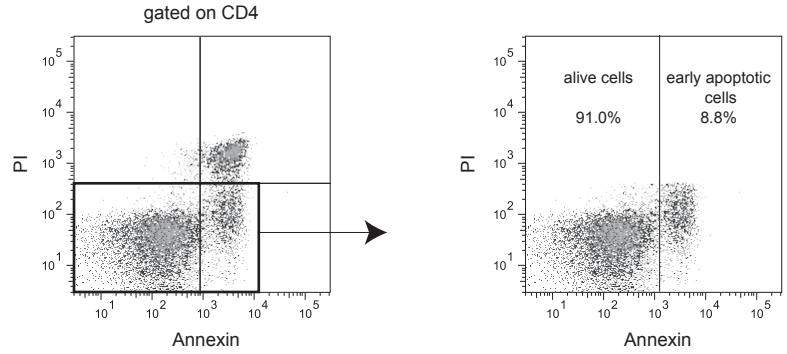
The latter effect was unique to duodenal myofibroblasts as tonsillar myofibroblasts preferentially secreted IL-6 and IL-1β after TLR3 but not TLR2 ligation. The amount of IL-6 and IL-1β protein release was highly variable between cell lines with IL-6 being consistently upregulated (variation 1.3 ng/ml- 32.9 ng/ml) after TLR stimulation, while IL-1β often remained below the threshold of detection. When comparing duodenal cells from non-CD patients with CD patients no significant difference was detectable in cytokine expression patterns (*Figure 4b*). It should be noted that mRNA levels of IL-2, IL-17, IL-21, IL-22 and IFN-γ were not detected in the supernatant of stimulated tonsillar- or duodenal myofibroblasts (data not shown). Recently, it has been reported that IL-1β inhibited IL-10 production in differentiating and memory $CD4^+$ T helper 17 cells (T_H17), whereas blockade of IL-1β *in vivo* led to increased IL-10 production by memory T_H17 cells.[21] As IL-10 mediated regulation has been implicated in maintaining tolerance to gluten,[22, 23] we investigated whether IL-1β could modulate cytokine production of T cells in our hands. Thereto, PBMC from a healthy DQ2 positive volunteer was activated with anti-CD3 anti-CD28 with or without IL-1β. At 72 hours of culture addition of IL-1β elicited increased IFN-γ and IL-21 and slightly decreased IL-10 production (*Figure 4c*). Next, we investigated whether activation of myofibroblasts would induce a similar enhancement of inflammatory cytokines by T cells. Thereto, TLR3-stimulated and non-stimulated duodenal myofibroblasts from CD and non-CD patients were co-cultured with

Figure 5 ***Duodenal myofibroblasts reduce early T cell apoptosis in vitro. (a,b)*** *Duodenal myofibroblasts from CD patients (n=3) and non-CD patients (n=4) were activated with IFN-γ for 48 hours thereafter cultured for 24 hours with or without poly I:C. A co-culture was initiated by adding anti-CD3 stimulated HLA-DQ2⁺-PBMC to the duodenal stromal cells. After 72 hours cells were harvested and apoptosis was measured using flowcytometric analysis with Annexin V and propidium iodide (PI).* ***(a)*** *Representative dot-plots of the gating strategy for measuring the percentage of early apoptotic cells* ***(b)*** *Percentage of early apoptotic cells of all duodenal myofibroblasts lines investigated. Results are presented as mean values ± range.*

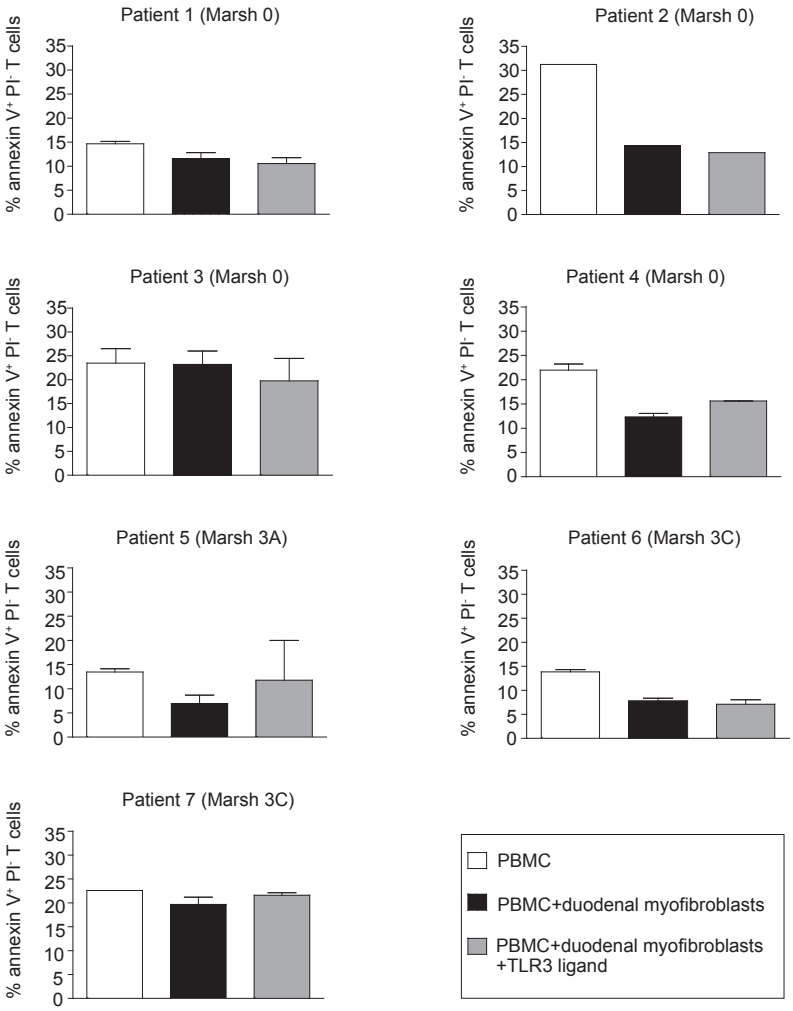
5a *PBMC*



PBMC + duodenal myofibroblast



5b



soluble anti-CD3 activated PBMC. At 72 hours of culture, cytokines secretion was determined in the culture supernatant. Unfortunately, the amount of IFN-γ and IL-10 released in the culture was highly variable in the cultures with 6 different duodenal myofibroblasts cell-lines (*Figure 4d*). Due to this no conclusions could be drawn.

Duodenal myofibroblasts reduce early T cell apoptosis in vitro

Both IL-1β and IL-6 are known to enhance T-cell survival by interfering with apoptosis induction.[24, 25] Therefore, we investigated if duodenal myofibroblasts could enhance T-cell survival in co-culture. PBMC obtained from a HLA-DQ2^{+} donor were co-cultured with seven different duodenal myofibroblasts from CD (n=3) and non-CD patients (n=4) that had been stimulated with or without poly I:C prior to co-culture. At 72 hours of culture the percentage of apoptosis in CD4^{+} T cells was determined using flow cytometric analysis. Gating strategies of the T-cell apoptosis assay are shown in *Figure 5a*. Late T-cell apoptosis could not be quantitated reliably as autofluorescence of myofibroblasts interfered. In four out of seven myofibroblast- T cell co-cultures reduced early T-cell apoptosis was observed (*Figure 5b*). Preincubation with TLR3 ligand did not further enhance the apoptotic effect. Reduced apoptosis was seen in co-cultures with non-CD (n=3) and CD (n=1).

Together, these data indicate that duodenal myofibroblasts can reduce early apoptosis of T cells and suggests myofibroblasts may play a role in enhancing survival of T cells in the lamina propria.

Discussion

In this study, we have identified how duodenal myofibroblasts are activated, determined their chemokine and cytokine profile and assessed their capacity to modulate T-cell activation and survival. In the cell lines tested, duodenal myofibroblasts from CD patients and non-CD patients responded variably. Therefore, larger numbers of cell-lines need to be analyzed to assess possible changes in myofibroblast function in CD patients.

All duodenal myofibroblasts had the capacity to sense innate signals via TLR2 and TLR3 leading to production of large amounts of chemokines and release of the inflammatory cytokines IL-6 and IL-1β. Predominantly TLR3 activation enhanced chemokine release by myofibroblasts. TLR3 recognizes double stranded RNA from viruses[26] or necrotic cells[27]. As such, in vivo, duodenal myofibroblasts may have the capacity to recruit immune cells in response to presence of viral products or cellular damage in the lamina propria underneath the epithelium. TLR3 has relevance for CD as an epidemiological study showed that repeated infections with rotavirus, a double-stranded RNA virus, may increase the risk of CD autoimmunity in childhood.[28] Moreover, injection of poly I:C, a synthetic TLR3 ligand, in mice results in CD-like

enteropathy associated with transient activation of tissue-transglutaminase-2[2] and villous atrophy[2,29] in the small intestine but not in the colon. Duodenal myofibroblasts are not unique in their ability to respond to TLR3 ligand since tonsillar myofibroblasts were also activated via TLR3. However, tonsillar myofibroblasts expressed TLR4 mRNA at higher levels and were activated by TLR4 stimulation with LPS while duodenal myofibroblasts were not. It has been shown that myofibroblasts derived from human colon express TLR2 and TLR4 and secrete CXCL-8 in response to stimulation with LPS.[30] As such, duodenal myofibroblasts may be adapted to their local microenvironment by selectively expressing TLR3 and down regulating TLR4 expression.

Besides expressing chemokines to attract cells, we show that duodenal myofibroblasts can reduce T-cell apoptosis *in vitro*. Such anti-apoptotic activity may play a role in the long-term survival of memory gliadin-specific inflammatory T cells in the lamina propria of CD patients. The mechanism behind this observation has yet to be identified. Cytokines such as IL-1β and IL-6 released by duodenal myofibroblasts may exert this anti-apoptotic activity in T cells within the CD lesion.

IL1-β is a proinflammatory cytokine with pleiotropic effects and is primarily produced by innate immune cells. Increased IL-1β levels have already been reported in chronically inflamed intestine of inflammatory bowel disease (IBD) patients.[31] Moreover, in a murine T-cell transfer model for colonic inflammation, IL-1β stimulation of $CD4^+$ T cells was required for the induction of intestinal inflammation.[32] Stimulation with IL-1β activates innate immune cells and promotes T-cell activation and survival.[24] Next to promoting survival we hypothesize that local IL-1β production by myofibroblasts could influence the cytokine profile of the effector memory T cells in CD. We showed that IL-1β selectively enhanced IFN-γ and IL-21 production by activated PBMC while IL-10 production was not increased. This is in agreement with previous data showing that IL-1β induces a pro-inflammatory cytokine profile of pathogen-induced human Th17 cells by inducing IFN-γ and suppressing IL-10 production while blocking of IL-1β increased IL-10 production.[21] Detection of IL-1β was variable in our myofibroblast cultures and depended on the density of the cells in culture (data not shown). Moreover, it is not clear whether IL-1β is produced as extensively in CD as in IBD however abundant numbers of $IFN\text{-}\gamma^+IL\text{-}21^+$ T cells can be detected in lamina propria of CD patients allowing a possible encounter with the inflammatory cytokine.[33,34]

Similar to IL-1β, IL-6 has also been reported to be elevated in myofibroblasts of patients with IBD.[35] Also IL-6 has the capacity to enhance T-cell survival *in vitro* and *in vivo*.[36] Duodenal myofibroblasts spontaneously produced substantial amounts of IL-6 without TLR stimulation suggesting that there is a low threshold for IL-6 release. Previously, IL-6 has been associated with a risk to develop CD. In particular, a functional polymorphism in the IL-6 promoter appeared to influence CD susceptibility in girls.[37] Next to stimulating T cell survival IL-6 may also modulate

the cytokine production of T cells as it is one of the co-factors required for T_H17-cell differentiation.[38]

Colonic myofibroblasts have been shown to have antigen presenting capacity and expression of MHC II molecules and exert costimulatory function.[16] It is questionable whether duodenal fibroblasts share this property. We have not been able to detect HLADQ2 on patient duodenal myofibroblasts rendering active antigen presentation unlikely. In contrast, costimulatory molecules PD-L1 and PD-L2 and the immunomodulatory enzyme COX-2 were found in duodenal myofibroblasts while CD40, CD80, CD86 were absent. Interestingly both COX-2 and PD-L activity have been associated with immune suppression rather than immune activation. Whether COX-2 and PD-L function in duodenal myofibroblasts contributes to the inhibition or maintenance of T-cell responses in intestinal inflammation remains to be established.

Previously, it has been shown that myofibroblasts from CD patients showed different morphology with a different collagen and TG2 pattern compared to non-CD patients.[39] We hypothesized that human intestinal myofibroblasts are functionally different in health and disease. However the number of patients in this study was too low to draw conclusions on differences between myofibroblasts from CD patients and non-CD patients.

Overall, the results of this study clearly show that duodenal myofibroblasts can be stimulated by TLR2 and TLR3 ligands but not TLR4 stimulation leads to potent chemokine release, inflammatory IL-6 and enhances T-cell survival. As such a possible role for duodenal myofibroblasts in enhancing inflammation in CD needs to be considered.

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6

Macrophages favor differentiation of IL-10-secreting type 1 regulatory T cells driving oral tolerance to gliadin

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To be submitted

ABSTRACT

Celiac disease is caused by a chronic inflammatory T-cell response against the dietary protein gliadin. Gliadin is an unusual food protein as it is insoluble, forms stable aggregates and is resistant to degradation. Using transgenic mice expressing human HLA-DQ2 and a gliadin-specific humanized T-cell receptor, we have previously shown that oral tolerance to gliadin is driven by a different mechanism than tolerance to the soluble protein ovalbumin. Ovalbumin ingestion induced Foxp3$^+$ regulatory T cells in the mesenteric lymph nodes (MLN) while gliadin induced tolerogenic interleukin-10-secreting type 1 regulatory T (Tr1) cells in the spleen. These findings imply that oral tolerance to gliadin develops outside of the mucosal immune system and occurs independently of Foxp3$^+$ regulatory T-cell differentiation. The aim of this study was to identify the mechanisms that drive gliadin antigen presentation and Tr1-cell differentiation in the spleen.

Fluorescently labeled gliadin was detected in MLN within 18 hours after oral feed but elicited poor T-cell proliferation. Because feeding smaller peptides led to substantial T-cell proliferation in the MLN, degradation appeared a pivotal step required for presentation of gliadin. As macrophages have strong degradation capacity, the effect of macrophage depletion on splenic gliadin presentation was assessed. Macrophage depletion prior to whole gliadin feed did not affect T-cell proliferation, but inhibited Tr1-cell differentiation arguing that macrophages may play a role in oral tolerance to gliadin. In agreement, *in vitro* splenic macrophages rather than dendritic cells favored differentiation of gliadin-specific Tr1 cells and expressed IL-27, a cytokine that mediates Tr1-cell induction.

These data indicate that protein degradation may be a decisive step in gliadin presentation and demonstrate that macrophages rather than dendritic cells favor the differentiation of gliadin-specific Tr1 cells.

Introduction

Celiac disease (CD) is driven by a chronic inflammatory T-cell response against the dietary protein gliadin, which results in small intestinal damage in genetically predisposed individuals. CD is a common food intolerance, affecting 1% of the Caucasian population. The only effective treatment is a strict gluten-free diet.[1]

Gluten, a mixture of glutenins and gliadins, is a storage food protein that can be found in wheat, barley and rye. Gluten is insoluble in water and resistant to degradation of gastrointestinal enzymes due to the high proline content (20%), thereby making it possible for large immunogenic peptides to reach the intestinal mucosal surface.[2, 3] In the intestine, gluten is transported across the epithelial barrier to the lamina propria where tissue transglutaminase 2 (TG2) facilitates the antigen binding of gluten peptides to the human leukocyte antigen (HLA) class II molecules by the introduction of negative charges in gluten peptides, a process called deamidation. In CD patients, deamidated gluten peptides are recognized and presented in the context of HLA class II molecules DQ2 or DQ8 to gluten-specific $CD4^+$ T cells.[4, 5] These ensuing differentiating gluten-specific $CD4^+$ T cells are inflammatory, produce IFN-γ and drive mucosal damage. Crucially, inflammatory gluten-specific $CD4^+$ T cells can be identified in CD patients but not in healthy HLA-DQ2 positive individuals who tolerate gluten.[6, 7] Despite the increasing knowledge on CD pathogenesis, it is still unknown why oral tolerance to gluten is lost in HLA-$DQ2^+$ CD patients while the majority of HLA-$DQ2^+$ individuals remain tolerant to gluten.

To identify which processes underlie oral tolerance to gluten we have made use of double transgenic mice that express HLA-DQ2 and an HLA-DQ2-restricted T-cell receptor (DQ2.gliadinTCR) specific for gliadin-γ1-epitope isolated from a CD patient. In these DQ2.gliadinTCR mice feeding deamidated gliadin induced no sign of CD pathology, but instead gliadin-specific tolerance was mounted in the spleen where suppressive IL-10-secreting type 1 regulatory T (Tr1) cells were formed.[8] This was unexpected as in general, oral tolerance to harmless soluble food proteins, such as ovalbumine (OVA), is dependent on antigen presentation in the intestinal mucosa-draining lymphoid tissue, an environment specifically equipped to favor differentiation of regulatory responses. In particular, after OVA feed, the antigen is transported to mesenteric lymph nodes (MLN) and Peyer's patches, presented by specialized mucosal antigen presenting cell (APC), which induce the differentiation of $Foxp3^+$ regulatory T (Treg) cells that drive protein-specific tolerance.[9-12] In contrast, the tolerogenic T-cell response to deamidated gliadin feed in DQ2.gliadinTCR mice did not occur under the strict control of mucosal-resident dendritic cells (DC) and the mucosal microenvironment but predominantly occurred in the spleen. In consequence, differentiating gliadin-specific T cells did not upregulate Foxp3 expression, but produced IFN-γ and IL-10.[8] These data established that oral tolerance to gliadin is mechanistically different from tolerance to other harmless food proteins and depends

on IL-10-secreting Tr1 cells that are formed in the spleen. One of the key factors for this unexpected mechanism of tolerance induction may be the unusual molecular characteristic of deamidated gliadin such as its negative charge, its insolubility and its tendency to form aggregates.

The aim of this study was to identify the mechanisms by which orally administered gliadin elicits gliadin-specific T-cell proliferation and differentiation of Tr1 cells in the spleen. In particular, we investigated the role of macrophages in this process as these cells contain higher levels of lysosomal proteases than DC and rapidly degrade internalized proteins.[13]

We report that macrophages play a pivotal role in the induction of gliadin-specific Tr1 cells in the spleen. *In vivo* depletion of macrophages during gliadin protein feed reduces the number of differentiating gliadin-specific Tr1 cells in the spleen. In contrast oral gliadin-peptide-induced Tr1-cell differentiation in the spleen was unaffected by macrophage depletion. *In vitro* cultures demonstrated that splenic macrophages determine the differentiation of gliadin-specific Tr1 cells by expressing IL-27. Our results demonstrate that degradation of gliadin proteins by macrophages may be an important step in antigen presentation and subsequent Tr1-cell differentiation in the spleen after gliadin feed.

Material and methods

Mice

HLA-DQ2 (DQA1*0501, DQB1*0201=HLADQ2.5) MHCII$^{\Delta/\Delta}$ transgenic (DQ2) mice and gliadin-TCR.MHCII$^{\Delta/\Delta}$ transgenic (gliadinTCR) mice, which have a transgenic T-cell receptor (TCR) specific for the DQ2-γ-I epitope were bred at the ErasmusMC. Single-transgenic DQ2 and gliadinTCR mice were maintained on a C57BL/6-DBA/2 mixed background. HLADQ2.gliadin-TCR.MHCII$^{\Delta/\Delta}$ double transgenic (DQ2.gliadinTCR) mice were obtained by crossing DQ2 mice with gliadinTCR mice. All mice were kept under specific pathogen-free housing conditions and the animal experimental committee of the Erasmus Medical Center approved the experiments. DQ2 mice, gliadinTCR mice and DQ2.gliadinTCR mice were bred and maintained on a gluten-free chow (Arie Blok BV,Woerden, the Netherlands).

Proteins and peptides

Crude gliadin from wheat (Sigma Aldrich, Zwijndrecht, the Netherlands) was dissolved in a 0.1 M NH_4HCO_3 2 M urea buffer (100mg/ml) and digested with 50μg/ml α-chymotrypsin (Sigma) at room temperature for 24 hours. Digestion was stopped by heating to 98˚C for 10 minutes. The chymotrypsin-treated gliadin (CT-gliadin) was centrifuged (5000g, 45min), filter-sterilized (0.45μm) and dialyzed against sterile PBS. Protein concentration was determined using a bicinchoninic acid assay (BCA

assay, Perbio Science, Etten-Leur, the Netherlands). To obtain chymotrypsin-digested deamidated gliadin (termed CT-TG2-gliadin), CT-gliadin was treated with guinea pig liver transglutaminase (TG2; 0.08U/mg) (Zedira GmbH, Darmstadt, Germany) for 16 hours at 37°C. For *in vivo* experiments, mice received a single gavage with 60 or 75 mg CT-TG2-gliadin or 3 mg of the synthetic TG2-gliadin-γ1-E peptide (termed TG2-gliadin peptide) (QPEQPQQSFPEQERPF) on 3 consecutive days by oral gavage (i.g.). For *in vitro* experiments, CT-TG2-gliadin protein (0.5 mg/mL) or TG2-gliadin peptide (5 μg/mL) was used.

