A DYNAMIC BALANCE

Regulatory and inflammatory T-cell responses in inflammatory bowel disease

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Regulatory and inflammatory T-cell responses in inflammatory bowel disease

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Parts of this chapter will be published as a review in Cells.

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GENERAL INTRODUCTION

The intestine is continuously exposed to harmless antigens from the diet and commensal bacteria, but also provides harmful pathogens access to the body. Invasion of intestinal tissue by gut-resident commensal bacteria and pathogens has serious health consequences including inflammation and sepsis. To ensure host defense, the intestinal tissue is protected by a complex and highly specialized network of innate and adaptive immune cells.[1]

Up to 60% of lymphocytes residing in the intestinal tissue are CD4$^+$ memory T cells that have the unique ability to exert memory to previously encountered antigens.[2] Upon re-exposure to antigen, CD4$^+$ memory T cells mount a rapid and highly efficient immune response. As a result, CD4$^+$ memory T-cell responses are essential for protective immunity against pathogens, but need to be tightly regulated to avoid inflammatory responses to commensal bacteria.[3] Uncontrolled inflammatory CD4$^+$ T-cell responses to commensal bacteria, as seen in patients with inflammatory bowel disease (IBD), can result in tissue damage and ensuing chronic intestinal inflammation.[4]

Regional control of mucosal immune responses in the intestine.

Regional Adaptation of the Mucosal Immune Response

Anatomically, CD4$^+$ T cells are located within both inductive and effector sites of the intestine. The mesenteric lymph nodes (MLN) and gut-associated lymphoid tissue (GALT) are "inductive sites", the main location for priming naive T- and B-cell responses.[1] The GALT consists of the macroscopically visible Peyer's patches (PP) of the small intestine and colonic patches in the colon and smaller structures referred to as solitary isolated lymphoid tissue follicles (SILT) in both small intestine and colon.[5] The mucosal epithelium and underlying lamina propria are the "effector sites" of the intestinal immune system, which harbor large populations of activated memory CD4$^+$ T cells and antibody-secreting plasma cells (Figure 1).

Antigen presenting cells (APCs) including dendritic cells (DCs) and macrophages are present in the organized lymphoid structures of the GALT and dispersed throughout the small intestinal and colonic lamina propria.[6] Antigens that cross the intestinal mucosa first encounter APCs in either the GALT or the lamina propria. There are several mechanisms that contribute to antigen uptake in the intestine. First, the follicle-associated epithelium of the Peyer's patches contains specialized M cells that can transport microbial antigens across the mucosal epithelium from the lumen to organized lymphoid structures.[7] This process is mediated through M-cell specific oligosaccharides and glycoproteins that allow selective microbial adherence and immunoglobulin A (IgA) receptors that recognize IgA-