Tracking of CT-TG2-gliadin protein

DQ2 mice received 3.2 mg CT-TG2-gliadin protein by gastric gavage (i.g.) or intravenously (i.v.). At 18 hours, spleens and MLN were isolated and embedded in cryopreservative solution (OCT, Tissue-Tek, Miles, Elkhart, IN). Immunohistochemical stainings were performed on 6 μm cryostat sections. Prior to staining, the samples were fixed with ice-cold acetone for 10 minutes and blocked with 10% normal mouse serum and 10% normal goat serum. Tissue sections were incubated using anti-gliadin primary antibody (Cusabio, rabbit polyclonal) and subsequently stained with secondary antibody goat-anti-rabbit fluorescently labeled with a DyLight-488 (Thermo Scientific, Fremont, USA). Control stainings were performed using no anti-gliadin antibody. Sections were mounted with medium for fluorescence containing DAPI (Vector Laboratories, Burlingame, USA). A conjugate control was performed for each immunostaining. Images were acquired analyzed using a Leica DM5500B upright microscope and LAS-AF image acquisition software (Leica Microsystems, Rijswijk, and the Netherlands).

For flow cytometry, CT-TG2-gliadin protein was labeled with Alexa Fluor 647 succinimidyl ester according to manufacturer's protocol (Invitrogen). DQ2 mice received 3.2 mg Alexa Fluor 647-labeled CT-TG2-gliadin by gastric gavage. Control mice received the same amount non-labeled CT-TG2-gliadin by gavage. At 18 hours after gavage, MLN were isolated and single cells suspensions were prepared by using Liberase TM (Roche, Germany) and DNAse (Sigma). Cells were analyzed with flow cytometric analysis for detection of Alexa Fluor 647 positive cells.

Adoptive transfer

Lymph nodes and spleens were obtained from DQ2.gliadinTCR mice. After erythrocyte lysis, the single cell suspension was enriched for $CD4^+$ cells by the 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 cells were fluorescently labeled with 5,6-carboxy-succinimidyl-fluoresceine-ester (CFSE) (Molecular Probes, Leiden, the Netherlands) at a final concentration of 5μM to follow their division profile. DQ2 acceptor mice received $1x10^7$ CFSE-labeled $CD4^+$ gliadin-TCRtg

T cells by intravenously injection in the tail vein. One day after transfer, DQ2 mice received once 75mg CT-TG2-gliadin protein or 3 consecutive days 3 mg of TG2-gliadin peptide i.g.. At 72 hours after the first feed, MLN and spleen were isolated and examined for the division of transferred $CD4^+$ gliadin-TCRtg T cells.

In vitro restimulation

For *in vitro* restimulation experiments, cells were purified from spleens or MLN of DQ2 acceptor mice. After erythrocyte lysis, the single cell suspension was enriched for $CD4^+$ cells by depletion of B cells and $CD8^+$ cells with rat-antibodies against B220 (clone 6B2) and CD8 (53.6.72) and anti-rat magnetic Dynabeads (Invitrogen). Next, $5x10^5$ $CD4^+$ gliadin-TCRtg T cells were restimulated with 0.5 mg/mL CT-TG2-gliadin or 5 μg/ml TG2-gliadin peptide for 24 hours. For intracellular detection of cytokines, cells were restimulated with 0.05 μg/ml phorbol 12-myristate 13-acetate (PMA; Sigma) and 0.1 μg/ml ionomycin (Sigma) and Monensin (Golgistop; BD) for 4 hours of culture.

Flow cytometry

The following antibodies were used for flow cytometry: 7-AAD (Molecular probes), anti-CD3 (BD, clone 145-2C11), anti-CD4 (BD, clone RM4-5), anti-CD45 (invitrogen; clone 30-F11), anti-CD8α (BD, clone 53-6.7), anti-CD11c (Biolegend, clone HL3), anti-CD103 (BD, clone M1/70), anti-CD11b (BD, clone M1/70), B220 (Biolegend clone RA3-6B2), anti-ICOS (Biolegend, clone C398.4A), anti-CD62L (BD, clone MEL-14), anti-TCR Vβ1 (Beckman Coulter, Woerden, the Netherlands; clone BL37.2). Anti-HLA-DQ (SPV-L3) was kindly provided by Dr. H. Spits, Amsterdam, the Netherlands.

Intracellular staining for Foxp3 (eBioscience, clone FJK-16S), anti-IL-10 (eBiosciences clone JES5-16E3), anti-IFN-γ (eBioscience; clone XMG1.2, Bergen op Zoom, the Netherlands) or the appropriate isotype controls was performed with the Foxp3 staining buffer kit, according to manufacturer's protocol (eBiosciences). Phenotype and cell division were measured on a FACSCanto™ II or FACSCalibur flow cytometer (BD). Data were analyzed using FlowJo software (BD).

Immunohistochemistry

At 72 hours after adoptive $CD4^+$gliadin-TCRtg T-cell transfer and gliadin feed as described above, spleens of DQ2 acceptor mice were isolated. Prior to staining, the samples were fixed with ice-cold acetone for 10 minutes and blocked with 10% normal mouse serum. Tissue sections were incubated using a primary antibody for anti-CD169 (acris antibodies, clone MOMA-1) or anti-CD11c primary antibody (Biolegend, clone N418). Immunoreactive sites were visible by staining 3,3'-diaminobenzidine tetrahydrochloride (Sigma) after CD169 staining. CD11c was subsequently stained with secondary antibody goat-anti-armenian hamster labeled with a DyLight-594

(Thermo Scientific, Fremont, USA) and the sections were mounted with medium for fluorescence containing DAPI (Vector Laboratories, Burlingame, USA). A conjugate control was performed for each immunostaining. Images were acquired and analyzed using a Leica DM5500B upright microscope and LAS-AF image acquisition software (Leica Microsystems, Rijswijk, the Netherlands).

Clodronate liposomes

Clodronate liposomes were prepared as described previously.[14] To deplete macrophages, DQ2 mice were injected intravenously with 200μL liposomes or saline as control.

In vitro T-cell differentiation

Spleens of DQ2 mice were isolated and single cells suspensions were prepared. By using Liberase TM and DNA-se spleens were digested. After erythrocyte lysis, $CD11c^+$ DC were isolated using anti-CD11c MACS beads (Miltenyi Biotec, Bergisch Gladbach, Germany). The negative fraction ($CD11b^+CD11c^{neg}$) after the MACS cell separation was plated in a 96-well tissue culture dish. After 2.5 hours incubation to allow adherence of macrophages, monolayers were washed to remove the non-adherent cells and adherent macrophages were incubated with medium.

$CD4^+$ gliadin-TCRtg T cells were obtained from spleens and lymph nodes from DQ2.gliadinTCR mice as described above. The enriched cells were fluorescently labeled with CFSE. A total of $2x10^4$ $DQ2^+$ cells, DC or macrophage or both, were incubated with $5x10^5$ CFSE-labeled $CD4^+V\beta1^+$gliadin-specific T cells in the presence of medium, CT-TG2-gliadin protein (0.5mg/ml), TG2-gliadin peptide (5μg/ml), IL-27 (25ng/ml) (R&D), rhTGFβ (20ng/ml) or 10nM retoinic acid for 24 or 72 hours as depicted in the figure legends. Cells were analyzed using flow cytometric analysis.

Real-time PCR

Splenic macrophages ($CD11b^+CD11c^{neg}$) or DC ($CD11c^+$) from DQ2 mice were isolated as described above. A total of $5x10^5$ cells were incubated with CT-TG2-gliadin (0.5mg/ml) or TG2-gliadin peptide (5μg/ml). After 24 hours, cells were isolated and total RNA was purified from the cell cultures using the NucleoSpin RNA XS kit (Machery-Nagel, Düren, Germany). Up to 1 μg RNA was reverse transcribed to single-stranded cDNA using a mix of random hexamers (2.5 μM) and oligo(dT) primers (20 nM). The reverse transcription reaction was performed in a total volume of 25 μl containing 0.2 mM of each dNTP (Amersham Pharmacia BioTech, Piscataway, NJ, USA), 200 U Moloney murine leukemia virus reverse transcriptase (M-MLV RT; Promega, Madison, WI, USA) and 25 U RNAsin (Promega). The conditions for the reverse transcription reaction were 37°C for 30 min, 42°C for 15 min and 94°C for 5 min. The cDNA was diluted five-fold and stored at –20°C. Real-time quantitative polymerase chain reaction (PCR) was performed using general fluorescence-based detection with SYBR green on an AbiPrismR

7900 Sequence Detection System (PE Applied Biosystems, Foster City, CA, USA). 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 $\Delta Ct = Ct_{target} - Ct_{cyclophillin}$. Specific primers were designed across different constant region exons resulting in the following primers: *Cyclophillin* forward 5'-AACCCCACCGTGTTCT-3', reverse 5'-CATTATGGCGTGTAAAGTCA-3'; *Il27* forward 5'-CGCAGGGA ATTCACAGT-3', reverse 5'AGCGAGGAAGCAGAGTCT-3'; *Cd11b* forward 5'-AAGGCTTTGGACAGAGTGT-3', reverse 5'-TGGGGGACAGTAGAAA CA-3'.

Statistics

Data are expressed as mean ± SD and analyzed using Student *t* test. $P < 0.05$ was considered significant.

Results

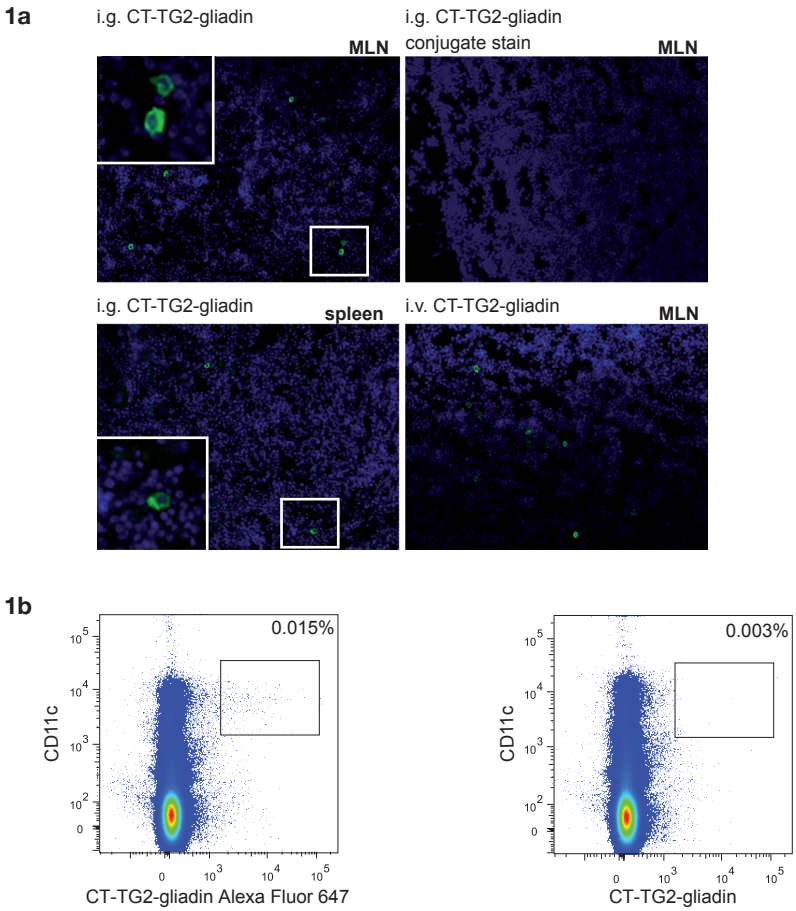
Gliadin protein is detected in CD11c$^+$ cells in the mesenteric lymph nodes but does not induce strong T-cell proliferation after oral ingestion

Previously, using the DQ2.gliadinTCRtg T-cell transfer model we have shown that orally administered CT-TG2-gliadin induced a dominant Tr1-cell proliferation in the spleen but not elicit proliferation in the MLN.[8] We hypothesized that the absence of T-cell proliferation in the MLN was related to an inability of APC to present whole CT-TG2-gliadin protein due to its resistance to proteolysis. This hypothesis would predict that the protein is detectable in the MLN of DQ2.gliadinTCR mice after oral ingestion. To investigate whether CT-TG2-gliadin is present in the MLN after gavage, DQ2 mice received CT-TG2-gliadin orally or intravenously as a positive control. After 18 hours, lymphoid organs and spleens were analyzed for presence of gliadin by immune fluorescence using an anti-gliadin antibody. After gavage, gliadin positive cells were detectable in the MLN and spleen while no signal was detected using conjugate control staining of the MLN after CT-TG2-gliadin feed (*Figure 1a*).

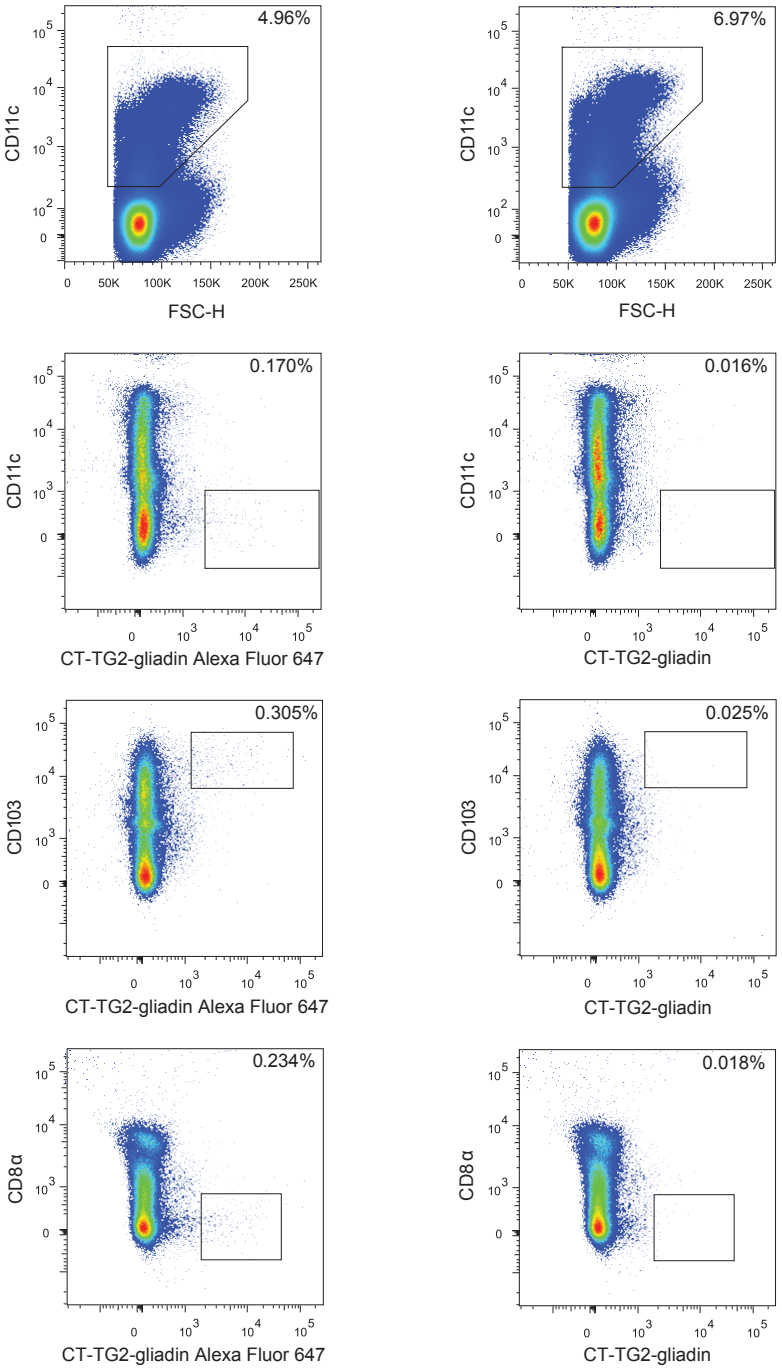
To further substantiate the presence of gliadin positive cells in cells of the MLN, mice were fed fluorescently labeled CT-TG2-gliadin or non-labeled CT-TG2-gliadin as a control. After 18 hours gliadin-positive cells in MLN were analyzed by flow cytometry. As shown in *Figure 1b*, Alexa Fluor 647-labeled gliadin is associated with CD11c$^+$ cells in the MLN. In particular, the CD11c$^+$ cells staining positive for Alexa Fluor 647 gliadin co-expressed CD103 but not CD8α or CD11b (*Figure 1c* and *Supplementary Figure 1S*). B220 positive B cells were negative for Alexa Fluor 647 gliadin in the MLN (*Figure 1d*).

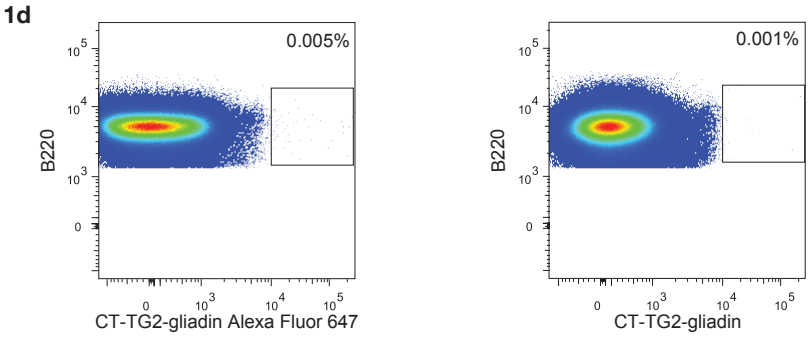
In conclusion, after CT-TG2-gliadin feed the protein is present in the MLN and is found to be associated with CD11c$^+$CD103$^+$ cells.