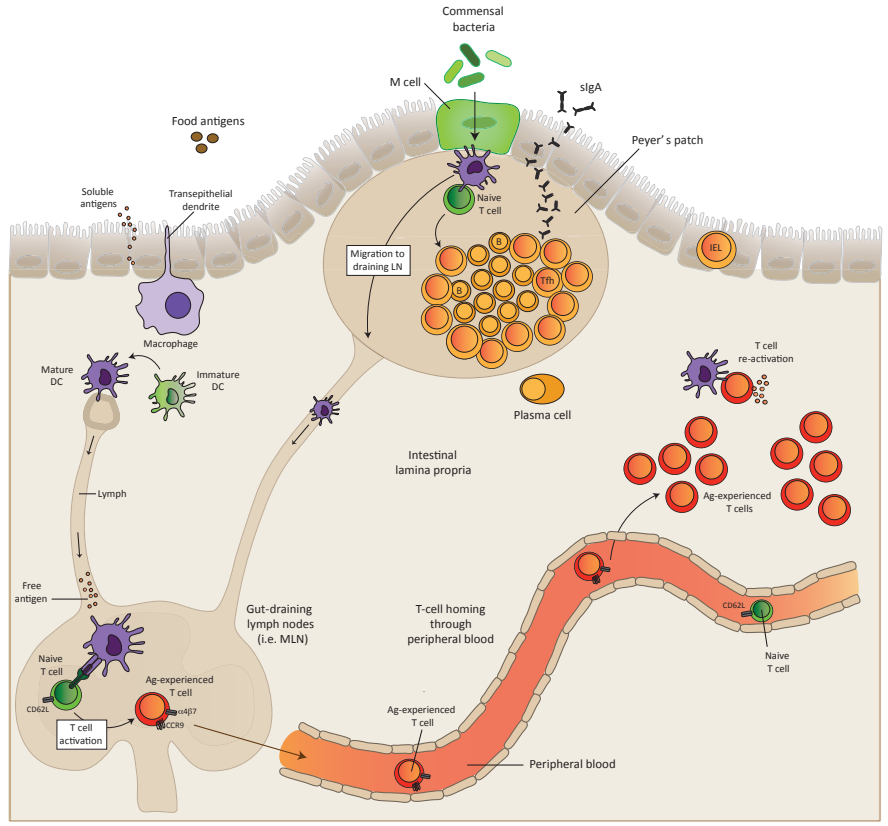

Figure 1. Regional control of mucosal immune responses in the intestine. CD4$^+$ T cells are located within both inductive and effector sites of the intestine. The mesenteric lymph nodes (MLN) and gut-associated lymphoid tissue (Peyer's patches in the small intestine; colonic patches in the colon) are "inductive sites", the main location for priming naive T- and B-cell responses. Antigens that cross the intestinal mucosa first encounter antigen presenting cells, including macrophages and dendritic cells (DCs) in either the GALT or the lamina propria. DCs migrate from the lamina propria to inductive sites in order to present intestinal antigen-derived peptides to naive CD4$^+$ T cells. Naive CD4$^+$ T cells recirculate from the peripheral blood into the gut-draining lymph nodes and GALT using the adhesion molecule CD62L. T-cell responses are initiated when a naive CD4$^+$ T cells encounter DCs expressing the appropriate peptide-MHC-complex. After recognition of cognate antigen and differentiation, antigen-experienced CD4$^+$ T cells lose CD62L expression and exit the lymph node to enter the blood. Through expression of the adhesion molecule $\alpha4\beta7$, CD4$^+$T cells enter the "effector sites" of the intestine where they reside as long-lived memory CD4$^+$T cells. Here, antigen-experienced CD4$^+$ T cells can be re-activated upon encounter of resident-DCs presenting antigen locally in the intestine. Abbreviation: sIgA, secretory IgA; Ag-experienced, antigen experienced.

coated bacteria.[7-9] Second, soluble antigens can diffuse through epithelial tight junctions and can be transferred across epithelial cells by transcellular routes. Third, luminal antigens are also captured by transepithelial projecting dendrites from macrophage-like CX3CR1$^+$ APCs in the Peyer's patches and lamina propria.[10, 11] M cells and CX3CR1$^+$ APCs transfer antigen to migratory DCs that migrate from the lamina propria to inductive sites in order to present antigen-derived peptides to naive CD4$^+$ T cells.[11-16] Tissue macrophages do not usually migrate to the GALT or MLN, but can contribute to adaptive immune responses by presenting processed antigen to effector T cells *in situ* in the lamina propria. Thus, intestinal APCs have a key role in the initiation of adaptive immune responses to intestinal antigens.