Figure 1 ***Whole gliadin protein is detectable in the MLN after gavage. (a)*** *DQ2 mice received 3.2mg CT-TG2-gliadin i.g. or intravenously. At 18 hours, MLN and spleen were isolated and stained with anti-gliadin or conjugate control and counterstained with DAPI. Representative immunofluorescence stainings on frozen sections of MLN and spleen. Green indicates the anti-gliadin staining and blue indicates the nuclei counterstaining by using DAPI. Original magnification: x 20. n=2 mice.* ***(b-d)*** *Mice received 3.2 mg CT-TG2-gliadin, either labeled with Alexa Fluor 647 succinimidyl ester or unlabeled.* ***(b,c)*** *At 18 hours, a single cell suspension of MLN were depleted for B- and CD4$^+$ T cells by using anti-B cells (6B2) and anti-CD4 (GK1.5), goat-anti-rat Dynabeads and stained for 7AAD, CD45, CD11c, CD11b, CD103, CD8α and analyzed for Alexa Fluor 647.* ***(b)*** *Representative dot plots of stainings gated on 7AAD^{neg}CD45$^+$, showing the expression of CD11c and Alexa Fluor 647.* ***(c)*** *Representative dot plots showing the expression of Alexa Fluor 647 in 7AAD^{neg}CD45$^+$CD11c$^+$ and subsequently gated for CD11b, CD103 and CD8α. Gates were set using the unlabeled CT-TG2-gliadin controls. N=3 per group.* ***(d)*** *At 18 hours, a single cell suspension of MLN was stained for B220 and analyzed for Alexa Fluor 647. Representative dot plots of stainings showing the expression of 7AAD^{neg}B220$^+$DQ2$^+$ cells.*



1c


4.96%
6.97%
CD11c
FSC-H
0.170%
0.016%
CT-TG2-gliadin Alexa Fluor 647
CT-TG2-gliadin
0.305%
0.025%
CD103
0.234%
0.018%
CD8α



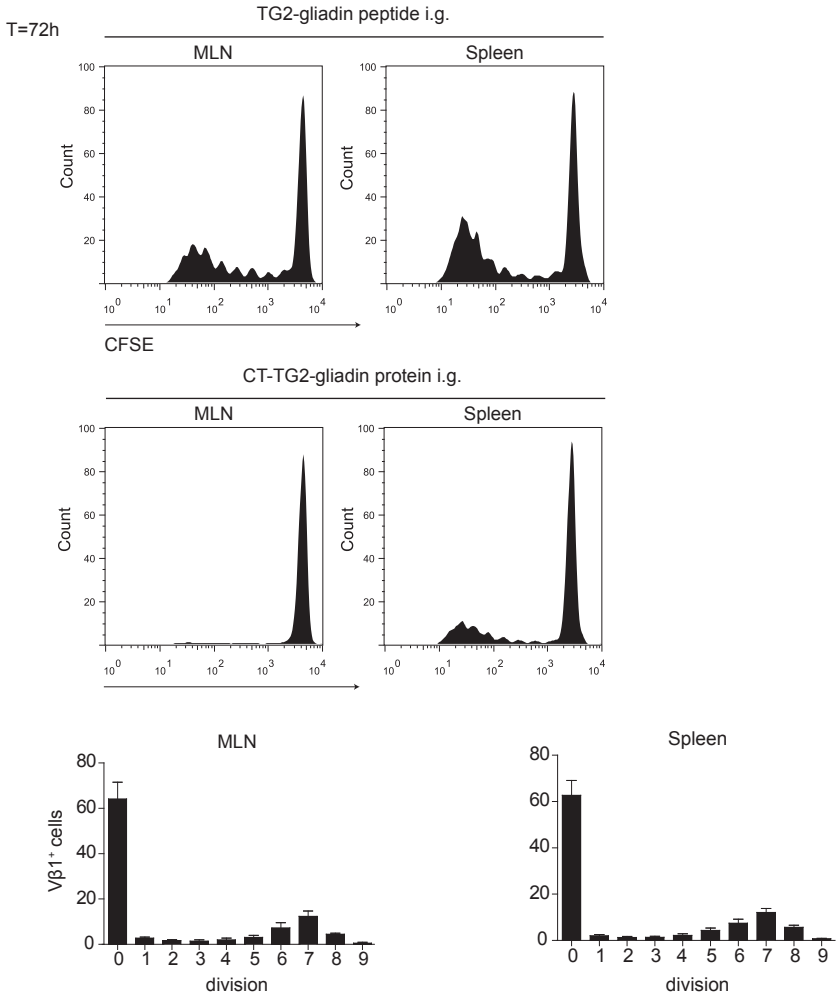
TG2-gliadin peptide is presented efficiently in the mesenteric lymph node

As, in contrast to the soluble protein OVA, CT-TG2-gliadin gavage induces preferential T-cell proliferation in the spleen rather than the MLN we hypothesize that proteolysis of gliadin into smaller peptides may be a requirement for antigen presentation to induce T-cell proliferation in the MLN. Consequently, the gliadin-specific T cell-response was investigated in the MLN after gavage of synthetic TG2-gliadin peptides using the DQ2.gliadinTCRtg T-cell transfer model as previously described.[8] In short, $CD4^+$ gliadin-TCRtg T cells were purified from DQ2.gliadinTCR mice, labeled with CFSE and intravenously injected in DQ2 acceptor mice. Starting the day after T-cell transfer, DQ2 mice raised on a gluten-free diet received either 3 mg TG2-gliadin peptide on 3 consecutive days or a single dose of 75mg CT-TG2-gliadin protein by gavage. At 72 hours after the first feed, MLN and spleen were isolated and examined for the division of transferred gliadin-specific T cells. Confirming earlier data,[8] CT-TG2-gliadin protein feed preferentially induced T-cell proliferation in the spleen when compared to the MLN (*Figure 2a*). However, in contrast to CT-TG2-gliadin, TG2-gliadin peptide induced T-cell proliferation in the gut-draining MLN as well as the spleen demonstrating that smaller fragments of gliadin can induce substantial T-cell proliferation in the MLN (*Figure 2a*).

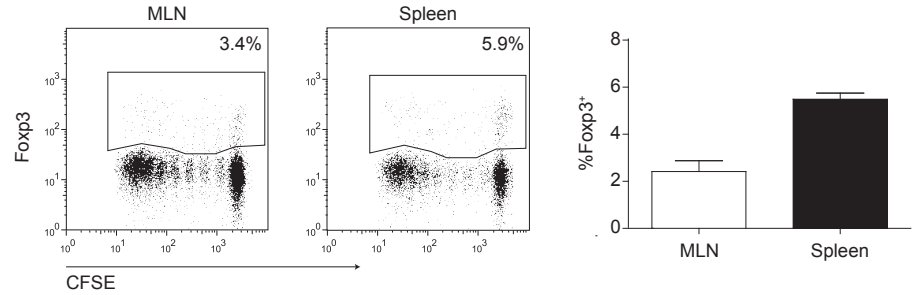
Within the mucosal environment of the MLN, presentation of the harmless food protein OVA by $CD103^+$ DC preferentially induces differentiation of naive T cells into $Foxp3^+$ regulatory T (Treg) cells.[9-12] Therefore, we expected that the TG2-gliadin peptide-induced T-cell response in the MLN had an enhanced frequency of $Foxp3^+$ T cells. However, TG2-gliadin peptide did not appear to induce preferential $Foxp3^+$ T-cell differentiation in the MLN as the percentage of gliadin-specific $Foxp3^+$ T cells in the MLN was lower than that in the spleen (*Figure 2b*). This was not due to the intrinsic inability of the transgenic MLN-DC to induce $Foxp3^+$ T cells, as gliadin-specific $Foxp3^+$ T cells differentiated *in vitro* in response to antigen presentation by MLN-DC cultured with TGF-β and retinoic acid (*Figure 2c*).

Figure 2 ***After oral gavage, gliadin peptide is presented efficiently in the MLN and spleen. (a-b)*** *DQ2 mice received 1×10⁷ CFSE-labeled CD4⁺ gliadin-TCRtg T cells followed by the oral administration of either 3 mg TG2-gliadin peptide on 3 consecutive days or a single gavage with 60mg CT-TG2-gliadin protein by gavage. At 72 hours MLN and spleens were isolated and analyzed by flow cytometry for division of transferred cells.* ***(a)*** *For each group a representative histogram plot showing CFSE dilution gated on CD4⁺Vβ1⁺ cells is depicted. Representative for n=10 mice.* ***(b)*** *Representative dot plots Foxp3 expression on dividing CD4⁺Vβ1⁺ cells from MLN and spleen at 72 hours after the first TG2-gliadin peptide feed. Representative for n= 5 mice.* ***(c)*** *Splenocytes from DQ2.gliadinTCR mice were cultured with 0.5 mg/ml CT-TG2-gliadin in the presence or absence of 20ng/mL recombinant human TGF-β with or without 10nM retinoic acid for 72 hours. Proliferation was assessed by CFSE dilution, Foxp3 and CD62L expression were determined by FACS analysis.*

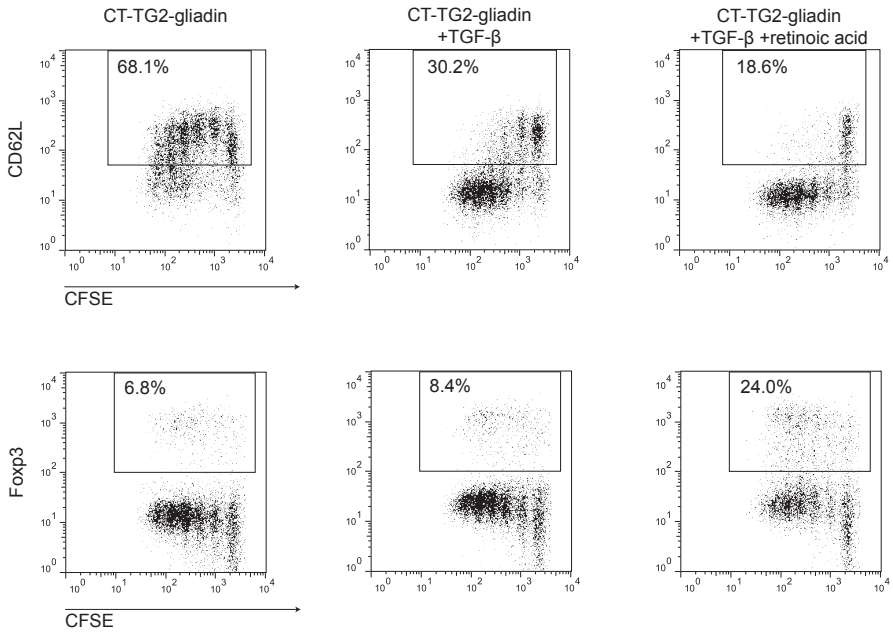
2a



2b



2c



In conclusion, in contrast to CT-TG2-gliadin protein, TG2-gliadin peptide is presented efficiently in the MLN suggesting that cleavage of gliadin may be a prerequisite for antigen presentation and subsequent proliferation of naive T cells. Indeed, since CT-TG2-gliadin protein feed solely leads to antigen presentation in the spleen and not in the MLN, this suggests that insufficient degradation of gliadin protein in the MLN may account for the absence of T-cell proliferation in response to CT-TG2-gliadin protein.

Macrophages determine the differentiation of splenic gliadin-specific Tr1 cells in vivo

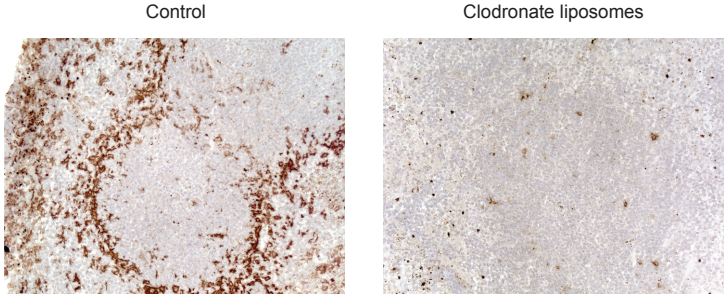
Macrophages, in particular marginal zone macrophages in the spleen, have a role in capturing and processing of antigen.[15] When compared to DC these macrophages have higher levels of lysosomal proteases and degrading capacity.[13] Therefore, we examined the contribution of macrophages on splenic gliadin presentation by depletion of macrophages with clodronate liposomes in the DQ2.gliadinTCRtg T-cell transfer model. Previously it was shown that infusion of clodronate liposomes effectively depleted macrophages by accumulation of clodronate liposomes in the cell leading to apoptosis.[16] DQ2 mice were intravenously injected with clodronate liposomes one day prior to $CD4^+$ DQ2.gliadinTCRtg T-cell transfer. Intravenous injection of saline was used as a negative control. As observed previously,[14] histological evaluation at 3 days after clodronate liposome treatment revealed that $CD169^+$ cells were efficiently depleted from the spleen (*Figure 3a*). $CD11c^+$ cells were nevertheless detectable in the spleen of liposome-injected mice demonstrating that clodronate liposomes treatment did not lead to the depletion of all $CD11c^+$ phagocytes (*Figure 3b*). After injection of clodronate liposomes or saline and subsequent transfer of $CD4^+$ gliadin-TCRtg T cells the mice were fed CT-TG2-gliadin or TG2-gliadin peptide. At 72 hours after feed the spleens were isolated and examined for the division of transferred gliadin-specific T cells. Flow cytometric analysis revealed that clodronate treatment did not inhibit proliferation of $CD4^+$ gliadin-TCRtg T cells in the spleen of mice after CT-TG2-gliadin feed as equal percentages of transferred cells had divided and the cells had undergone the same number of divisions (*Figure 3c,d*). As expected, proliferation in response to TG2-gliadin peptide feed also was unchanged after macrophage depletion (*Figure 3c,e*).

At 72 hours, both responding $CD4^+$ gliadin-TCRtg T cells and non-responding cells were isolated from the spleen and restimulated *in vitro* with TG2-gliadin peptide. At 24 hours after *in vitro* restimulation, the expression of ICOS reflecting cellular activation and the percentage of cytokine-secreting gliadin-specific T cells were analyzed by flow cytometry. Depletion of macrophages before CT-TG2-gliadin feed resulted in decreased ICOS expression (*Figure 3d*) and elicited an approximately three-fold reduction in the percentage of IL-10- and IFN-γ -producing gliadin-specific T cells in the spleen (*Figure 3f*). In contrast, macrophage depletion before TG2-gliadin peptide feed did not affect the percentage of cytokine secreting cells nor reduced the activation of the gliadin-specific T cells (*Figure 3e, g*).

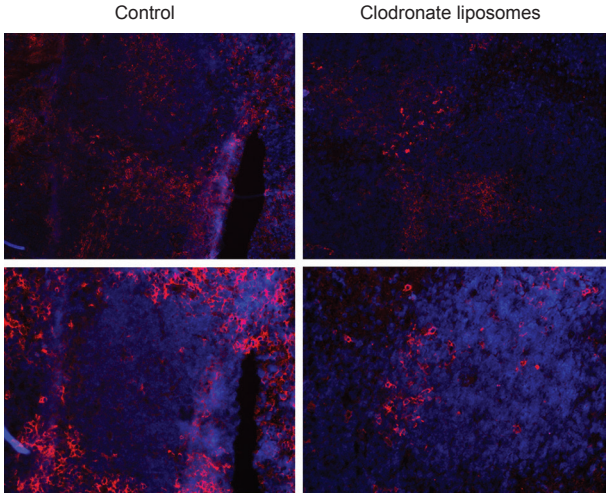
In conclusion, in mice fed with CT-TG2-gliadin, splenic macrophages are required for ICOS expression by activated T cells and drive the differentiation of IL-10- and IFN-γ-producing splenic gliadin-specific T cells. Macrophages are not essential for these gliadin-specific T-cell responses when mice are fed TG2-gliadin peptide inferring that macrophage-driven cleavage of CT-TG2-gliadin regulates the differentiation of gliadin-specific Tr1 cells in the spleen.

Figure 3 ***Macrophages determine the differentiation of splenic gliadin-specific Tr1 cells in vivo. (a-g)*** *DQ2 mice received 1x 10^7 CFSE-labeled $CD4^+$ gliadin-TCRtg T cells follow by the oral administration of TG2-gliadin peptide 3 mg and 6 mg during 2 consecutive days or 75mg of CT-TG2-gliadin protein the next day. 24 hours prior to the T-cell transfer mice were either injected with 200µL clodronate liposomes or saline (control). At 72 hours after the first feed, spleens were isolated and single cell suspensions were analyzed by flow cytometry for division of transferred cells.* ***(a)*** *Representative CD169 stainings on spleen sections of control (left) and clodronate liposome (right) injected mice. N=8 control mice, n=5 clodronate liposome mice.* ***(b)*** *Representative CD11c stainings on spleen sections of control (left) and clodronate liposome (right) injected mice.* ***(c)*** *For each group a dot plot showing CFSE dilution gated on $CD4^+V\beta1^+$ cells is depicted.* ***(d,e)*** *Histograms of dividing and $ICOS^+$ cells gated on $CD4^+V\beta1^+$ cells. (n=2-5 per group)* ***(f,g)*** *Quantitative analysis of the percentage of cytokine producing cells within the dividing restimulated $CD4^+V\beta1^+$cell population after CT-TG2-gliadin feed* ***(f)*** *or TG2-gliadin peptide feed.* ***(g)*** *Each symbol represents one mouse. Horizontal bars represent mean values.*

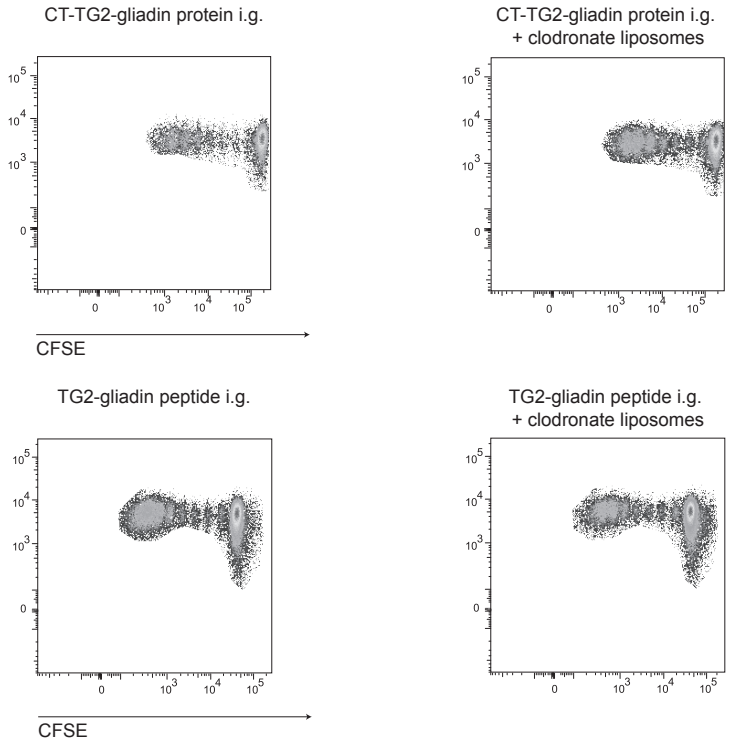
3a *CD169*



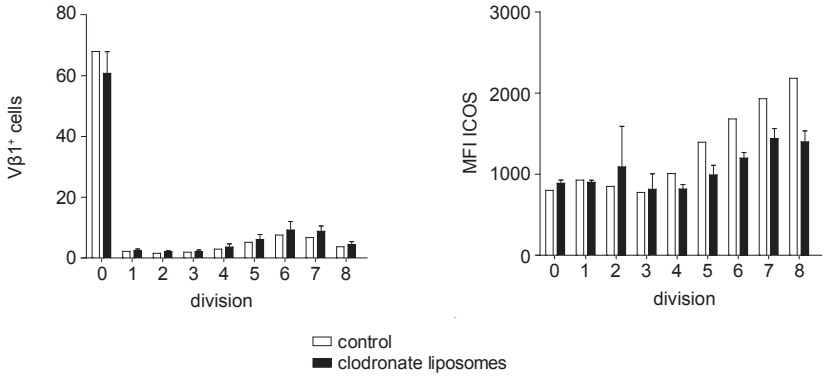
3b *CD11c*



3c *Spleen T=72h*



3d *CT-TG2-gliadin protein*



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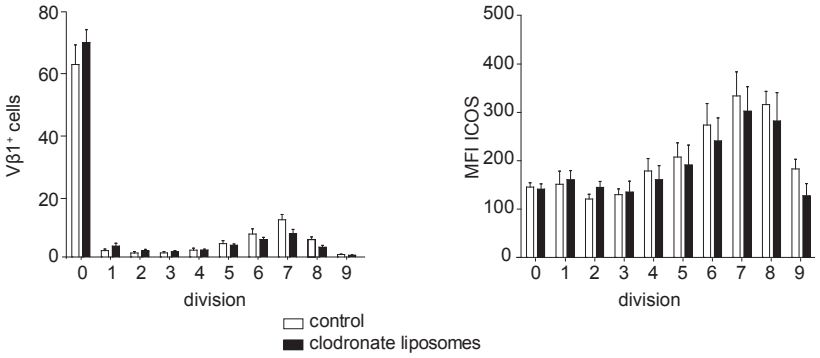

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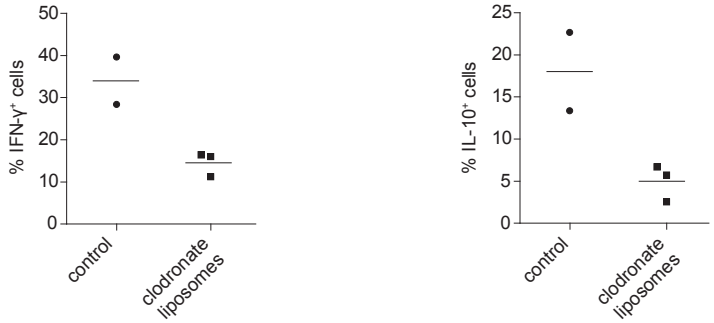
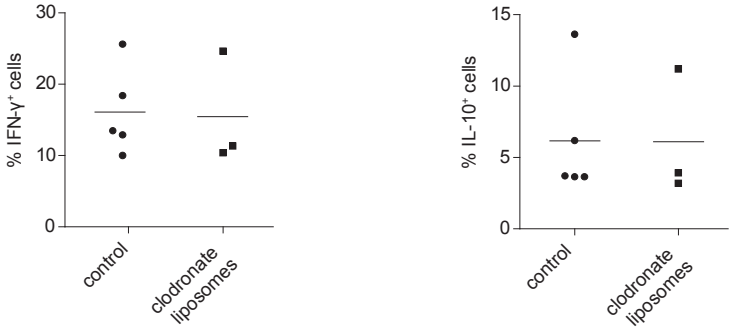


3g *TG2-gliadin peptide*



Macrophages determine the differentiation of gliadin-specific Tr1 cells in vitro

To demonstrate that macrophages are required for directing the phenotype of the proliferating gliadin-specific T cells, we developed an *in vitro* model for Tr1-cell differentiation. In short, naive $CD4^+$ gliadin-TCRtg T cells from DQ2.gliadinTCR mice were co-cultured with freshly isolated splenic macrophages ($CD11c^{neg}CD11b^+$) or splenic DC ($CD11c^+$) from DQ2 mice in the presence of CT-TG2-gliadin or TG2-gliadin peptide. The ability of splenic macrophages and splenic DC to induce Tr1-cell differentiation in response to CT-TG2-gliadin or TG2-gliadin peptide was assessed by flow cytometry at 72 hours of culture. As it is possible that macrophages and DC cooperate in inducing the Tr1 cell phenotype cultures with macrophages only, DC only and mixtures of macrophages and DC were used. After 72 hours, CT-TG2-gliadin loaded DC induced a higher frequency of T cells going into division with a mean of approximately 80% while macrophages loaded with CT-TG2-gliadin induced approximately 70% of T cells to divide. Once dividing, the T cells underwent approximately 5 divisions irrespective of the nature of the APC (*Figure 4a*). However, culture with CT-TG2-gliadin loaded macrophages consistently induced increased percentages of IFN-γ and IL-10 producing gliadin-specific T cells when compared to DC alone. Mixtures of DC and macrophage cultures improved the proliferative capacity when compared to macrophages alone but differentiation of IFN-γ- and IL-10-producing cells was lower than in the cultures stimulated with CT-TG2-gliadin loaded macrophages only (*Figure 4a,b*).