Intestinal DCs and macrophages have divergent functional properties. In the steady state, the specific microenvironment of the gut, including epithelial-cell derived factors and bacterial products, alters the functional properties of DCs residing in the intestine.[17] This is illustrated by the observation that DCs isolated from Peyer's patches produce higher levels of the regulatory cytokine interleukin 10 (IL-10) compared to splenic DCs after CD40-mediated stimulation.[18] Moreover, DCs from the lamina propria and MLN are more efficient than splenic DCs at inducing expression of the transcription factor forkhead box P3 (Foxp3)[19, 20], which is indispensable for the differentiation and function of regulatory CD4$^+$ T cells.[21-24] Thus, the conditioning of intestinal DCs and macrophages in steady state maintains a tolerogenic state in the intestine by skewing CD4$^+$ T-cell differentiation in favor of a regulatory phenotype. During inflammation, infiltration of microbiota beneath the epithelial-cell layer leads to enhanced production of chemokines and pro-inflammatory cytokines. As a result, Ly6C^{high} monocytes and DC precursor cells are recruited to the intestine.[17, 25] This shifts the balance from a tolerogenic immune response that is induced by conditioned macrophages and DCs to an inflammatory response induced by freshly-recruited unconditioned macrophages and DCs. As a result, an inflammatory CD4$^+$ T-cell response directed against the invading pathogens is generated. Taken together, CD4$^+$ T cells responding to intestinal antigens are considerably affected by the context in which antigen presentation occurs.

Thus, intestinal immune responses arise from a dynamic process with constant changes in antigen exposure, inflammatory and anti-inflammatory cytokines, and selective participation of cell types that act according to their location. In the GALT and MLN, where priming of naive CD4$^+$ T cells occurs, this information is integrated and serves as a 'rheostat' that determines the precise effector function of the CD4$^+$ T-cell response that is generated.

Priming and Migration of Mucosally-Imprinted CD4$^+$ T cells

The GALT allows for tissue-restricted priming to intestinal antigens and regionalization of the intestinal immune response. Naive CD4$^+$ T cells migrate from the peripheral blood into