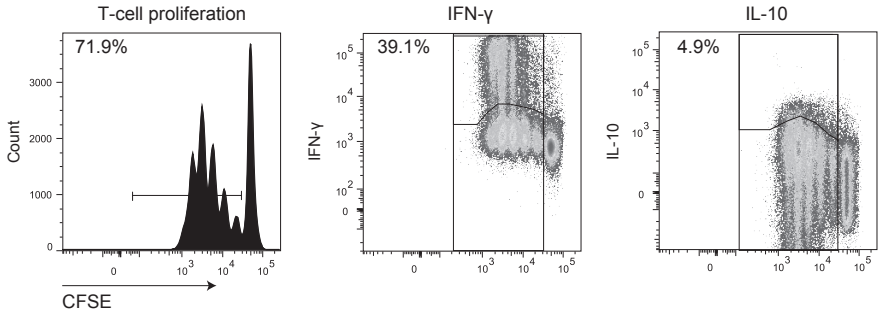
These findings show that antigen presentation by macrophages drives the differentiation of Tr1 cells in response to CT-TG2-gliadin protein *in vitro*.

IL-27 expressed by macrophages induces IL-10 and IFN-γ production by gliadin-specific T cells in vitro

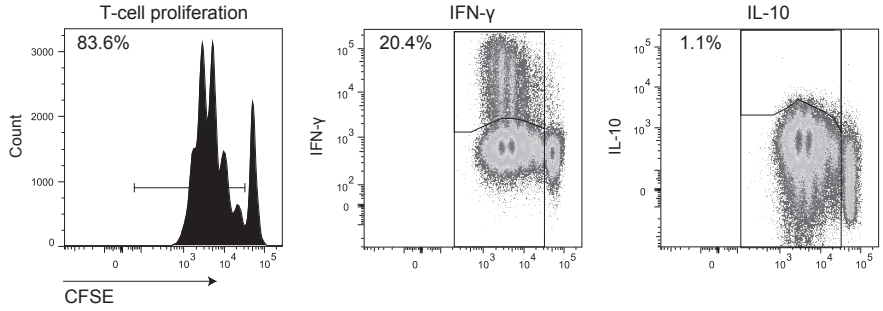
The innate immune cell-derived cytokine IL-27 has been reported to promote the induction of Tr1 cells.[17, 18] Therefore, we assessed whether recombinant IL-27 could induce Tr1-cell differentiation in response to CT-TG2-gliadin loaded macrophages or DC. We observed a 4-fold increased percentage of IFN-γ-producing gliadin-specific T cells in the culture with CT-TG2-gliadin loaded macrophages when compared to control in response to IL-27. In cultures with CT-TG2-gliadin loaded DC the addition of IL-27 resulted in a 2-fold increase of IFN-γ gliadin specific T cells compared to control. The addition of IL-27 to the culture of CT-TG2-gliadin loaded macrophages led to a significant increase in IL-10-producing gliadin-specific T cells. A smaller increase on the IL-10-producing gliadin-specific T cells was seen in the culture with DC after addition of IL-27 (*Figure 5a,b*). Next, we assessed whether macrophages expressed more *Il27* mRNA than DC. Macrophages expressed higher levels of *Il27* mRNA after overnight incubation with CT-TG2-gliadin protein compared to medium or incubation with TG2-gliadin peptide Moreover, *Il27* mRNA expression was significantly higher in CT-TG2 gliadin-loaded macrophages than in

Figure 4 ***Macrophages determine the differentiation of splenic gliadin-specific Tr1 cells in vitro. (a,b)*** *$CD4^+$ gliadin-TCRtg T cells were purified from spleens, labeled with CFSE and stimulated in vitro with CT-TG2-gliadin-loaded splenic-derived macrophages or DC or both. At 72 hours T-cell proliferation and T-cell cytokine production were assessed by flow cytometric analysis.* ***(a)*** *Representative histograms and dot plots showing T-cell proliferation, IFN-γ- and IL-10-producing cells. Gated on $CD4^+Vβ1^+$T cells and the appropriate isotype control antibodies.* ***(b)*** *Quantitative analysis of the percentage of IFN-γ- and IL-10-producing cells within the dividing $CD4^+Vβ1^+$cell population. Horizontal bars represent mean values ± SD (n=3 per group). *P <0.05, Student t-tests.*

4a *Macrophages CT-TG2-gliadin*



DC CT-TG2-gliadin



4b *CFSE*

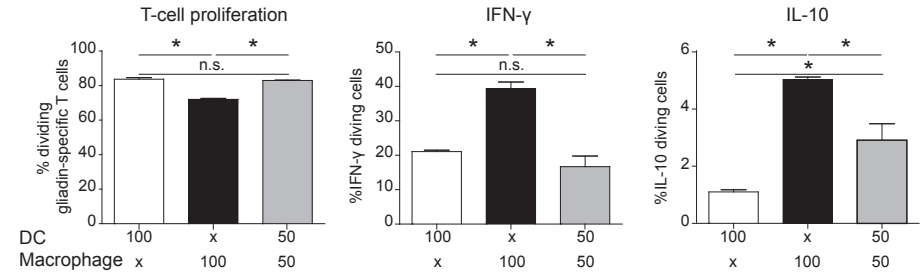
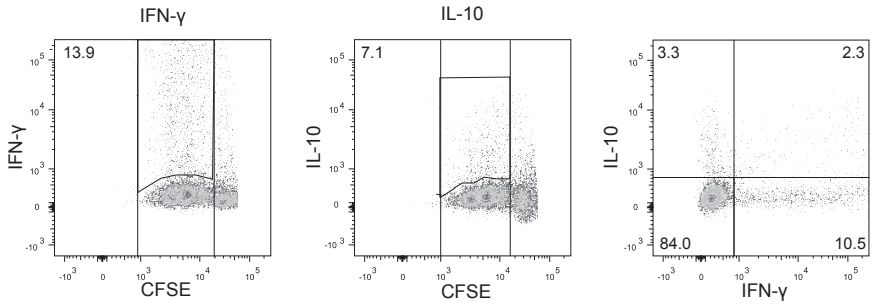
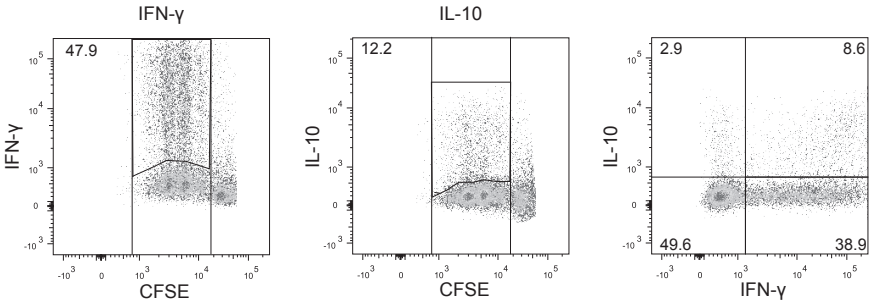


Figure 5 ***Il27 expressed by macrophages induces IL-10 and IFN-γ production of gliadin-specific T cells in vitro. (a,b)*** *$CD4^+$ gliadin-TCRtg T cells were purified from spleens, labeled with CFSE and stimulated in vitro with CT-TG2-gliadin-loaded splenic-derived macrophages or DC with or without 25ng/mL recombinant IL-27. At 72 hours T-cell proliferation and T-cell cytokine production were assessed by flow cytometric analysis.* ***(a)*** *Representative dot plots showing T-cell proliferation, IFN-γ- and IL-10-producing cells. Gated on $CD4^+$ T cells and the appropriate isotype control antibodies.* ***(b)*** *Quantitative analysis of the percentage of cytokine producing cells within the dividing $CD4^+$ cell population. Horizontal bars represent mean values ± SD (n=3 per group).* ***(c,d)*** *Splenic-derived macrophages or DC from DQ2 mice were cultured for 24 hours in the presence of CT-TG2-gliadin (0.5mg/mL) or TG2-gliadin peptide (5µg/mL). Il27* ***(c)*** *and Cd11b* ***(d)*** *expression relative to cyclophillin was determined by real-time PCR analysis. Horizontal bars represent mean values ± SD (n=4-11 per group). *P <0.05, Student t-tests.*

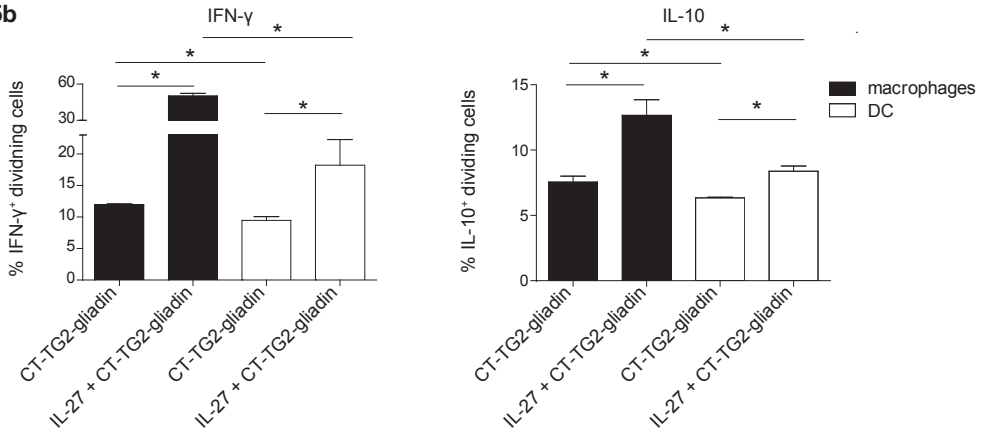
5a *Macrophages CT-TG2-gliadin*



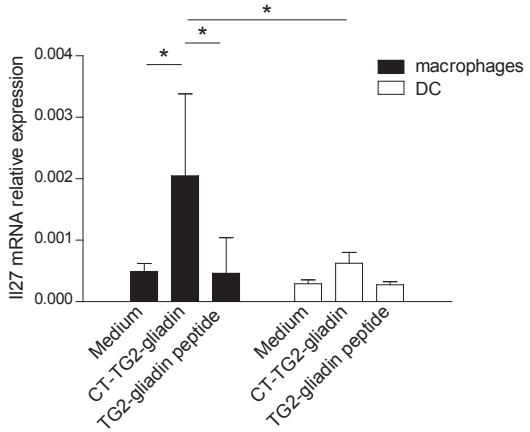
Macrophages CT-TG2-gliadin + IL-27



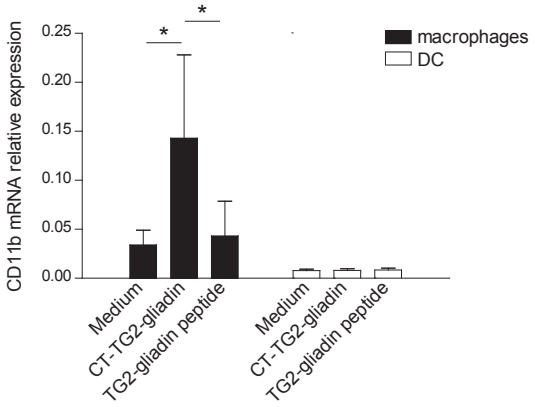
5b


IFN-γ
% IFN-γ+ dividning cells
60
30
20
15
10
5
0
CT-TG2-gliadin
IL-27 + CT-TG2-gliadin
CT-TG2-gliadin
IL-27 + CT-TG2-gliadin
IL-10
% IL-10+ dividing cells
15
10
5
0
macrophages
DC
CT-TG2-gliadin
IL-27 + CT-TG2-gliadin
CT-TG2-gliadin
IL-27 + CT-TG2-gliadin

5c


Il27 mRNA relative expression
0.004
0.003
0.002
0.001
0.000
macrophages
DC
Medium
CT-TG2-gliadin
TG2-gliadin peptide
Medium
CT-TG2-gliadin
TG2-gliadin peptide

5d


CD11b mRNA relative expression
0.25
0.20
0.15
0.10
0.05
0.00
macrophages
DC
Medium
CT-TG2-gliadin
TG2-gliadin peptide
Medium
CT-TG2-gliadin
TG2-gliadin peptide

CT-TG2 gliadin-loaded DC (*Figure 5c*). In order to establish whether *Il27* mRNA expression correlated with macrophage activation, mRNA expression of the CR3 receptor beta integrin *Cd11b* was assessed. As observed for *Il27*, macrophages that had been incubated with CT-TG2-gliadin overnight expressed significantly more *Cd11b* mRNA than CT-TG2-gliadin-loaded DC (*Figure 5d*). In conclusion, expression of *Il27*, a factor that drives Tr1-cell differentiation, is specifically induced in macrophages but not DC upon incubation with CT-TG2-gliadin and addition of IL-27 potentiates gliadin-specific Tr1-cell differentiation *in vitro* with a more potent effect on T cells co-cultured with CT-TG2-loaded macrophages than DC.

Discussion

In this study, we report that splenic macrophages are crucial for the differentiation of gliadin-specific Tr1 cells that mediate oral tolerance to gliadin in DQ2.gliadinTCR mice. Although splenic DC have the capacity to elicit gliadin-specific T-cell proliferation *in vitro* and *in vivo*, these cells did not induce Tr1-cell differentiation as effectively. Macrophages have increased capacity for lysosomal proteolysis when compared to DC.[13] In agreement, depletion of macrophages *in vivo* inhibited the splenic Tr1-cell response to oral CT-TG2-gliadin while macrophage depletion did not alter the splenic T-cell response to TG2-gliadin peptide feed. Upon incubation with CT-TG2-gliadin but not TG-gliadin peptide macrophages expressed *Il27*, a factor that mediates gliadin-specific Tr1-cell differentiation while DC did not have this capacity. Together, these data reveal a novel mechanism of oral tolerance induction to an unusual food protein.

Previously we have shown that, in mice, oral tolerance to gliadin is mechanistically different from oral tolerance to the soluble food protein OVA. While oral tolerance to OVA is mounted in the gut-draining MLN by the conversion of naive T cells into suppressive $Foxp3^+$ T_{REG} cells, tolerance to oral gliadin was mediated via induction of suppressive IL-10-secreting Tr1 cells in the spleen.[8] The unexpected finding that an immune response to a food protein was mounted in the spleen rather than in a gut-draining lymphoid tissue and the unusual molecular characteristics of deamidated gliadin, led us to believe that degradation of gliadin is a pivotal step in the differentiation of Tr1 cells and the subsequent development of oral tolerance to gliadin. Therefore, we first established that insufficient degradation of gliadin was causing the suboptimal development of a T-cell response in the MLN after gliadin feed. As expected using immunohistochemistry and feeding fluorescently-labeled CT-TG2-gliadin the labeled protein was detectable in $CD103^+CD11c^+$ APC within the MLN showing that the antigen normally drained to the gut-draining lymphoid tissue but apparently did not induce a productive T-cell response. However, productive T-cell responses in the MLN could only be obtained after feeding TG2-gliadin peptide suggesting

that degradation of the peptide could impose gliadin presentation in the MLN after gliadin feed. Dietary gluten contains a number of epitopes that were identified as TCR binding in CD patients.[19] The common feature of these epitopes is the presence of high levels of proline and glutamine residues, making the gluten protein resistant to degradation.[3,20] Macrophages, and particularly those in the splenic marginal zone, are equipped with high amounts of lysosomal proteases such as Cathepsin S, Cathepsin B and Cathepsin D while other APC such as DC and B cells express these to a lower degree.[13] Therefore, we envisaged that splenic Tr1-cell differentiation after CT-TG2-gliadin feed involved macrophages. To demonstrate that differentiation of Tr1 cells, after CT-TG2-gliadin feed, is depended on macrophages we depleted macrophages *in vivo* by giving mice clodronate liposomes intravenously. As shown previously, this technique induces apoptosis CD169 positive marginal zone metallophilic macrophages that are situated at the border of follicles and the marginal zone inducing a cellular depletion up to 48 hours after injection.[16] Crucially, macrophage depletion dramatically reduced the differentiation of Tr1 cells after CT-TG2-gliadin feed demonstrating that macrophages are pivotal for the tolerogenic Tr1-cell response to gliadin. Intriguingly, the clodronate liposome treatment did not abolish proliferation of T cells. A possible explanation for this finding is that despite absence of CD169 staining after injection the ablation of macrophages was not complete allowing residual cells to degrade sufficient amounts of antigen to maintain proliferation. Alternatively, splenic DC populations have the capacity to induce this proliferation but lack the capacity to drive the differentiation of these cells into IL-10- and IFN-γ-secreting cells.

Our results show that gliadin presentation leading to T-cell proliferation can be performed by APC other than macrophages, but macrophages are essential for providing the qualitative costimulatory signals leading to the differentiation of IL-10-secreting Tr1 cells after presentation.

Recent reports have identified IL-27 as a driving factor for the induction of IL-10-producing Tr1 cells in mouse and human. IL-27 and its target transcription factor c-Maf, the cytokine IL-21 and the costimulatory receptor ICOS coordinately act together to promote Tr1-cell differentiation. Mice deficient for either c-Maf, IL-21 or ICOS have decreased capacity for IL-27 induced differentiation of Tr1 cells.[18] In agreement, addition of recombinant IL-27 to our *in vitro* cultures drastically potentiated the number of IFN-γ- and IL-10-secreting gliadin-specific Tr1-cells, but the effect was more potent in co-culture of T cells with macrophages than with DC. Moreover, in these *in vitro* experiments CT-TG2-gliadin loaded macrophages expressed *Il27* mRNA, while CT-TG2-gliadin loaded DC expressed low *Il27* mRNA. In addition, TG2-gliadin peptide did not induce *Il27* mRNA expression in macrophages. These results demonstrate that specifically macrophages but also recognition of the non-degraded gliadin form are prerequisites for IL-27 induction. It is unclear why splenic macrophages rather than DC preferentially express *Il27*. We hypothesize that the

two processes of IL-27 production and lysosomal degradation may be connected. IL-27 is shown to inhibit acidification of the lysosomes in human macrophages.[21] Such a process may modulate the duration a protein is present in the cells[13] and affect the outcome of the T-cell response with reduced digestion allowing for a superior immune response[22]. From human studies we also know that macrophage-derived IL-27 promotes the differentiation of IL-10-producing cells in the spleen. In patients with human visceral leishmaniasis, splenic Tr1 cells are an important source for IL-10. In the plasma of these patients elevated levels of IL-27 are found and isolated splenic cells showed significantly elevated *IL27* mRNA levels compared with patients after treatment. Splenic $CD14^+$ macrophages were the main source of *IL27* mRNA.[23]

Whether ICOS-ligation also is a pivotal factor in the macrophage driven Tr1-cell induction is unclear. In our *in vivo* experiments, depletion of macrophages reduced the expression of ICOS on dividing T cells however *in vitro* T cells that were primed with CT-TG2-gliadin-loaded DC expressed ICOS to a similar degree as CT-TG2-gliadin-loaded macrophages (data not shown), allowing a possible role for ICOS in the differentiation process.

Previously, we have shown that IL-10-secreting gliadin-specific Tr1 cells, which are generated in the spleen mediate oral tolerance to gliadin, have low IL-2 release and show an 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 and the population consists of cells that secrete either one or both of these cytokines. The IFN-γ secretion would argue denoting them as having a T helper 1 phenotype. In this manuscript we often denote these cells as Tr1 cells because of their suppressive function but we certainly do not exclude that this phenotype is changeable with regard to the production of IL-10. As such, in a particular phase of differentiation T helper 1 cells can develop into IL-10-secreting cells that have the same phenotype. Repetitive TCR stimulation may be a key factor in this process.[24, 25]

Clearly our data show that even when presented in the MLN TG2-gliadin-γ1-peptide does not favor induction of $Foxp3^+$ T_{REG}-cell differentiation in DQ2.gliadinTCR mice. This is in agreement with findings in a DQ2- gliadin α-II epitope-dependent mouse model. [26] As shown in *in vitro* culture, once presented in HLA-DQ2 in the context of specific mucosal cofactors such as TGF-β and retinoic acid mucosal $Foxp3^+$ T_{REG}-cell differentiation does occur. We hypothesize that failure of Foxp3 induction is related to the phenotype of the APC in the MLN as specific subsets of $CD11c^+CD103^+$ cells may favor Foxp3 expression.

The results obtained in this mouse model support a role for macrophages in the induction of tolerance to gluten. Macrophages seem to be of crucial importance to generate gluten-specific tolerogenic T-cell responses and promote the differentiation of T cells into anti-inflammatory IL-10-producing Tr1 cells in the healthy intestine.