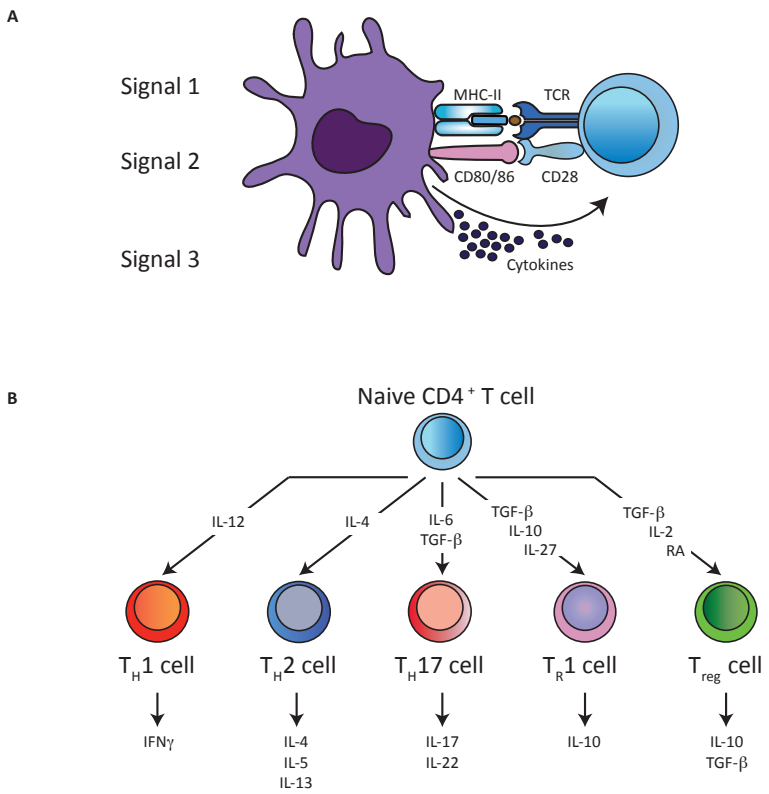

Figure 2. CD4$^+$ T-cell activation and differentiation. (A) CD4$^+$ T cells differentiate in response to appropriate TCR signaling (signal 1), co-stimulation (signal 2) and the surrounding cytokine environment (signal 3). Many co-stimulatory and co-inhibitory receptors determine consequences of functional TCR signaling thus modulating the degree of activation, differentiation and effector function of CD4$^+$ T cells.[132] **(B)** Upon interaction with cognate antigen presented by APCs, CD4$^+$ T cells can differentiate into a variety of functionally different effector subpopulations, including T helper (Th)1, Th2, Th17, peripherally-derived Treg (Treg) and CD4$^+$Foxp3^{neg} T regulatory 1 cells (Tr1). The cytokine environment, which induces lineage-specific transcription factors during differentiation of CD4$^+$ T cells, plays a central role in skewing naive CD4$^+$ T cells toward a dominant CD4$^+$ Th-cell population with concomitant effector function.[103] It should be noted that CD4$^+$ Th cells can be plastic in nature as cells from different Th subpopulations may (transiently) share phenotypic and functional features.[103]

gut-draining lymph nodes and Peyer's patches using the lymphoid tissue homing receptors CD62L and chemokine receptor C-C motif receptor 7 (CCR7).[26] As depicted in Figure 2A, activation, proliferation and differentiation of naive CD4$^+$ T cells in gut-draining lymph nodes is dependent on cognate T-cell receptor (TCR) signaling (signal 1), co-stimulation (signal 2) and the soluble cytokines mostly provided by the APC (signal 3, see Figure 2).[27] After recognition of cognate antigen on the surface of APCs and during their differentiation,

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antigen-experienced CD4+ T cells downregulate CD62L and CCR7 and initiate expression of integrins and selectin ligands that allow selective migration to intestinal tissues.[28, 29] After priming, antigen-experienced CD4+ T cells exit the lymph node, enter the blood and migrate to the intestinal lamina propria or enter the epithelial layer where they reside as long-lived memory CD4+ T cells (Figure 1).

Signals from the APC and microenvironment in the MLN drive the acquisition of integrins and chemokine receptors required for preferential T-cell homing towards the intestine. CD4+ T cells activated in the MLN upregulate expression of the integrin $\alpha4\beta7$ and the chemokine C-C motif receptor 9 (CCR9).[30, 31] Intestinal APC-derived retinoic acid (RA) is a crucial factor promoting induction of $\alpha4\beta7$ and CCR9 on responding CD4+ T cells.[32, 33] Epithelial cells in the small intestine produce chemokine CCL25, the ligand for CCR9, and lamina propria venules express the integrin $\alpha4\beta7$ ligand mucosal vascular addressin cell adhesion molecule 1 (MADCAM1).[34-36] Hence, circulating CD4+ T cells that express both $\alpha4\beta7$ and CCR9 are recruited to the small intestinal lamina propria.[37] Expression of integrin $\alpha4\beta7$ also contributes to accumulation of CD4+ T cells in the large intestine.[38, 39] Chemokine receptors involved in regulation of CD4+ T-cell homing to the colon at steady state are less well defined, although recently, it was discovered that G protein coupled receptor 15 (GPR15) directs CD4+ T cells to the colon.[40, 41] During intestinal inflammation, CCR6 and its ligand CCL20 also contribute to CD4+ T-cell recruitment to the inflamed small and large intestine, demonstrating that CD4+ T cells can use alternative homing receptors during inflammation when compared to steady state.[42] Signals from the APC and MLN microenvironment determine the regionalization of the mucosally-imprinted CD4+ T-cell response irrespective of the T-cell phenotype and function. Thus, both regulatory and inflammatory CD4+ T cells are imprinted to home to the intestine.

The migration of mucosally-imprinted CD4+ T cells after their egress from gut-draining lymph nodes offers a unique opportunity to study ongoing intestinal CD4+ T-cell responses in peripheral blood. However, these mucosally-imprinted CD4+ T cells are not easily identified in peripheral blood. Using murine experiments we have established that proliferating antigen-specific CD4+ T cells in the MLN downregulate CD62L and upregulate CD38 expression. Intestinal APC-derived RA and TGF-β drive the reduction of CD62L and increase CD38 expression on differentiating mucosal CD4+ T cells. This demonstrates that, similar to $\alpha4\beta7$ and CCR9, the CD62L^{neg}CD38+ phenotype is regulated by signals from the APC and microenvironment.[43, 44] Imprinting of the CD62L^{neg}CD38+ phenotype is induced regardless of regulatory or inflammatory T-cell function, as suppression of Foxp3+ Treg differentiation does not affect the imprinting of the CD62L^{neg}CD38+ phenotype.[43]

In both mice and humans, the mucosally-imprinted CD62L^{neg}CD38+ phenotype is retained by CD4+ T cells in the circulation, allowing to distinguish these cells from other CD4+ effector T cells in the peripheral blood.[43, 45] In agreement with their microenvironmental

imprinting, circulating CD38+ effector T cells (defined as CD4+CD62L^{neg}CD38+), comprising 4-10% of the total CD4+ T-cell pool, are enriched in cells expressing the CCR9 and $\alpha4\beta7$ compared to CD38^{neg} effector T cells (defined as CD4+CD62L^{neg}CD38^{neg}).[43] Conversely, cells expressing the skin-homing receptor cutaneous leukocyte-associated antigen (CLA) are almost absent in the CD38+ effector T-cell population but enriched in the CD38^{neg} effector T-cell population. Moreover, specificity for intestinal luminal antigen is contained within this population, as after oral gluten challenge all gluten-specific CD4+ T cells in peripheral blood of celiac disease patients have the CD38+ effector phenotype.[43] This distinctive phenotype lowers the threshold for detection of intestinal antigen-specific CD4+ T cells and provides a new approach to non-invasively monitor intestinal CD4+ T-cell responses in peripheral blood.[43]

Intestinal CD4+ T-cell function: Regulatory Yin versus Inflammatory Yang.

Both regulatory and inflammatory CD4+ T cells differentiate from naive CD4+ T cells upon antigen encounter by intestinal APCs in the GALT and intestinal draining lymph nodes.[14, 16, 44, 46, 47] As a consequence, intestinal CD4+ T-cell populations can be functionally divided into regulatory and inflammatory populations. Mucosal application of a harmless antigen will not exclusively elicit regulatory CD4+ T-cell populations but also generate low frequencies of inflammatory CD4+ T-cell populations thus maintaining the capacity to eradicate harmless antigens upon breaching of the mucosal barrier. As such, a predominant regulatory immune response creates an overall tolerant state to contact with harmless antigens at steady state, while still allowing for the generation of an inflammatory immune response when tissue perpetration occurs.

Regulatory CD4+ T cells: "Yin"

The healthy intestinal lamina propria is home to a large number of CD4+ memory T cells with a regulatory phenotype, defined by their functional capacity to suppress an inflammatory T-cell response.[48] The majority of regulatory CD4+ T cells in the intestine are Foxp3+CD4+ regulatory T cells denoted as Tregs.[49] Tregs are classified into thymus-derived Treg (tTreg) and peripherally-derived Treg (pTreg), which differ in developmental origin and signals required for their development.[50] Thymus-derived Treg arise during CD4+ T-cell differentiation in the thymus under the influence of relatively high avidity interactions of the TCR with self-antigens. The nuclear factor Helios and cell surface protein neuropillin (NRP1) are constitutively expressed by tTreg.[51-53] Conversely, pTreg differentiate from naive CD4+ T cells after activation under tolerogenic conditions in secondary lymphoid tissues and do not express Helios and NRP1. Recent murine data has shown that intestinal Tregs can be divided into three subsets on the basis of RAR-related orphan receptor γt (RORγt) and GATA-binding protein 3 (GATA3) expression: microbiota-induced RORγt+ pTreg