This raises the question whether loss of tolerance to gluten in CD might be a result of defective macrophage induced regulation.

Acknowledgements

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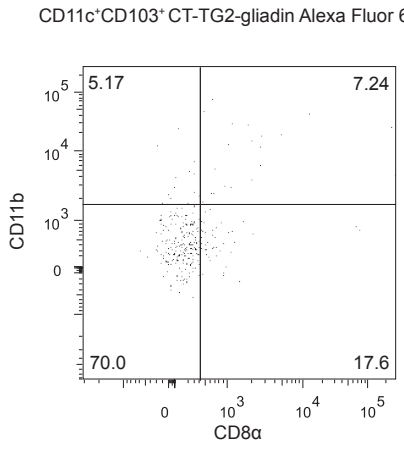
Supplementary figure

Figure S1 *Mice received 3.2 mg CT-TG2-gliadin, either labeled with Alexa Fluor 647 succinimidyl ester or unlabeled. At 18 hours, a single cell suspension of MLN were depleted for B- and CD4$^+$ T cells by using anti-B cells (6B2) and anti-CD4 (GK1.5), goat-anti-rat Dynabeads and stained for 7AAD, CD45, CD11c, CD103, CD11b, CD8α and analyzed for Alexa Fluor 647. One representative dot plot is showing the expression of CD8α and CD11b in 7AAD^{neg}CD45$^+$CD11c$^+$CD103$^+$Alexa Fluor 647$^+$ cells.*



7

General discussion

The main objective of this thesis was to identify defects in the regulation of T-cell responses in the inflamed intestine of CD and IBD patients. In this chapter the main findings of our studies are reported and placed in a broader perspective.

Beneath the single layer of epithelial cells, the balance between tolerance and immunity is tightly regulated. Breakdown of intestinal tolerance to food proteins or commensal microbiota results from a disturbance of the balance between inflammatory activity and regulatory activity in the intestinal environment.

This balance can be disturbed due to an imbalance in the adaptive T cell compartment comprising T effector cells and T regulatory (T_{REG}) cells. Decreased absolute numbers of T_{REG} cells could cause the imbalance. Alternatively, defective T_{REG}-cell function or increased resistance of effector T cells to T_{REG} cell-suppression could be of importance.

The role of T_{REG} cells in intestinal inflammation

Various T-cell populations have anti-inflammatory functions of which Foxp3$^+$ T_{REG} cells and Foxp3^{neg} Tr1 cells are of special importance in the intestine.[1]

Foxp3$^+$ T_{REG} cells

Deletions and loss of function mutations in the *Foxp3* gene result in fatal inflammatory disease in mice, and Immune dysregulation, Polyendocrinopathy, Enteropathy, X-linked (IPEX) syndrome in human.[2, 3] In this syndrome the intestine is the most affected organ and the intestinal pathology results from inability to regulate the CD4$^+$ effector T-cell responses.[2] IPEX patients have severe intestinal inflammation. The intestinal inflammation is characterized by villous atrophy of the small intestine and lymphocyte and plasma cell infiltration in the lamina propria of the small intestine and colon.[3, 4] IPEX reveals the importance of Foxp3$^+$ T_{REG} cells in maintaining intestinal homeostasis. Foxp3$^+$ T_{REG} cells can be divided on the basis of their origin in thymus-derived natural T_{REG} cells and peripherally-induced T_{REG} cells.[5]

Decreased numbers of T_{REG} cell populations could be an explanation for the loss of tolerance in chronic intestinal disease.

Foxp3$^+$ T_{REG} cells in small intestinal inflammation

In **chapter 2** we showed that frequencies of mucosally-induced Foxp3$^+$ T_{REG} cells are unaltered in the circulation of CD patients. Using expression of CD62L^{neg}CD38$^+$ we compared the mucosally-induced T_{REG} cell populations in pediatric CD patients, adults with treated CD and adults with refractory CD. These data establish that there is no gross defect in mucosal Foxp3$^+$ T_{REG}-cell induction in CD.

In contrast to the inducible Foxp3$^+$ T_{REG} cells, increased frequencies of natural Foxp3$^+$ T_{REG} cell population were detected in subgroups of adult CD patients. Since we did not observe this in the early-onset pediatric CD patients, we hypothesize that

these systemic changes may be related to a particular phase of disease with prolonged exposure to non-specific chronic inflammatory mediators. This is supported by the observation that an increase of natural Foxp3$^+$ T_{REG} cells is present in adult patients with cancer[6, 7] and autoimmune diseases[8-10]. On the basis of murine tolerance models with ovalbumin it has been shown that natural T_{REG} cells are dispensable for protein specific oral tolerance.[11] Therefore we hypothesize that the increase in natural T_{REG} cells in CD is non-specific and likely not causative in disease pathogenesis.

Confirming earlier reports,[12-15] increased numbers of Foxp3$^+$ cells were found in histological intestinal samples of CD patients compared to non-CD patients, indicating that recruitment of Foxp3$^+$ T cells to the inflamed tissue is intact or even increased.

It is currently unknown, whether inducible T_{REG} cells are important for gliadin-specific tolerance in human or mice. From tetramer staining on human gluten-specific CD4$^+$ T cells in peripheral blood we know that the percentage of circulating gluten-specific mucosally-induced T_{REG} cells is extremely low and difficult to detect with current methods making it challenging to study whether there are differences in frequencies of induced T_{REG} cells in diseased and non-diseased individuals.[16] From murine oral tolerance models with ovalbumin, it has been established that inducible T_{REG} cells are crucial for oral tolerance to dietary antigen.[17] In the murine system inducible Foxp3$^+$ T_{REG} cells differentiate from naive T cells in the MLN under the control of CD103$^+$ DC that secrete TGF-β and retinoic acid.[18] This T_{REG}-cell induction can be overruled when retinoic acid acts in synergy with DC derived IL-15, a cytokine abundantly expressed in the lamina propria of CD lesions.[19 20] Using IL-15 transgenic mice, it was shown that the number of inducible Foxp3$^+$ cells was lower than wild type mice after oral ovalbumin feed. In addition, the inhibition of T_{REG}-cell differentiation was accompanied by the induction of a CD4$^+$ T_H1-cell response by inducing IL-12 and IL-23 production in MLN DC.[19] In a humanized HLA-DQ8 CD mouse model, it has been suggested that the cytokine IL-15 prevents the induction of Foxp3$^+$ T_{REG} cells.[21] In HLA-DQ8-IL-15 transgenic mice it has been shown that in the context of high IL-15 presence in the lamina propria, retinoic acid favored IFN-γ production in the lamina propria, increased the anti-gliadin antibody production and increased the numbers of intra-epithelial lymphocytes after gluten challenge. These data suggest that IL-15 alters the cytokine production of the T cells and may prevent Foxp3 induction.[19] However, it has not been directly shown in the HLA-DQ8 or in HLA-DQ8-IL-15 transgenic mice that the induction of T_{REG} cells is prevented after gluten feed. Therefore, it is unclear if IL-15 prevents loss of induced T_{REG} cells in CD and whether this is gluten-specific. Moreover, from the humanized gluten fed HLA-DQ2 mouse models for CD, namely HLA-DQ2-gliadin α2 epitope-dependent and the HLA-DQ2-gliadin γ1 epitope-dependent mouse model, it is known that tolerance to gliadin is not dependent on induction of Foxp3$^+$ T_{REG}-cell differentiation suggesting that multiple forms of regulation of gluten-specific T-cell responses could potentially exist.[22, 23]

Transgenic mice models have improved our understanding of the complex pathogenesis of CD. However, disadvantage of these models is the specific HLA-type and different T cell receptors for gliadin peptide used. An approach to make the transgenic models more powerful would be to increase the number of gliadin-specific T cell receptors with the most immunodominant peptides in both HLA-DQ2 and DQ8 transgenic mice. Evaluating oral tolerance in these different transgenic mice could provide novel insights into the regulation of gluten-specific immune responses and CD pathogenesis.

The CD phenotype does not resemble the classical phenotype of Foxp3 deficient IPEX patients. If diminished Foxp3$^+$ T_{REG}-cell function is important in the loss of tolerance to gluten, which remains to be proven, it appears to be uniquely restricted to the small intestine and dependent on a single antigen, i.e. gluten. Factors that could influence local Foxp3$^+$ T_{REG}-cell function are pro-inflammatory cytokines (e.g. IL-15[24], IL-21[25]), site-specific composition of antigens (food or microbiota), site-specific antigen presenting cells and resident tissue cells. Future studies are needed to understand the relative contribution of these factors in small intestine and colon.

Foxp3$^+$ T_{REG} cells in colon inflammation

With the exception of IPEX, there is limited evidence suggesting that intestinal inflammation of IBD patients is associated with decreased numbers of Foxp3$^+$ T_{REG} cells. Changes in peripheral blood Foxp3$^+$ T_{REG} cells and lamina propria Foxp3$^+$ cells have been reported in IBD patients.[26-29] Maul *et al.*[27] found that both ulcerative colitis (UC) and Crohn's disease (Crohn) patients had decreased numbers of T_{REG} cells in peripheral blood during active disease. In intestinal tissue of these actively diseased IBD patients the numbers of T_{REG} cells was not different compared to patients with other inflammatory disease. Most studies support these data as they describe an expansion of Foxp3$^+$ T_{REG} cells in the mucosa of IBD patients in both inflamed and non-inflamed tissue of the gut.[26, 28, 29] We showed in **chapter 3** that even in defective IL-10 regulation there is normal influx of Foxp3$^+$ T cells in inflamed colonic tissue. It is unknown if there is a numerical lack of circulating mucosally-induced Foxp3$^+$ T_{REG} cells in IBD patients. Therefore, it warrants re-investigation using our mucosal cell surface markers in IBD patients.

Despite the presence of T_{REG} cell at sites of inflammation, it can be questioned whether these T_{REG} cells are functionally suppressive. Several groups have isolated T_{REG} cells from patients with IBD and tested their suppressive function *in vitro*. All studies showed that T_{REG} cells of IBD patients, either isolated from the peripheral blood or the intestinal tissue, were equally suppressive compared to controls.[26, 27, 29-31] These results suggest that there may be a non-intrinsic defect of T_{REG} cell function in IBD. Local environmental factors like pro-inflammatory cytokines IL-15[19], IL-6[32], IL-21[33] or other innate cell types (e.g. DC[34], macrophages) may limit T_{REG}-cell function.

Tr1 cells

Besides Foxp3$^+$ T_{REG} cells, the intestine contains other CD4$^+$Foxp3^{neg} T cells that have regulatory capacity. The best-described CD4$^+$Foxp3^{neg} T cells are the peripherally-induced IL-10-secreting type 1 regulatory T (Tr1) cells. Tr1 cells were first described as IL-10- and IFN-γ-producing T cells that arose *in vitro* after culture with IL-10.[35] The term Tr1 cells is used for all IL-10-secreting T cells that secrete little IL-2 and have suppressive activity that is IL-10 dependent.[36] IL-27 and its target transcription factor c-Maf, the cytokine IL-21 and the co-stimulatory receptor ICOS coordinately act together to promote Tr1-cell differentiation.[37] Currently, no specific surface marker for Tr1 cells has been identified and these cells are less easily identified than Foxp3$^+$ T_{REG} cells.

Tr1 cells in celiac disease

Tr1 cells are important regulatory cells in tolerance to the food protein gluten (or gliadin) in transgenic mice that express HLA-DQ2 and have a gliadin-specific humanized T-cell receptor (DQ2.gliadinTCR mice). Feeding these DQ2.gliadinTCR mice deamidated gliadin induces no sign of CD pathology, but instead gliadin-specific tolerance was mounted in the spleen where IL-10- and IFN-γ- producing cells were formed. These gliadin-specific Foxp3^{neg} T cells secreted large amount of IL-10- and IFN-γ and resemble Tr1 cells, as they secreted little IL-2 and were suppressive in a delayed type hypersensitivity model.[23] In **chapter 6** we showed that innate cells, namely splenic macrophages, play a role in tolerance induction to gluten in these DQ2.gliadinTCR mice. As *in vivo* depletion of macrophages inhibited splenic Tr1-cell differentiation after gliadin protein feed. In contrast, after smaller gliadin-peptide feed Tr1-cell differentiation in the spleen was unaffected by macrophage depletion. *In vitro* cultured splenic macrophages rather than DC favored differentiation of gliadin-specific Tr1 cells by expressing IL-27. When compared to DC, macrophages have higher levels of lysosomal proteases and degrading capacity.[38] These results demonstrated that degradation of gliadin by macrophages is an important step in antigen presentation and subsequent Tr1-cell differentiation in the spleen after gluten protein feed in DQ2.gliadinTCR mice.

Gluten-specific IL-10-producing Tr1 cells can be isolated from intestinal biopsies from CD patients on a gluten-free diet and they have been suggested to inhibit gluten-specific inflammatory producing T-cell responses.[39] The findings from the DQ2.gliadinTCR mouse model together with the fact that Tr1 cells can be isolated from the mucosa of treated CD patients favor the hypothesis that IL-10-producing Tr1 cells are important in the maintenance of tolerance to gluten.

Whether there are decreased numbers of Tr1 cells in active CD is currently unknown. In **chapter 4** we showed that in restimulated lamina propria T cells of CD patients, IL-10-producing CD4$^+$ T cells were virtually non-detectable. It would be interesting to know whether this is due to do defective induction of IL-10-producing

T cells and whether non-CD individuals or treated CD patients have higher numbers of IL-10-producing cells compared to active CD patients in the lamina propria. In addition to this, it would be interesting to know whether active CD patients have fewer numbers of mucosally-induced ($CD62L^{low}CD38^{+}$) IL-10-producing T cells in peripheral blood compared to non-CD subjects. To address these questions one would need to enumerate these Tr1 cells. This is technically difficult as there is no exclusive marker and the cells are much more anergic than effector T cells.

In conclusion, impaired tolerance in CD patients may be related to dysregulation in the Tr1-cell response and the balance between the pro-inflammatory cytokine IFN-γ and the immunosuppressive IL-10 is lost. If CD is the result of a dysregulated Tr1 cell-response it is again the question why there is only a small intestinal phenotype associated with this dysregulation. In mice, regional differences in phenotype of T_{REG} cells have been reported. For example, $Foxp3^{neg}IL\text{-}10^{+}$ Tr1 cells are the predominant source of IL-10 in the small intestine, whereas IL-10-producing $Foxp3^{+}$ T_{REG} cell are more abundantly present in the colon (*Figure 1*).[40] More knowledge regarding regional differences between small intestine and colon may have consequences for understanding loss of tolerance in CD and IBD.

T-cell regulation by cytokines

Loss of tolerance can be explained by defective T_{REG}-cell function or increased resistance of effector T cells to T_{REG} cells by the local inflammatory cytokine milieu.

Alterations in cytokine networks are important in autoimmune tissue pathology and can result in intestinal inflammation. Cytokines are small soluble proteins that can alter the behavior or properties of the producing cell or other cells in the intestinal environment.

The healthy intestinal mucosa contains an array of cytokines secreted by immune cells- such as neutrophils, macrophages, dendritic cells (DC), lymphocytes and mast cells. In this thesis we focused on the role of the suppressive cytokine IL-10 and the inflammatory cytokine IL-21 and their role in intestinal inflammation.

Interleukin 10

IL-10 is a potent anti-inflammatory cytokine that plays a key role in limiting inflammatory immune responses against pathogens. In the intestine IL-10 is produced by T cells, B cells, monocytes, DC, macrophages as well as certain non-hematopoietic cells.[41] IL-10 signals via a heterodimeric receptor complex consists of two alpha (IL-10RA) and two beta (IL-10RB) chains.[42] Mice deficient for IL-10 develop spontaneous colitis upon colonization with bacteria.[43] Also in humans, patients with mutations in the IL-10 signaling pathway develop severe intestinal inflammation.[44, 45] Furthermore genome-wide association studies have associated polymorphisms in the

Figure 1 ***Schematic overview of cellular interactions of T_{REG} cells with other immune cells during intestinal inflammation.***

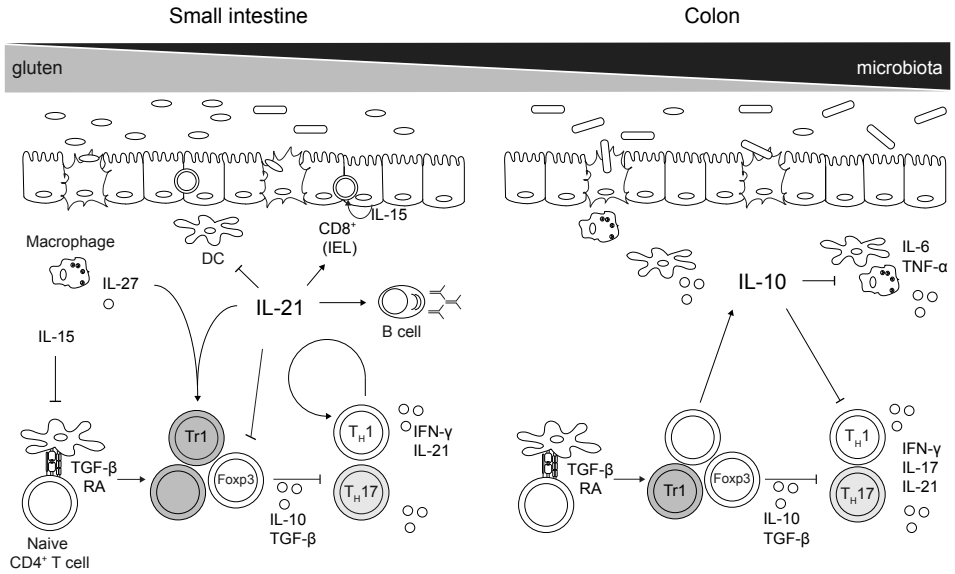
Both IL-10-secreting Tr1 cells and Foxp3$^+$ T_{REG} cells regulate intestinal homeostasis. Tr1 cells are the prominent source of IL-10 in the small intestine whereas IL-10-producing Foxp3$^+$ T_{REG} cells are more abundantly present in the colon. TGF-β and RA induce Foxp3$^+$ T_{REG}-cell differentiation, whereas IL-27 induces Tr1-cell differentiation. T_{REG} cells produce IL-10 and TGF-β that suppress effector T_H cells (T_H1, T_H17) in intestinal inflammation. Effector CD4$^+$ T cells produce the pro-inflammatory cytokines IFN-γ and IL-21 in small intestinal inflammation in CD and IFN-γ, IL-17 and IL-21 in colonic inflammation in IBD.

(left) *The dual role of IL-21 in small intestinal CD inflammation. IL-21 has pro-inflammatory actions. IL-21 induces T_H1 cells to produce IFN-γ, stimulates B-cell survival and function, activates CD8$^+$ T cells and inhibits the suppressive function of Foxp3$^+$$T_{REG}$ cells. On the other hand, IL-21 has anti-inflammatory actions. IL-21 induces Tr1-cell differentiation, induces IL-10 production by CD4$^+$ T cells and inhibits DC maturation.*

(right) *The role of IL-10 in colonic inflammation. Besides T_{REG}-cell derived IL-10, myeloid sources of IL-10 are important for suppressive function. IL-10 controls both T_H1 and T_H17 cells and DC-derived pro-inflammatory cytokine production IL-6 and TNF-α production.*

The question whether IL-10 and IL-21 are differentially regulated in the small intestine versus the colon is still unanswered and requires more investigation.

TGF-β, transforming growth factor beta; RA, retinoic acid; IL, interleukin; Tr1, type 1 regulatory T cell; T_{REG}, regulatory T; T_H, T helper; IFN-γ, interferon gamma; DC, dendritic cell; IEL, intra-epithelial lymphocyte; TNF-α, tumor necrosis factor alpha; CD, celiac disease; IBD, inflammatory bowel disease



IL-10 pathway with IBD.[46-48] Although the genetic defect, the clinical presentation and the treatment of early-onset colitis due to defective IL-10 signaling are known, the precise mechanisms by which IL-10 regulates immune responses and intestinal homeostasis are lacking. In **chapter 3** we investigated the functional consequences of defective IL-10 signaling on innate and adaptive immune responses by using the immune cells and intestinal biopsies of an IL-10RA-deficient patient with early-onset colitis. Prior to detection of the *IL-10RA* mutation clinical remission of intestinal disease was achieved with thalidomide, intravenous immunoglobulin (IVIG) and colchicine.

Consequence of defective IL-10 signaling on innate cells

In **chapter 3**, our data shows that the numbers of circulating $CD11c^{+}HLA\text{-}DR^{+}$ DC of an IL-10RA-deficient patient was within the normal range of pediatric IBD patients and pediatric controls. Functionally, mature IL-10RA-deficient monocyte-derived DC displayed hyper-reactive cytokines secretion after bacterial ligand stimulation, without affecting the co-stimulatory molecule expression. This differential regulation of cytokines and co-stimulatory molecules by endogenous IL-10 is remarkable, as previous *in vitro* neutralization studies proposed a role for IL-10 in both autocrine co-stimulation and cytokine release.[49, 50] Nevertheless, our data are consistent with observations in $CD11c^{+}$ DC-specific IL10RA-deficient mice, in which only DC cytokine release, and not co-stimulatory molecule expression, was affected after bacterial ligand stimulation.[51] Disparate regulation of co-stimulatory molecules and cytokines by endogenous IL-10 may possibly be explained by the use of different inhibitory signaling cascades, as IL-10-mediated regulation involves direct and indirect pathways which may act sequentially or are activated depending on the phase of cellular maturation.