(Helios^{neg}NRP1^{neg}), food antigen-induced RORγt^{neg} pTreg (Helios^{neg}NRP1^{neg}) and self-antigen induced GATA^{+} tTreg (Helios^{+}NRP1^{+}). These subsets illustrate that tTreg and pTreg have great functional diversity and non-redundant roles in orchestrating intestinal tolerance.[54-56]

The *in vivo* induction of pTreg is particularly important in the intestine. When compared to splenic DCs, DCs from the GALT and intestinal draining nodes such as MLN favor peripheral induction of pTregs in response to food and microbial antigens through TGF-β and, in the upper gastrointestinal tract, with RA-mediated signals.[14, 19, 44, 57-59] Colonization with commensal bacteria such as Altered Shaedler Flora (ASF) and fecal suspensions from specified pathogen free (SPF) mice increases the frequency of Foxp3^{+} cells in the total CD4^{+} T-cell pool.[60-62] Most of the colonic Tregs in conventionally-housed mice express low levels of Helios, indicating that these Tregs are likely of peripheral origin.[60, 63] Interestingly, analysis of the colonic TCR repertoire of Tregs demonstrated that colonic Treg cells utilize TCRs that are unique to the colon and different from those used in other organs.[63] The majority of colonic Treg TCRs recognized colonic contents or bacterial isolates indigenous to the mouse colony.[63] These colonic Treg TCRs did not facilitate tTreg-cell development after intrathymic transfer, inferring that colonic antigen-specific Tregs differentiate from naive CD4^{+} T cells peripherally in the intestine in response to microbiota of the host.[63]

Evidence for a key role for Foxp3^{+} Treg in maintenance of tolerance to intestinal microbiota came from the T-cell transfer mouse model.[64-72] In this model, transfer of naive CD45RB^{high}CD4^{+} T cells isolated from the spleens of healthy donor mice into immunodeficient SCID mice causes a chronic intestinal inflammation with characteristics similar to those of human IBD.[65] Cotransfer of the reciprocal mature CD45RB^{low} subset prevents colitis development.[65] The intestinal inflammation in this murine model is dependent upon the presence of bacteria, as it does not occur under germ-free conditions and can be ameliorated by antibiotic treatment of the SCID recipients.[71] Together, these data demonstrate that the healthy immune system contains microbiota-specific inflammatory CD4^{+} T cells that are normally regulated by regulatory CD4^{+} T cells. Further phenotypic characterization of the regulatory CD4^{+} T cells present in the CD45RB^{low} subset showed that they expressed high levels of CD25 and expressed the transcription factor Foxp3.[22, 23, 67] It is now widely accepted that Tregs are crucial to antagonize inflammatory CD4^{+} T cells in the intestine through multiple mechanisms, including IL-2 scavenging, the production of the cytokines such as IL-10[66], IL-35[73] and TGF-β[69, 70], and high expression of coinhibitory receptors such as cytotoxic lymphocyte antigen 4 (CTLA-4) and programmed cell death-1 (PD-1).[67, 68] In humans, mutations affecting the *Foxp3* gene result in immune dysregulation, polyendocrinopathy, enteropathy, and X-linked syndrome (IPEX).[74, 75] Although IPEX patients display autoimmunity in multiple organs, almost all individuals with IPEX develop autoimmune enteropathy, emphasizing the key role of Tregs in preventing intestinal inflammation.[76, 77]