Similar to IL-10RA-deficient DC, cultured IL-10RB-deficient macrophages secreted more proinflammatory cytokines after restimulation with LPS compared to controls.[52] In contrast, these deficient macrophages showed elevated levels of co-stimulatory molecules.

From recent mouse studies, we know that macrophage-derived IL-10 is dispensable for gut homeostasis as mice lacking IL-10 expression in macrophages do not develop spontaneous colitis and severity of induced colitis was similar as wild type mice.[53] These data indicate that other cells can overcome the deficiency of macrophage-derived IL-10 and provide IL-10 in the intestinal immune cells. In contrast, loss of IL-10RA expression in macrophages impaired the homeostatic function of these macrophages and these mice develop colitis with accumulation of T cells and neutrophils in the lamina propria and increased serum levels of IL-6 and IL-17.[53] These data illustrate that IL-10 signaling in macrophages is pivotal for intestinal homeostasis. In addition, in a mouse transfer-model where wild-type T cells were transferred in an IL-10RB-deficient Rag2 knockout, a mice lacking the ability to make B and T cells, exaggerated

T-cell responses develop. Following transfer of wild type $CD4^+$ T cells into IL-10RB-deficient mice, T_H1 cytokines IFN-γ, IL-6, IL-12 and IL-1β were elevated in colon with increased numbers of $CD4^+IFN\text{-}\gamma^+$ T cells in the lamina propria compared to mice without the deficiency.[52] In addition, loss of IL-10RB signaling impaired the generation and function of tolerogenic macrophages and their ability to secrete IL-10.

These data support our observation; IL-10 is a critical homeostatic conditioning agent in the colon to prevent hyperactivation of colon-resident DC and macrophages in the gut. As defective IL-10R signaling on innate cells leads to enhanced cytokine production this results in defective immune regulation contributing to severe intestinal inflammation.

Consequence of defective IL-10 signaling on T cells

During remission, the composition of peripheral blood T cell subsets did not differ between the IL10RA-deficient patient and pediatric IBD patients and controls (**chapter 3**). Moreover, $Foxp3^+$ cells were detected in the inflamed intestinal tissue of the patient, indicating normal $Foxp3^+$ T_{REG}-cell recruitment to the intestinal lesions.

Although IL-10 deficiency in mice does not impair the ability of T_{REG} cells to prevent colitis in a T cell-transfer model, IL-10 deficient T_{REG} cells are less able to abrogate established colitis or prevent inflammation induced by triggering microorganisms *Helicobacter hepaticus*.[54, 55] These results suggest that regulation of intestinal responses does not absolutely require IL-10 production by T_{REG} cells, but IL-10 becomes mandatory in the presence of a strong microbial stimulus or established intestinal inflammation. Moreover the colitis in IL-10 deficient T_{REG} mice is less severe than in complete $IL\text{-}10^{-/-}$ knockout mice, indicating that IL-10 produced by other cells contributes to intestinal tolerance.

Investigating the T effector cells of our IL-10RA deficient patient (**chapter 3**), showed comparable amounts of IFN-γ and IL-17 of CD3-activated PBMC with healthy controls. These data indicate that during remission, IL10RA-deficient T cells were not hyper-activated by T-cell receptor ligation. Exogenous IL-10 regulated both IFN-γ and IL-17 production in healthy activated T cells *in vitro*. In agreement, the inhibitory effects of exogenous IL-10 on IFN-γ and IL-17 production were clearly defective in the IL10RA-deficient patient PBMC. Moreover, the patients' intestinal lesions at diagnosis contained $CD3^+$ T cells with co-localizations of IL-21+, IL-17A+ and T-bet+ cells. In the absence of functional IL-10RA, inflammatory lesions contain both infiltrating T_H1–like and T_H17-like cells demonstrating the importance of IL-10 signaling in limiting mucosal inflammatory T_H1 and T_H17 cell responses. Whether IL-10 regulates T_H1 and T_H17 cells directly or indirectly via antigen presenting cells or by favoring T_{REG} cell-mediated suppression remains unknown.[56, 57] In mice, T_H17 cells, but not T_H1 cells, express IL-10R[58]; therefore, treatment of IL-10R deficiency may require both DC inhibition and T-cell suppression to fully inhibit T cell-driven inflammation.

The role of IL-10 in intestinal inflammation

Based on the data above we can conclude that IL-10 signaling is crucial for intestinal homeostasis. The described IL-10RA-deficient patient in **chapter 3**, presented with symptoms of severe colitis and perianal fistulizing disease. To date, of the described patients only one IL-10RA-deficient patient showed small intestinal involvement.[59] It could be, as it was the case in our patient, that at time of diagnosis it was not standard to perform an esophagogastroduodenoscopy if there were no specific symptoms. IL-10-deficient mice developed chronic enterocolitis upon colonization with bacteria.[43] This enterocolitis involved the entire intestine, the duodenum, proximal jejunum, and proximal colon being most severely affected. The jejunum showed villous atrophy, crypt hyperplasia with infiltrative lymphocytes in the lamina propria. The colon showed atrophy of the mucosa with reduced numbers of goblet cells. The question if IL-10 signaling is differentially regulated in the human small intestine versus the colon is still unanswered and requires more investigation.

This patient with a primary immunodeficiency represents a unique model for studying the mechanisms by which the immune system attempts to prevent inflammatory responses by IL-10. Our study identifies distinctive roles for IL-10 in regulating DC and T-cell responses in IBD and contributes to a better understanding of IBD pathophysiology, which may lead to novel therapeutic targets (*Figure 1*).

Interleukin 21

IL-21 is expressed by $CD4^+$ T cells[60], folliculair T helper cells[61], natural killer T cells[62] and neutrophils[63]. Of the $CD4^+$ T helper (T_H) subsets, T_H1, T_H2 and T_H17 cells are capable to produce IL-21 *in vitro*.[60]

IL-21 exerts its biological effect via the IL-21 receptor (IL-21R) and the common γ -chain.[60] The IL-21R is expressed on hematopoietic immune cells such as T, B, NK, macrophages and dendritic cells (DC).[33] In addition the IL-21R is also expressed on non-hematopoietic cells, i.e. epithelial cells.[64] In summary, IL-21 is considered as a pleiotropic cytokine that has effects on both innate and adaptive immunity cells and is mainly expressed by activated $CD4^+$ T cells.

Effect of loss of IL-21

Mice deficient for IL-21 ($IL\text{-}21^{-/-}$) or the IL-21R ($IL\text{-}21R^{-/-}$) showed normal lymphoid development including normal B cell numbers, development of $CD4^+$ T cell subsets and no gross organ defects. However, upon immunization with T cell dependent antigen, $IL\text{-}21R^{-/-}$ and $IL\text{-}21^{-/-}$ mice had increased levels of IgE with lower levels IgG_1 than wild type animals.[65, 66] In addition, $IL\text{-}21R^{-/-}$ mice showed defective $CD4^+$ T-cell differentiation with increased numbers of $Foxp3^+$ cells and decreased numbers of T_H17 cells upon stimulation of naive $CD4^+$ T cells *in vitro*.[67]

Recently, loss-of-function mutations in the *IL-21* and *IL-21R* genes have been

identified in human pediatric immunodeficiency patients. Patients with mutations in the *IL-21R* gene have a severe and life-threatening phenotype and develop cryptosporidial infections associated with chronic cholangitis and liver failure.[68] Patients with a mutation in the *IL-21* gene presented with severe early-onset colitis, which was fatal in 2 out of 3 patients.[69] In analogy to the observations in IL-21 and IL-21 deficient mice, the underlying defect results from impaired B-cell proliferation and immunoglobulin class switching in humans.

Effect of an excess of IL-21

IL-21 transgenic mice (IL-21-Tg) have a shortened life span and are smaller in size but have no gross organ abnormalities or distinctive intestinal malformation.[65, 70] Death occurs due to defective hematopoiesis leading to an accumulation of myeloid cells and reduced erythropoiesis in bone marrow. Massive accumulation of $CD8^+$ memory T cells and a reduction of naive T cells was observed in spleen and lymph nodes of these mice.[70]

IL-21 plays a critical role in development of systemic lupus erythematosus (SLE) in BXSB-*Yaa* mice. These mice have elevated expression of IL-21 with multiple abnormalities, including hypergammaglobulinemia, autoantibody production, reduced frequencies of marginal zone B cells and monocytosis, renal disease leading to premature morbidity. Induction of IL-21R deficiency through genetic manipulation in these mice resulted in absence of the disease.[71] A different SLE-murine model with high IL-21 levels is the Sanroque mouse.[72] Sanroque mice have a mutation in *Roquin* resulting in less degradation of ICOS mRNA. Consequently, increased ICOS expression on T cells results in excessive numbers of T folliculair helper cells, germinal centers, excessive production of IL-21 and high titers of autoantibodies. The Sanroque mice display an SLE-like phenotype, but showed no intestinal pathology (own observations, not published). Both these murine models suggest that high or deficient IL-21 levels are associated with autoimmunity but do not result in intestinal pathology by itself. The effects IL-21 over expression in humans are not known, there are incidental reports that elevated serum IL-21 can be found in patients with psoriasis[73] and SLE[74, 75]. Whether this is causally related or a consequence of the disease is unknown.

The role of IL-21 in small intestinal inflammation

$CD4^+$ T cells with specificity for dietary gluten drive the mucosal inflammation in CD via the secretion of proinflammatory cytokines. In particular IFN-γ is thought to play a role in intestinal epithelial damage.[76, 77] IL-21 has received increasing attention as a potential player in CD pathophysiology, since genome-wide association studies of CD identified risk variants in the *IL-2/IL-21* gene region.[78] We showed in **chapter 4** that compared to non-diseased controls, biopsies of CD patients showed increased numbers of IL-21 positive cells in the lamina propria. This was already observed in mildly inflamed tissue of pediatric CD patients. *Ex vivo*, isolated lamina propria lymphocytes

and intra-epithelial lymphocytes from CD patients were shown to produce IL-21 (**chapter 4** and ref. [79]). In addition, gluten-reactive $CD4^+$ T cells derived from biopsies of CD patients have been shown to produce IL-21.[80]

Given the role of T_H17 cells in chronic tissue-specific inflammation it has been investigated whether these cells are the source of IL-21 in CD. Polyclonal T cell lines derived from CD biopsies contain IL-17-secreting cells; however, the gluten-reactive T cells that produced IL-21 and IFN-γ were IL-17 negative.[80] We showed in **chapter 4** that IL-21 secretion occurs independently of IL-17 release in early onset CD. This was confirmed by polyclonal restimulation of freshly isolated lamina propria cells from newly diagnosed pediatric patients with phorbol ester and calcium ionophore, and demonstrated that a large majority of lamina propria IL-21-secreting $CD4^+$ T cells produced IFN-γ; however, few IL-17 producing cells were detected.

Within CD lesions, we could detect, besides $CD4^+$ T cells, a clear population of neutrophils as a source of IL-21. Whether these IL-21-secreting neutrophils in the celiac lesion have a specific function in CD-specific B cell antibody production will need further investigation.

The effect of high IL-21 production on intestinal inflammation *in vivo* is currently unknown. It has been demonstrated that blockage of IL-21 in *ex vivo* organ cultures of biopsies from CD patients resulted in inhibition of the IFN-γ production and the T_H1 transcription factor T-bet.[81] This suggests that gluten-specific IFN-γ- IL-21-producing T cells create a positive autocrine feedback loop and enhance the production of IFN-γ. If IL-21 produced by lamina propria T cells has a role in the massive $CD8^+$ intra-epithelial lymphocyte accumulation or the autoantibody production seen in CD is still unknown.

The current literature on IL-21 focused mainly on the pro-inflammatory and positive autocrine feedback. Too little is known of negative feedback and anti-inflammatory self-regulation of IL-21.

The role of IL-21 in colonic inflammation

The role of IL-21 in colonic inflammation has been investigated IBD patients and murine models. Genetic variation within the *IL-21* gene region is associated with UC.[82,83] Biopsies of UC and Crohn patients showed enhanced levels of IL-21 compared to controls. Moreover, increased numbers of IL-21 expressing lamina propria T cells have been found in UC and Crohn patients in comparison to controls. These $CD4^+$ T cells in the lamina propria from IBD patients express IL-21 and IFN-γ. In activated T cell cultures derived from the lamina propria of IBD patients, blocking the IL-21R resulted in down regulation of IFN-γ secretion.[84] Within patient comparison of circulating numbers of $IL\text{-}21^+$-producing $CD4^+$ T cells showed an increase during disease remission compared to active disease[85], suggesting that IL-21 has no direct inflammatory effect in the intestine.

$IL\text{-}21^{-/-}$ mice showed milder inflammation of the colon after dextran sulfate (DSS) or 2,4,6-trinitrobenzene sulfonic acid (TNBS) induced colitis. Blockade of

the IL-21R with an IL-21R/Fc fusion protein was shown to attenuate DSS-induced colitis.[86] These data all suggest a pro-inflammatory effect of IL-21 in the intestine. However, there is a possible anti-inflammatory effects of IL-21 on innate cells should not be overlooked. As such, murine T-cell transfer colitis revealed greater numbers of colonic DC in RAG2$^{-/-}$IL-21R$^{-/-}$ than in RAG$^{-/-}$ mice associating with increased colitis pathology.[87] This indicates that IL-21 production may be a way to eliminate DC leading to maintenance of immune tolerance.

IL-21 and IL-10

The effect of IL-21 on immune suppression remains an area of controversy. Both induction of immune suppression by IL-10 as well as limiting T_{REG} cell-mediated immune suppression have been described (*Figure 1*).

IL-21 mediates suppressive effects via its induction of IL-10. IL-21 increases IL-10 expression in TCR-stimulated naive CD4$^+$ T cells and induces IL-10 in pre-activated T cells.[88] In addition, IL-21R$^{-/-}$ mice showed decreased IL-10 production and in the IL-21 transgenic mice increased levels of IL-10 are present.[89] Moreover, sufficient IL-21 signaling is crucial for the generation and amplification of Tr1 cells.[37] Gluten-specific Tr1 cell clones can be isolated from the intestine of treated CD patients, and they are thought to play a role in the suppression of the gluten-specific T-cell responses.[39] Based on these findings one could expect low IL-21 production in small intestinal inflammation. By contrast, in **chapter 4** we showed enhanced numbers of IL-21 positive cells in lesional tissue of CD or IBD patients. In addition, isolated duodenal lamina propria T cells from CD patients showed increased IL-21 production and almost no IL-10 production. One could suggest that in CD IL-21 fails to induce IL-10 and thereby fails to down-regulate the immune response. In **chapter 3** we showed that IL-21 is highly expressed in absence of IL-10. High numbers of IL-21$^+$ cells were present in colonic inflammation of an IL-10RA-deficient patient with early onset IBD. It is unclear whether the net effect of IL-21 in presence of deficient IL-10 signaling is pro- or anti- inflammatory. Impaired IL-10 signaling may result in absence of down regulation of IL-21 and an increased pro-inflammatory response. This observed IL-21 up-regulation could be a cause or consequence of intestinal inflammation. In contrast to induction of IL-10-producing Tr1 cells, and thereby mediating an anti-inflammatory effect, IL-21 can inhibit the suppressive function of CD4$^+$CD25$^+$ T_{REG} cells on effector CD4$^+$ T cells in humans.[25] Moreover, IL-21R$^{-/-}$ mice showed increased numbers of CD4$^+$ Foxp3$^+$ T cells upon stimulation of naive CD4$^+$ T cells *in vitro*.[67] These results implicate a pro-inflammatory effect of IL-21, either by inhibiting the development or the function of T_{REG} cells. Since there are no data available on direct effects of IL-21 on Foxp3$^+$ T_{REG} cells, it remains unknown if IL-21 directly inhibits T_{REG}-cell function or acts indirectly via the effects on T effector cells. These data indicate that changes in the IL-21/IL-10 interplay may have an important role in the pathophysiology of intestinal inflammation as seen in IBD and CD.

T-cell regulation by myofibroblasts

At the resolution of the primary immune response, a small number of memory T cells are retained in the lamina propria to form a pool of antigen-specific memory cells that allows an accelerated response upon antigenic rechallenge. Little is known how these memory T cells are preserved in the intestinal lamina propria. We hypothesize that intestinal stromal cells contribute to chronic inflammation in CD by sustaining memory T-cell survival. Intestinal stromal cells, including myofibroblasts, have long been considered as passive structural cells and research has focused on their role in tumorgenesis, fibrosis and wound healing rather than the immunological function. Growing evidence suggest that non-hematopoietic stromal cells have multiple roles in the intestinal immune system.[90] In **chapter 5** we provide additional support for the role of duodenal myofibroblasts as immune cells. We assessed how myofibroblasts are activated, determined their chemokine and their cytokine profile and measured their capacity to modulate T-cell activation and survival. Myofibroblasts sensed innate signals via TLR2 and TLR3 leading to production of large amounts of chemokines and release of inflammatory cytokines like IL-6 and IL-1β. We reported that duodenal myofibroblasts expressed chemokines to recruit immune cells and reduced T-cell apoptosis *in vitro* and may play a role in enhancing survival of memory gliadin-specific inflammatory T cells in the lamina propria of CD patients. The mechanism behind this observation has yet to be identified. Cytokines such as IL-1β and IL-6 released by duodenal myofibroblasts may exert this anti-apoptotic activity in T cells within the CD lesion.

IL-1β is a proinflammatory cytokine with pleiotropic effects and is primarily produced by innate immune cells.[91] Increased levels of IL-1β production by lamina propria mononuclear cells have been reported in inflamed colon of IBD patients.[92-97] Moreover, in a murine T-cell transfer model for colonic inflammation, IL-1β stimulation of $CD4^+$ T cells was required for the induction of intestinal inflammation in mice. As such, transfer of IL-1R-deficient T cells showed less severe colitis with reduced influx $CD4^+$ T cells in the inflamed tissue compared to wild type T cells.[98] These data suggest a role of IL-1β in promoting intestinal inflammation. Stimulation with IL-1β activates innate immune cells and promotes T-cell activation and survival.[99] Next to promoting survival we hypothesize that local IL-1β production by myofibroblasts could influence the cytokine profile of the effector memory T cells in CD. In **chapter 5** we showed that IL-1β selectively enhanced IFN-γ and IL-21 production by activated PBMC while IL-10 production was not increased. This is in agreement with previous data showing that IL-1β induces a pro-inflammatory cytokine profile and inhibits IL-10 production.[100] In pathogen-induced human T_H17 cells, IL-1β primed IFN-γ production and inhibits IL-10 production. Moreover neutralization of IL-1β increased IL-10 production. IL-1β could be seen as a pro-inflammatory regulator of T_H17 cells and a strong inhibitor of IL-10 production of memory T cells.[100] Increased serum

levels of IL-1β have been described in CD patients compared to controls[101], however it is not clear whether IL-1β is produced as extensively in the lamina propria of CD patients as in IBD patients.[97] Nevertheless, abundant numbers of IFN-γ$^+$IL-21$^+$ T cells can be detected in lamina propria of CD patients suggesting a possible encounter with the inflammatory cytokine IL-1β. (**chapter 4** and ref.[80]) It would be interesting to investigate whether IL-1β production by myofibroblasts, macrophages or DC block the IL-10 production of memory gluten-specific T cells and increase their IFN-γ production leading to intestinal inflammation.

IL-6 has been reported to be elevated in myofibroblasts of patients with IBD.[102] Similar as IL-1β, IL-6 has the capacity to enhance T cell survival *in vitro* and *in vivo*.[103]. Besides stimulating T-cell survival IL-6 may also modulate the cytokine production of T cells as it is one of the co-factors required for T_H17-cell differentiation.[104]

It has previously been shown that myofibroblasts from CD patients showed different morphology compared to non-CD patients.[105] We hypothesized that human intestinal myofibroblasts are functionally different in health and disease. However the number of patients in **chapter 5** was too low to draw conclusions on differences between myofibroblasts from CD patients and non-CD individuals.

In conclusion, understanding the immune function of duodenal myofibroblasts is far from complete. However, our data clearly show that duodenal myofibroblasts can be stimulated by TLR2 and TLR3 ligands, which leads to potent chemokine release, inflammatory cytokine release (IL-6 and IL-1β) and enhanced T-cell survival. As such a possible role for duodenal myofibroblasts in enhancing inflammation in CD needs to be considered.

T-cell regulation by macrophages

As mentioned above, we showed in **chapter 6** that macrophages play a role in induction of tolerance of gluten by induction of Tr1-cell differentiation. This raises the question whether loss of tolerance to gluten in CD may be a result of defective macrophage induced regulation. The role of intestinal macrophages in T_{REG}-cell induction remains to be elucidated. Macrophages and DC are critical for the establishment and maintenance of gut homeostasis.[106, 107] However, heterogeneity in phenotype, origin and function has led to confusion over the classification between macrophages and DC, especially in mucosal tissue.[108, 109] Much attention has been paid to intestinal DC but far less to intestinal macrophages. For example, there is an important role for intestinal CD103$^+$ DC in oral tolerance by driving the differentiation of gut-homing Foxp3$^+$ T_{REG} cells.[110] Although lamina propria macrophages are non-migratory and therefore unlikely to initial priming of naive T cells, they do express MHCII molecules and can facilitate the secondary expansion of Foxp3$^+$ T_{REG} cells in the lamina propria and maintain their suppressive capacity in the lamina propria.[111, 112] With CX_3CR1-deficient mice,

lacking gut-resident macrophages, it has been shown that oral tolerance to protein is CX_3CR1 and IL-10 dependent due to impaired survival of antigen-specific T_{REG} cells.[111] Moreover, it has been reported that IL-10 production by intestinal $CD11b^+$ innate immune cells, likely macrophages, is required for T_{REG} cell maintenance in the lamina propria. However, it was recently shown that CX_3CR1 macrophage-derived IL-10 was dispensable for gut homeostasis and maintenance of colonic T_{REG} cells, as mice deficient for IL-10 specific in CX_3CR1 macrophages have no difference in numbers of the intestinal T_{REG} cells compared with wild type controls.[53] Potentially due to more sources of IL10-producing cells. In contrast loss of IL-10RA signaling in CX_3CR1 macrophages resulted in severe colitis.

Mice with deficient IL-10RB signaling on innate cells ($IL\text{-}10RB^{-/-}Rag2^{-/-}$ mice) showed colitis upon transfer of wild type naive $CD4^+$ T cells with increased numbers of IFN-γ-producing T cells and reduced numbers of induced $Foxp3^+$ T_{REG} cells in the lamina propria and MLN. T_{REG}-cell maintenance was assesses by transferring $CD4^+Foxp3^+$ T cells in the $Rag1^{-/-}IL10RB^{-/-}$ mice and showed comparable levels of T_{REG} cells in the lamina propria and MLN between IL10 R-deficient mice and $Rag1^{-/-}$ mice.[52] Suggesting that IL-10 signaling in macrophages is not required to maintain T_{REG} cells in the lamina propria. In addition to the mouse data, in 7 patients with severe early onset IBD harboring causal mutations in the *IL-10RA* or *IL-10RB* gene there is an aberrant generation of anti-inflammatory macrophages with diminished IL-10 expression and decreased generation of inducible $Foxp3^+$ T_{REG} cells *in vitro* culture with IL-10RB-deficient macrophages.[52]

Since mucosal macrophages are resident in the lamina propria and do not migrate to the MLN, it remains to be proven if macrophages play a major role in initiating adaptive immune responses by priming naive $CD4^+$ T cells.[113,114] However, it is possible that macrophages may act locally in the lamina propria, skewing or maintaining T cells that have been primed previously in the MLN.

Concluding remarks

As discussed above, the immunological processes that maintain tolerance in the intestine are complex and incompletely understood. We focused on the identification of T-cell responses that play a dominant role in either induction or regulation of chronic intestinal inflammation. Knowledge on normal homeostasis and specific T-cell responses in the inflamed intestine can give additional insights in pathophysiology. Our findings promote knowledge on intestinal immune regulation, which is urgently required to advance our ability to classify the varying clinical pathologies and understand variation in treatment response of CD and IBD patients. This may be relevant for initiating treatment regimens based on the underlying pathophysiology (IBD) or late effects of the disease (e.g. refractory CD, intestinal T-cell lymphoma after refractory CD, malignancy in IBD).

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Summary

As described in **chapter 1**, the intestinal immune system protects the mucosal surfaces from pathogenic microorganisms and on the other hand maintains tolerance towards dietary antigens and non-pathogenic microorganisms. The immune system continuously tailors these inflammatory and tolerogenic responses to maximize host defense without unnecessary collateral damage, a balance denoted as intestinal homeostasis. $CD4^+$ T cells play a central role in the intestinal immune system. The balance between inflammatory $CD4^+$ effector T cells and the function of T_{REG} cells in the intestinal mucosa is crucial for homeostasis as uncontrolled T-cell responses can lead to chronic inflammatory intestinal disorders like celiac disease (CD) and inflammatory bowel disease (IBD). CD is an uncontrolled inflammatory $CD4^+$ T-cell response to the food protein gluten leading to chronic inflammation of the small intestine. Uncontrolled inflammatory $CD4^+$ T-cell responses to commensal non-pathogenic bacteria are associated with IBD.

Changes in numbers of T_{REG} cells could be indicative of defects in mucosal homeostasis in CD. In **chapter 2** we investigated whether numeric deficiency of mucosally-induced ($CD62L^{neg}CD38^+Foxp3^+$) or natural ($CD62L^{neg}CD38^+Foxp3^+$) T_{REG} cells could be detected in peripheral blood of CD patients. In particular, we compared pediatric CD, aiming to select for disease at onset, with adult CD. No differences between naturally occurring and mucosally-induced $Foxp3^+$ T_{REG} cells were seen between pediatric CD patients and controls. Adult refractory and treated CD patients showed increased percentages of naturally-occurring circulating $CD62L^+Foxp3^+$ T cells, but normal mucosal-induced $CD62L^{neg}CD38^+$ T cell frequencies. In contrast, duodenal biopsies did reveal enhanced numbers of $Foxp3^+$ cells in both pediatric and adult CD patients. This implies that CD patients have a normal frequency of circulating mucosal-induced T_{REG} cells and that changes in naturally occurring $Foxp3^+$ T_{REG} cells may be associated with a particular stage of inflammation rather than the initiation of disease.

Interleukin-10 (IL-10) is crucial for maintaining intestinal homeostasis, as IL-10 receptor (IL-10R) deficiency causes severe infantile-onset IBD. Currently, it is unclear how immune responses are affected by IL-10R deficiency. In **chapter 3** we characterized the immunological consequences of IL-10R deficiency in a now 11-year-old girl who presented with infantile-onset IBD and was diagnosed with an *IL10RA* mutation at 9 years of age. In contrast to recently reported cases disease remission could be achieved without hematopoietic stem cell transplantation, allowing in-depth analysis of the mechanism by which IL-10 controls immune responses and maintains intestinal immune homeostasis. Clinical remission was achieved while receiving thalidomide and intravenous immunoglobulins.

The IL10RA-deficient immune system developed normally with respect to numbers and phenotype of circulating cells. Despite normal co-stimulatory molecule expression, LPS-stimulated monocyte-derived dendritic cells (DC) released higher levels of TNF-α and IL-6 *in vitro*. IL-10 was required to control DC cytokine production and anti-CD3-driven IFN-γ and IL-17 release by PBMC. This agreed with high numbers of IL-17$^+$ and T-bet$^+$ cells in intestinal biopsies at disease onset. *In vitro*, immunoglobulins suppressed anti-CD3-driven IL-17 and IFN-γ release, while thalidomide inhibited LPS-mediated TNF-α production by PBMC.

In summary, the immune system can develop normally in absence of IL-10 signaling but shows aberrant inflammatory cytokine release by DC and uncontrolled IFN-γ and IL-17 responses.

In **chapter 4** we identified the contribution of two pro-inflammatory cytokines interleukin (IL-) 21 and IL-17A, to the onset of CD and the severity of lesion formation in a cohort of pediatric CD patients with different histopathological scores. Immunohistochemical analysis showed high numbers of IL-21-producing cells in small intestinal biopsies from pediatric CD patients while no increased numbers of IL-17 secreting cells were found. These increased IL-21-producing cells were already observed in Marsh 1-2 lesions. In addition, freshly isolated lamina propria cells revealed a large population of IL-21-secreting CD4$^+$ T cells that did not secrete IL-17A but mostly secreted IFN-γ. Biopsies from adult CD patients also contained high numbers of IL-21 positive cells. However, a subgroup of adult patient biopsies with Marsh 3C lesions contained concomitantly increased numbers of IL-21- and IL-17- secreting cells. As damage in the duodenal tissue leads to increased contact with microbe-associated molecular patterns, we hypothesized that microbial sensing by Toll-like receptors (TLR) may modulate the T-cell derived IL-17 and IL-21 secretion. Co-stimulation with the TLR3 ligand during polyclonal T-cell activation significantly increased IL-21 secretion, while TLR2 ligand selectively enhanced IL-17A release. In conclusion, our data demonstrate an IL-17A-independent increase in IL-21 production by CD4$^+$ T cells is characteristic for pediatric CD and suggest that selective encounter of TLR ligands in lesional tissue may modify the quantity of T-cell derived cytokine release.

In **chapter 5** we studied the phenotype and immune function of duodenal myofibroblasts. We hypothesized that intestinal myofibroblasts may contribute to the chronic inflammation in CD by enhancing memory T-cell viability in the lamina propria. We isolated duodenal myofibroblasts of CD and non-CD patients, as confirmed by CD90 and α-smooth muscle actin expression. Duodenal myofibroblasts expressed sensing, cell adhesion and regulatory molecules. All duodenal myofibroblasts tested had the capacity to sense innate signals via TLR2 and TLR3 leading to production of large amounts of chemokines and release of the inflammatory cytokines IL-6 and IL-1β. Predominantly TLR3 activation enhanced chemokine release by myofibroblasts. Besides expressing chemokines to attract cells, we show that duodenal myofibroblasts reduced T-cell apoptosis *in vitro*. Such anti-apoptotic activity may play a role in the long-term survival of memory gliadin-specific inflammatory T cells in the lamina propria of CD patients. The mechanism behind this observation has yet to be identified. Cytokines such as IL-1β and IL-6 released by duodenal myofibroblasts may exert this anti-apoptotic activity in T cells within the CD lesion. Next to promoting survival, IL-1β could affect the cytokine profile of the effector memory T cells as exogenous IL-1β altered the IFN-γ, IL-21 and IL-10 secretion by T cells. However, as duodenal myofibroblasts from CD patients and non-CD patients tested responded variably, larger numbers of cell-lines should be analyzed to assess possible changes in myofibroblast function in CD patients. In conclusion, myofibroblasts can participate in intestinal immune responses by expressing chemokines and cytokines. Their exact role in CD remains to be elucidated.

Transgenic mice expressing human HLA-DQ2 and a gluten-specific T-cell receptor can be used to study gluten-specific T-cell responses. Using these transgenic mice, we have previously shown that tolerance to gluten is induced and driven by tolerogenic interleukin-10 secreting type-1 regulatory T (Tr1) cells in the spleen. In **chapter 6** we aimed to identify mechanisms that drive gluten antigen presentation and Tr1-cell differentiation. Because feeding smaller gluten peptides led to substantial T-cell proliferation in the mucosal-draining lymph nodes, gluten degradation appeared a pivotal step required for mucosal lymph node presentation and induction of T_{REG} cell-differentiation. As macrophages have a strong degradation capacity, the effect of macrophage depletion on splenic gluten presentation was assessed. Macrophage depletion prior to whole gluten feed did not affect T-cell proliferation, but inhibited Tr1-cell induction arguing that macrophages play a role in oral tolerance to gluten. In *in vitro* cultures, splenic macrophages, rather than dendritic cells, favored differentiation of gluten-specific Tr1 cells by expressing IL-27. In conclusion, protein degradation may be a decisive step in gluten presentation and macrophages rather than DC favor the differentiation of gluten-specific Tr1 cells through an IL-27 dependent mechanism.

In **chapter 7** the main findings of the studies described are reported and placed in a broader perspective.

Samenvatting

Introductie

De mucosa (het slijmvlies) van ons maag-darm kanaal is in direct contact met de buitenwereld en wordt continu blootgesteld aan grote hoeveelheden lichaamsvreemde antigenen (moleculen die een immuunreactie kunnen opwekken) uit voedsel en van micro-organismen. Het immuunsysteem van de darm beschermt de mucosa tegen schadelijke micro-organismen en blijft aan de andere kant tolerant voor antigenen van voedsel en niet-schadelijke bacteriën, inclusief het eigen microbioom (darmflora). Deze mucosale tolerantie is een strak gereguleerd proces en is afhankelijk van actieve onderdrukking van immuunreacties. De balans tussen afweerreactie tegen ziekmakende bacteriën en het behoudt van tolerantie geeft een evenwicht dat homeostase wordt genoemd.

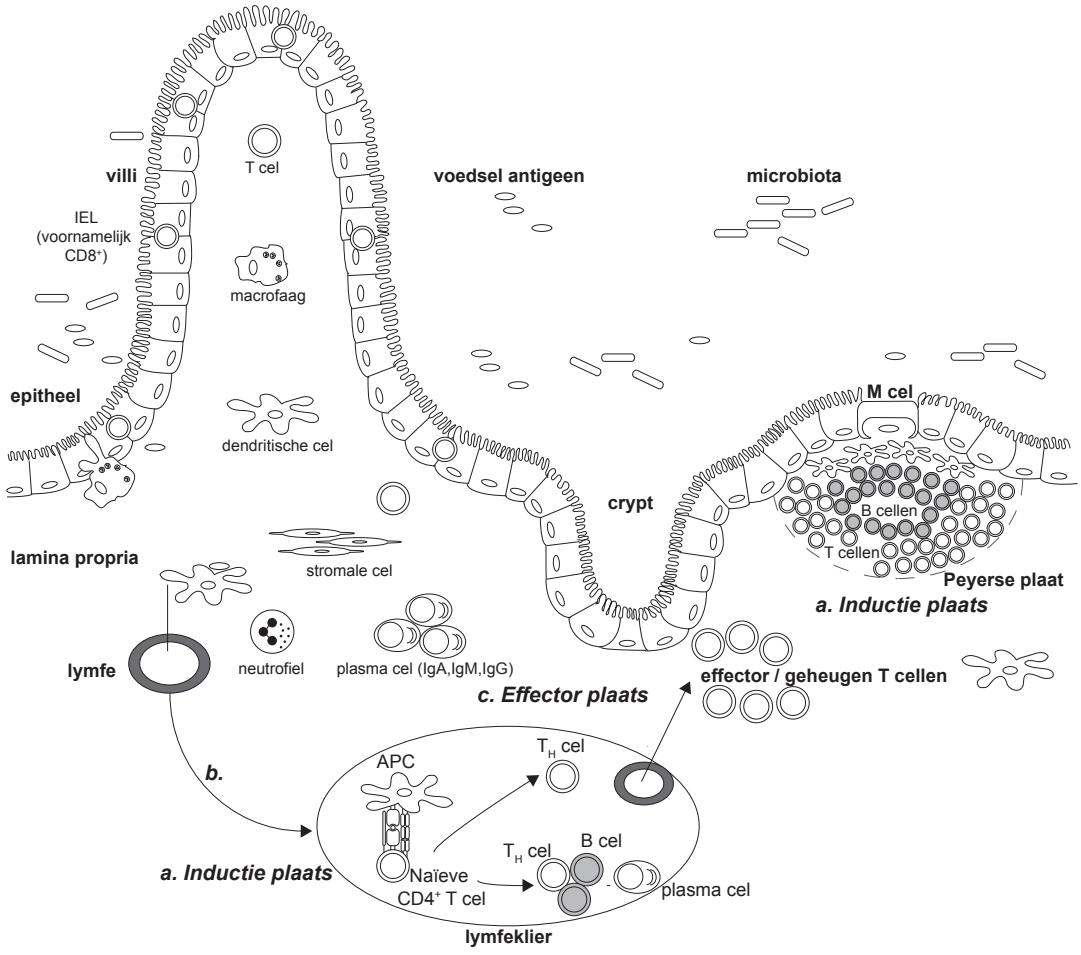
De homeostase wordt verstoord wanneer de tolerantie verloren gaat en een ongecontroleerde afweerreactie ontstaat tegen onschuldige antigenen, welke een chronische ontsteking veroorzaken. Een voorbeeld van een ongecontroleerde reactie tegen een voedsel antigeen is coeliakie. Bij coeliakie is er sprake van een chronische ontsteking van de dunne darm die wordt veroorzaakt door een afweerreactie op inname van gluten eiwit. Ongewenste afweerreacties tegen eigen darmbacteriën veroorzaken inflammatoire darmziekten (in het Engels Inflammatory Bowel Disease, IBD).

Organisatie van het immuunsysteem van de darm (*Figuur 1*)
Slechts een enkele laag epitheel cellen scheidt de grote hoeveelheden antigenen van voedsel en bacteriën in het darmlumen met de steriele omgeving van het darmweefsel. Om de grote hoeveelheid antigenen te reguleren bevat de darm het grootste deel van ons immuunsysteem en is het de plek waar de meeste witte bloedcellen (afweercellen) zich bevinden. Antigenen uit het darmlumen kunnen worden getransporteerd over het epitheel en stimuleren het immuunsysteem. De lamina propria onder het epitheel bevat veel antigeen presenterende cellen (APC) zoals dendritische cellen (DC) en macrofagen. Deze cellen verzorgen de eerstelijns afweer door het vangen en verwerken van het antigeen.

Het immuunsysteem van de darm wordt onderverdeeld in inductie en effector plaatsen. De inductie plaatsen bestaan uit de darm-geassocieerde lymfoïde weefsels (in het Engels Gut-Associated Lymphoid Tissue, GALT) en de lokale darm drainerende lymfeklieren (mesenteriale lymfeklieren). De GALT bestaat uit Peyerse platen en geïsoleerde lymfeklier follikels. APC brengen het antigeen naar de GALT en mesenteriale lymfeklieren en scheiden cytokines uit om een immuunreactie op gang te brengen. Cytokines zijn kleine oplosbare eiwitten die belangrijk zijn bij de regulatie van cel-cel interacties. Naïeve, niet eerder aan antigeen blootgestelde, T- en B-cellen uit perifeer bloed komen ook de GALT of lymfeklier binnen en worden geactiveerd door de APC. De T cel receptor herkent het peptide afkomstig van het antigeen welke gepresenteerd wordt door de APC in een Major Histocompatibility complex (MHC). Dit resulteert in activatie, celdeling en differentiatie van naïeve T cellen naar een grotere hoeveelheid van antigeen-specifieke effector en geheugen T cellen. Na activatie, migreert een deel van de T cellen terug naar het bloed om vervolgens uit te treden in de lamina propria of tussen de epitheel cellen van de darm. De plek waar gedifferentieerde antigeen-specifieke effector cellen hun acties uitvoeren worden effector plaatsen genoemd. Het migratie proces wordt geleid door een profiel van specifieke eiwitten (adhesie moleculen en chemokines) op het cel oppervlak, waarmee ze naar de juiste plek worden geleid.

Effector T cellen
Afhankelijk van het type antigeen en de omgeving van cytokines, sturen de APC de naïeve voorloper T cellen naar de nodige T cel subset. Naïeve T cellen hebben 3 signalen van de APC nodig om actief te worden, te delen en te differentiëren tot een specifieke T cel subset. Signaal 1 is de verbinding van het peptide-MHC complex van de APC met de T cel receptor welke zorgt voor T-cel activatie. Signaal 2 komt van de co-stimulatoire moleculen aanwezig op de APC en de T cel, welke T cel vermeerdering en overleving stimuleren of remmen. Cytokines geven signaal 3. Cytokines sturen de differentiatie van de naïeve T cel naar een specifieke cytokine-producerende subset. $CD4^+$ T cellen kunnen naar verschillende effector T helper (T_H) cel subsets differentieren, zoals T_H1, T_H2, T_H17 en de regulatoire T (T_{REG}) cellen, elk met een andere func-

Figuur 1 ***Organisatie van het immuunsysteem van de darm.*** *(**a**) De inductie plaatsen bestaan uit de darm geassocieerde lymfoïde weefsels (in het Engels gut-associated lymphoid tissues, GALT) dat onder het epitheel ligt en de verderop gelegen lokale darm drainerende lymfeklieren. De GALT bestaat Peyerse platen en geïsoleerde lymfe follikels. Op inductie plaatsen, naïeve B- en T- cellen differentiëren na antigeen presentatie door antigeenpresenterende cellen (APCs). (**b**) APCs brengen het antigeen naar de GALT en mesenteriale lymfeklieren. Na APC-T cel interactie, differentiëren naïeve T cellen tot effector T_H cellen. Gedifferentieerde T_H cellen migreren naar de effector plaatsen. (**c**) Effector plaatsen zijn de plekken waar effector en geheugen T cellen hun functie uitvoeren als ze zijn gedifferentieerd. Effector plaatsen bestaan uit het epitheel, het onderliggende bindweefsel en de lamina propria. APC antigeen presenterende cel; DC dendritische cel; T_H T helper cel, IEL intra-epitheliale lymfocyt*



tie. Deze effector T_H cellen kunnen worden onderscheiden op basis van hun cytokine profiel en expressie van een specifieke transcriptie factor, zoals bijvoorbeeld Foxp3. De selectieve cytokine productie door de verschillende T_H cellen geeft de cel een precieze immuunfunctie om bacteriën te doden, hulp te bieden aan B cellen, het activeren van $CD8^+$ T cellen of om de niet-specifieke afweer cellen te activeren. Met de vorming van effector T cellen worden gelijk geheugen T cellen gevormd. Deze geheugen T cellen zijn lang levende cellen en geven bij een tweede contact met hetzelfde antigeen een snellere herkenning en efficiëntere immuunreactie.

Regulatoire T cellen

Naast activerende T_H cellen zijn er T_{REG} cellen die immuunreacties kunnen onderdrukken. T_{REG} cellen werken direct door cel-cel contact of indirect door de uitscheiding van cytokinen, zoals interleukine (IL)-10 en Transforming Growth Factor (TGF)-β, welke T-cel deling kunnen remmen. T_{REG} cellen in het bloed of in de darm kunnen afkomstig zijn uit de thymus of zijn ontstaan uit naïeve voorloper T cellen die gedifferentieerd zijn tot T_{REG} cellen. De T_{REG} cellen uit de thymus worden ook wel natuurlijke T_{REG} cellen genoemd en spelen met name een rol in het behoudt van tolerantie tegen lichaamseigen antigenen en het voorkomen van auto-immuniteit. Perifeer geïnduceerde T_{REG} cellen ontstaan uit naïeve T cellen in lymfeklieren. De darmomgeving stimuleert het ontstaan van perifeer geïnduceerde T_{REG} cellen. Twee subsets van geïnduceerde T_{REG} cellen zijn belangrijk in de darm, de $Foxp3^+$ T_{REG} cellen en de $Foxp3^{neg}$ IL-10-producerende type 1 regulatoire T (Tr1) cellen.

Mucosale tolerantie

Mucosale tolerantie tegen onschuldige antigenen is afhankelijk van actieve onderdrukking van antigeen-specifieke immuunreacties. De belangrijkste mechanismen om tolerantie te induceren zijn het vormen van niet-reagerende (= anerge) T cellen, verwijdering van antigeen-specifieke T cellen en het ontstaan van antigeen-specifieke T_{REG} cellen. De darm heeft een bevoorrechte omgeving voor het ontstaan van tolerantie tegen onschuldige antigenen uit het darmlumen. De meest bekende spelers in deze tolerogene omgeving zijn cytokines, enzymen en metabolieten (bijvoorbeeld TGF-β, IL-10, cyclooxygenase-2 en retinol zuur) als mede een aantal celtypen (zoals DC, macrofagen, stromale cellen en epitheel cellen).

Mucosale tolerantie van de darm is het best onderzocht in experimenten met voedsel eiwitten zoals het kippeneiwit ovalbumine. Vanwege de grote verscheidenheid aan microbiota zijn antigeen-specifieke T cellen specifiek voor bacteriële antigenen uit de darmflora moeilijker te onderzoeken, maar er wordt aangenomen dat tolerantie tegen darmbacteriën via dezelfde mechanismen ontstaan als tegen voedseleiwitten.

Verstoring van darm homeostase

Coeliakie

In 1940 was het de Nederlandse kinderarts Willem-Karel Dicke die ontdekte dat gluten de oorzaak was van coeliakie en hij introduceerde het glutenvrij dieet als effectieve behandeling. Gluten eiwitten zijn aanwezig in tarwe, gerst en rogge. Coeliakie is de meest voorkomende intolerantie tegen een voedseleiwit en komt in ongeveer 1-2% van de bevolking voor. Coeliakie patiënten hebben klachten als diarree, gewichtsverlies, steatorroe (vetontlasting) en andere symptomen van ondervoeding zoals bloedarmoede en vitaminedeficiënties. De huidige behandeling is een levenslang gluten-vrij dieet, welke resulteert in compleet herstel van de dunne darm mucosa. Een zeldzame, ernstige complicatie voor coeliakie patiënten is dat ze niet meer reageren op het gluten-vrij dieet en refractaire coeliakie ontwikkelen. Een deel van deze refractaire coeliakie patiënten heeft een hoog risico op de ontwikkeling van een dunne darm maligniteit, een zogenaamd enteropathie geassocieerd T cel lymfoom.

Immunologisch wordt coeliakie gedreven door een inflammatoire T-cel reactie tegen gluten eiwitten resulterend in dunne darm ontsteking in genetisch belaste mensen. De dunne darm ontsteking wordt gekenmerkt door villus atrofie (afwezigheid van villi), crypt hyperplasie (verdikking van de crypt) en toename van T- en B- cellen in de lamina propria en tussen de epitheel cellen. Er is een sterke genetische gevoeligheid voor coeliakie. Bijna alle coeliakie patiënten dragen de *HLA-DQ2* of *HLA-DQ8* genen. In de context van deze HLA-moleculen wordt het gluten gepresenteerd door de APC aan de $CD4^+$ T cel. Inflammatoire gluten-specifieke T cellen kunnen worden geïsoleerd uit darmweefsel van coeliakie patiënten en niet in gezonde HLA-DQ2 positieve individuen. Deze $CD4^+$ gluten-specifieke T cellen produceren de pro-inflammatoire cytokines interferon (IFN)-γ en IL-21, waarvan gedacht wordt dat deze cytokines de mucosale schade veroorzaken. Van muizenstudies weten we dat dragerschap van *HLA-DQ2/DQ8* genen samen met inflammatoire gluten-specifieke T cellen niet genoeg zijn om coeliakie te krijgen, dus meer factoren zijn betrokken bij verlies van tolerantie en het ontstaan van de ontstekingsreactie.

Ondanks de toegenomen kennis omtrent het ontstaan van coeliakie is het nog steeds onduidelijk waarom bepaalde mensen met *HLA-DQ2* genen coeliakie ontwikkelen en waarom anderen met dezelfde genen niet. Het is opmerkelijk dat 25% van de West-Europese populatie *HLA-DQ2* genen heeft en slechts 1% coeliakie ontwikkeld.

Inflammatoire darm ziekten

IBD is een chronische recidiverende ziekte gedreven door een inflammatoire T-cel reactie tegen darm microbiota. IBD wordt onderverdeeld in de ziekte van Crohn en colitis ulcerosa. De incidentie in de VS en Europa is ongeveer 5-15 gevallen per 100.000 mensen per jaar. De meeste IBD patiënten presenteren zich met diarree, rectaal bloedverlies en buikpijn. Als patiënten zich als kind presenteren kan er tevens sprake zijn

van groeivertraging. De ziekte van Crohn wordt gekenmerkt door darmlaesies die zich kunnen uitbreiden tot diepe ontstekingsreacties over het gehele maag-darm kanaal. Colitis ulcerosa laesies zijn meer oppervlakkig en beperken zich tot de dikke darm (colon). In tegenstelling tot de aaneengesloten ontsteking in colitis ulcerosa, is bij de ziekte van Crohn is ontstoken en gezond weefsel afwisselend aanwezig. IBD is op dit moment niet te genezen en behandeling omvat levenslange afweeronderdrukkende therapie waarbij ook chirurgische interventies nodig kunnen zijn.

De precieze oorzaak van IBD is onbekend, maar drie factoren spelen een oorzakelijke rol. Deze factoren zijn genetische belasting, de microbiota en het immuunsysteem van de darm. Er wordt gedacht dat IBD het gevolg is van een uit de hand gelopen inflammatoire T-cel reactie tegen darmflora dat ontstaat in een genetisch belast persoon.

$CD4^+$ T cellen spelen een belangrijke rol in het ontstaan van IBD omdat de darmontsteking veel T cellen en T-cel-afkomstige cytokines bevat en diermodellen met darmontsteking afhankelijk zijn van de aanwezigheid van T cellen. Het beste bewijs van T cel betrokkenheid is de effectiviteit van T-cel remmende therapie in de meeste IBD patiënten.

In een gezonde darm worden inflammatoire T-cel reacties tegen darmflora actief gereguleerd door T_{REG} cellen en anti-inflammatoire cytokines als TGF-β en IL-10. Mensen en muizen met defecten in het *Foxp3* gen, de transcriptiefactor van T_{REG} cellen, hebben ernstige ontstekingsreacties in het gehele lichaam inclusief een ernstige darmontsteking. Ook mensen en muizen met defecten in het *IL-10* gen of genen coderend voor eiwitten in de IL-10 signaleringsroute hebben een ernstige darmontsteking.

Ondanks het duidelijke verband tussen IBD en T-cel reacties behoeft het verder onderzoek hoe immunologische reacties tegen darmflora zijn gereguleerd, IBD veroorzaken en hoe in homeostase darmflora-specifieke T cellen zijn gereguleerd.

Samenvatting proefschrift

Zoals hierboven beschreven spelen T cellen een belangrijke rol in de ontwikkeling van coeliakie en IBD. De balans tussen de functie van T_{REG} cellen en effector $CD4^+$ T cellen is cruciaal voor homeostase. Betere kennis over T-cel reacties is essentieel om verstoringen in normale lichaamsfuncties die leiden tot coeliakie en IBD beter te begrijpen en voor de ontwikkeling van nieuwe therapieën. Dit proefschrift heeft zich erop gericht om beter inzicht te krijgen in defecte regulatie van T-cel reacties die een darm ontsteking tot gevolg hebben.

Veranderingen in het aantal T_{REG} cellen kan een indicatie zijn voor de afwijkende homeostase in coeliakie. In **hoofdstuk 2** onderzochten we of er numerieke verschillen bestonden in twee T_{REG} cel populaties in bloed van coeliakie patiënten, namelijk de

mucosaal geïnduceerde (gekenmerkt door de markers ($CD62L^{neg}CD38^{+}Foxp3^{+}$) of de natuurlijke T_{REG} cel populaties. Om te kijken naar het vroege ontstaan van coeliakie werden kinderen met coeliakie vergeleken met gezonde controles en is er gekeken in het bloed van volwassen coeliakie patiënten. Er werd geen verschil gevonden in het aantal natuurlijke T_{REG} cellen en de mucosaal geïnduceerde T_{REG} cellen als kinderen met coeliakie werden vergeleken met controle kinderen. Volwassen refractaire en behandelde coeliakie patiënten lieten verhoogde aantallen natuurlijke T_{REG} cellen zien, maar een normaal aantal mucosaal geïnduceerde T_{REG} cellen. In biopten werden verhoogde aantallen $Foxp3^{+}$ cellen gezien in zowel kinderen als volwassen coeliakie patienten. Dit impliceert dat coeliakie patiënten in hun bloed normale aantallen mucosaal geïnduceerde T_{REG} cellen hebben en dat veranderingen in natuurlijke T_{REG} cellen zijn geassocieerd met een specifiek stadium van de ontsteking en niet met het ontstaan van de ziekte in een vroeg stadium.

IL-10 is cruciaal voor het behoud van darm homeostase, want genetische defecten in de IL-10 signaleringsroute leiden tot ernstige IBD op zeer jonge leeftijd. Het is onbekend hoe immuunreacties worden beïnvloed door IL-10 receptor defecten. In **hoofstuk 3** karakteriseerden we de immulogische gevolgen van een IL-10 receptor defect bij een elf jarig meisje met een mutatie in het IL-10 receptor gen die zich presenteerde met IBD op de leeftijd van drie maanden. In tegenstelling tot de standaard behandeling met beenmergtransplantatie, werd remissie (vermindering van ziekteverschijnselen) bereikt met medicamenteuze therapie middels thalidomide en intraveneuze immuunglobulines. Dit maakte het mogelijk om te onderzoeken hoe IL-10 immuunreacties controleert en darm homeostase behoudt.

Een immuunsysteem met een IL-10 receptor deficiëntie ontwikkelt zich normaal met betrekking tot het aantal en fenotype van circulerende immuun cellen. Ondanks normale expressie van co-stimulatoire moleculen, maakten bacterie gestimuleerde DC veel meer pro-inflammatoire cytokines als IL-6 en TNF-α in celkweek. IL-10 was nodig om de cytokine productie door DC te remmen en de T cel-afkomstige cytokines IFN-γ (T_H1) en IL-17 (T_H17) te remmen. Overeenkomstig werden er in de darmlaesies, in biopten genomen ter diagnose, veel T_H1 en T_H17 positieve cellen gevonden. Bovendien vonden we dat immuunglobulines de T cel cytokines IL-17 and IFN-γ remde, terwijl thalidomide de bacterie gestimuleerde TNF-α productie remde. Samenvattend, het imuunsysteem ontwikkeld zich normaal in afwezigheid van een IL-10 receptor maar vertoont afwijkende inflammatoire cytokine afgifte van DC en ongecontroleerde T_H1 en T_H17-cell reacties.

In **hoofdstuk 4**, identificeerde we het aandeel van de pro-inflammatoire cytokines IL-21 en IL-17 in het ontstaan van coeliakie in een groep van kinderen met coeliakie met verschillende histopathologische scores (Marsh scores). Immunohistochemie toonde een verhoogd aantal IL-21-producerende cellen in dunne darm biopten van kinderen

met coeliakie zonder dat IL-17-producerende cellen verhoogd waren. Dit verhoogde aantal cellen was al aanwezig in coeliakie patiënten met milde histopathologische afwijkingen (Marsh score 1 en 2). T cellen geïsoleerd uit de dunne darm toonden een grote populatie IL-21-producerende $CD4^+$ T cellen die geen IL-17 maar IFN-γ uitscheidde. Biopten van volwassen coeliakie patiënten bevatte ook veel IL-21 positieve cellen. In een groep van volwassen coeliakie patiënten, met Marsh score 3C, bevatte de darmlaesie naast IL-21-producerende cellen ook IL-17 positieve cellen. Omdat dunne darmschade tot gevolg heeft dat er toename is van contact met microben, veronderstellen we dat microbiële aftasting met zogenaamde toll like receptoren (TLR) de cytokine productie beïnvloedt. Stimulatie van de T cel met een TLR3 stimulus leidde tot toegenomen IL-21 productie, terwijl TLR2 stimulatie leidde tot verhoogde IL-17 productie. Concluderend, onze data laten zien dat een IL-17-onafhankelijke verhoogde IL-21 productie door $CD4^+$ T cellen karakteristiek is voor coeliakie en suggereert dat selectieve TLR stimuli de T-cel afkomstige cytokine productie kan sturen.

In **hoofdstuk 5** hebben we de immuunfunctie van myofibroblasten afkomstig uit de dunne darm onderzocht. Myofibroblasten zijn stromale cellen die structuur geven aan de darm. We veronderstelde dat myofibroblasten een rol spelen in de chronische ontstekingsreactie in coeliakie door de overleving van geheugen T cellen te stimuleren. Hiervoor isoleerde we myofibroblasten uit dunne darm biopten van coeliakie patiënten en personen zonder coeliakie. Alle dunne darm myofibroblasten konden TLR2 en TLR3 signalen waarnemen wat leidde tot de productie van grote hoeveelheden chemokines en pro-inflammatoire cytokines als IL-6 en IL-1β. Naast de expressie van chemokines om cellen aan te trekken, zorgden myofibroblasten ook voor minder T cel dood in kweek. De geteste myofibroblast cellijnen reageerden erg variabel. Daarom moeten meer cellijnen worden getest om een verstoorde myofibroblast functie in coeliakie aan te tonen of uit te sluiten. Deze studie laat wel zien dat myofibroblasten kunnen deelnemen aan immuunreacties in de darm.

In **hoofdstuk 6** hebben we een muismodel gebruikt om gluten-specifieke T-cel reacties te onderzoeken. In een eerdere studie hebben we laten zien dat transgene muizen (muizen die genen dragen van mensen) met het menselijke *HLA-DQ2* gen en een gluten-specifieke T cel receptor, geen coeliakie ontwikkelen na het eten van gluten. Deze muizen zijn tolerant voor gluten door de vorming van Tr1 regulatoire cellen. Opvallend was dat deze cellen werden gevormd in de milt en niet in de lymfeklieren van de darm. We hebben in dit hoofdstuk gekeken naar welke mechanismen verantwoordelijk zijn voor de vorming van deze T_{REG} cellen door gebruik te maken van het muismodel. Omdat het geven van kleinere eiwitfragmenten (peptiden) wel T-cel deling gaf in de lymfeklieren van de darm denken we dat gluten degradatie een belangrijke stap is voor antigeen presentatie in de darm. Macrofagen kunnen eiwitten goed degraderen. Door het uitschakelen van de macrofagen vonden we dat macrofa-

gen nodig waren voor de vorming van Tr1 cellen in zowel het diermodel als in celkweekmethoden. Deze studie laat zien dat gluteneiwit degradatie een belangrijke stap is voor gluten presentatie en dat macrofagen een belangrijke rol spelen in differentiatie naar tolerogene Tr1 cellen.

In **hoofdstuk 7** worden de belangrijkste bevindingen van de beschreven studies besproken en geplaatst in een breder perspectief.

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List of publications

du Pré MF, van Berkel LA, Ráki M, **van Leeuwen MA**, de Ruiter LF, Broere F, Ter Borg MN, Lund FE, Escher JC, Lundin KE, Sollid LM, Kraal G, Nieuwenhuis EE, Samsom JN. *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*. 2011 Jun;106(6):1147-59

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van Leeuwen MA, Tindemans I, Nugteren S, Raatgeep HC, Groeneweg M, Samsom JN *Characterization of human duodenal myofibroblasts and their interaction with T cells.* In preparation.

van Leeuwen MA, Costes LMM, van Berkel LA, Kozijn AE, du Pré MF, Raatgeep HC, van Rooijen N, Koning F, Samsom JN *Macrophages favor differentiation of IL-10-secreting type 1 regulatory T cells driving oral tolerance to gliadin.* To be submitted.

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PhD portfolio

Name PhD student: Marieke Anne van Leeuwen
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Research school: Molecular Medicine Postgraduate School
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Courses and seminars

2009	Course Animal Experimentation, Erasmus MC, Rotterdam
2009	Basic course for legislation and organisation for clinical researchers (BROK), Erasmus MC, Rotterdam
2010	Advanced Molecular Immunology, Erasmus MC, Rotterdam
2011	Advanced Immunology, postdoctoral course, Amsterdam
2011	Workshop Adobe Photoshop, Illustrator and Indesign, MolMed, Rotterdam
2012	Biomedical English writing and communication
2012	Young Investigators Forum ESPGHAN
2009-2013	Research seminars and journal clubs, Laboratory of Pediatrics and department of Pediatric Gastroenterology, Erasmus MC
2011-2013	Research seminars, T cell Consortium, Erasmus MC

Scientific presentations and meetings

2009	Oral, Dutch society of Immunology Winterschool, Noordwijkerhout
	Poster, 9th Research Day Pediatrics, ErasmusMC, Rotterdam
2010	Oral, Dutch Society of Immunology Winterschool, Noordwijkerhout
	Oral, 10th Research Day Pediatrics, ErasmusMC, Rotterdam
	Oral, Celiac Disease Consortium, Utrecht
	Oral, European Mucosal Immunology Group meetings, Amsterdam
	Poster, 14th MolMed Day, Rotterdam
2011	Poster, Dutch Society of Immunology Winterschool, Noordwijkerhout
	Poster, 15th International Congress of Mucosal Immunology, Paris, France
	Oral, 14th International Coeliac Disease Symposium, Oslo, Norway
	Poster, annual meeting of the Dutch Society of Gastroenterology
	Poster, 15th MolMed Day, Rotterdam

2012	Oral, Dutch Society of Immunology Winterschool, Noordwijkerhout
	Poster, ESPGHAN 2012, Stockholm, Sweden
	Oral, annual meeting of the Dutch Society of Gastroenterology
	Poster, 16th MolMed Day, Rotterdam
2013	Oral, Dutch Society of Immunology Winterschool, Noordwijkerhout
	Oral, Masterclass Mucosal Immunology meeting, Rotterdam
	Oral, annual meeting of the Dutch Society of Gastroenterology
	Poster, 17th MolMed Day, Rotterdam
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About the author

Marieke Anne van Leeuwen was born on November 17th, 1982 in Dodewaard. In 2001 she graduated at the 'Merlet College Land van Cuijk' in Cuijk (athenaeum-bèta) and she started in the same year, via admittance exams, with studying medicine at the Erasmus University Rotterdam. During the second year of medical school she started a master of clinical epidemiology at the National Institute for Health Sciences for which she attended the Summer School of Harvard University in Boston. In July 2005, she completed her master research project (supervised by Prof.dr. S.W.J. Lamberts and dr. J.W. Koper) and she obtained her bachelor degree in medicine and master degree in clinical epidemiology. After obtaining her medical degree in September 2007 she started working at the Reinier de Graaf Gasthuis in Delft as a resident in pediatrics. In March 2009 she started with her PhD project as described in this thesis, in the Laboratory of Pediatric Gastroenterology at the Erasmus MC in Rotterdam, under supervision of Dr. Janneke Samsom (promotor: Prof.dr. J.C. Escher). From July 2013 till July 2014 she worked as a resident in pediatrics at the Erasmus MC Sophia Children's Hospital. In May 2015 she will start with her residency training in pediatrics at the University Medical Center Groningen (head: Prof.mr.dr. A.A.E. Verhagen).

Marieke van Leeuwen lives together with Mark Eijgelsheim and together they have a daughter Olivia (2012). They are expecting their second child in February 2015.