GENERAL INTRODUCTION AND OUTLINE OF THIS THESIS

The regulatory cytokine IL-10 has an essential role in maintaining intestinal homeostasis, as mice genetically deficient in IL-10 develop severe intestinal inflammation.[78] Under conventional housing conditions, IL-10-deficient mice develop microbiota dependent inflammation in both the small and large intestine, demonstrating a clear role for IL-10 in both intestinal compartments.[78] Under SPF conditions, disease in IL-10-deficient mice is limited to the colon and the presence of *Helicobacter hepaticus* is required for colitis development.[79, 80] Under germfree conditions, the development of colitis is not observed in IL-10-deficient mice.[81] Together, these data show that stimulation of the immune system by commensal microbiota is critical for the development of disease in IL-10-deficient mice. In humans, the essential role of IL-10 in intestine inflammation is clearly demonstrated in patients with defects in IL-10 signaling, who tend to develop colitis with an even earlier onset and higher penetrance than IPEX patients.[82-85]

The production of IL-10 by Tregs is essential to restrain local inflammation in the intestine, as Treg-cell specific ablation of a conditional IL-10 allele (induced by Cre recombinase knocked into the Foxp3 gene locus) causes spontaneous colitis but no systemic autoimmunity.[86] The capacity of Tregs to produce IL-10 is likely facilitated by the environment at mucosal interfaces, as intestinal Treg produce higher levels of IL-10 compared to Tregs from the spleen and other organs.[60] In addition to Tregs, during intestinal inflammation IL-10 can be produced by myeloid cells[87-89], including macrophages and DCs, and other adaptive immune cells, including B cells and CD4^{+}Foxp3^{neg} T-cell populations. In the T-cell transfer model of colitis, IL-10 produced by CD11b^{+} myeloid cells in recipient hosts is needed to prevent induction of colitis.[88] This demonstrates that, in addition to Treg-mediated production of IL-10, IL-10 production by innate immune cells is critical for regaining mucosal homeostasis after transient inflammation. Recent data have shown that IL-10 primarily acts on APCs as deficient IL-10 receptor signaling in APCs causes *Helicobacter* induced colitis which is not compensated by IL-10 signaling in T cells.[90-92] Loss of IL-10R-dependent signaling in APCs elicits differentiation of inflammatory CD4^{+} T cells that are colitogenic.[90-92] Taken together, these data highlight the crucial role of IL-10 in intestinal Foxp3^{+} Treg effector function and ensuing maintenance of tolerance to commensal microbiota.

However, not only Foxp3^{+} Treg secrete IL-10 in the intestine. The best studied IL-10-producing CD4^{+}Foxp3^{neg} T-cell population are T regulatory type 1 (Tr1) cells, which have been shown to inhibit inflammatory T-cell responses and colitis in an IL-10-dependent manner.[93-95] Underscoring the regional differences in intestinal immune responses, Tregs and IL-10-producing CD4^{+}Foxp3^{neg} T cells exert regulatory function in different intestinal compartments. In the colonic lamina propria, nearly all IL-10 producing cells are Foxp3^{+} Tregs. Conversely, in the small intestine, CD4^{+}Foxp3^{neg} T cells are the predominant source of IL-10.[49, 96-98] In line with this compartmentalization of intestinal regulatory T-cell subsets,

mice that have a specific deletion of IL-10 in Foxp3$^+$ Treg develop inflammation specifically in the colon, but not in the small intestine.[86] This illustrates that CD4$^+$Foxp3$^+$ Tregs and IL-10-producing CD4$^+$Foxp3^{neg} T cells carry out nonredundant functions at different intestinal locations. In the T-cell transfer model of colitis, both Foxp3$^+$ Tregs and Tr1 cells are able to prevent colitis[93, 99, 100], demonstrating that both subtypes have the functional capacity to suppress inflammatory CD4$^+$ T cells irrespective of the inflammatory location. Therefore, the compartmentalization of Foxp3$^+$ Treg and IL-10-producing CD4$^+$Foxp3^{neg} T cells in the intestine may be a result of the difference in the environment during the phase of regulatory CD4$^+$ T-cell induction.[49] In line with this hypothesis, repetitive anti-CD3 treatment induces Tr1-like cells in the epithelial compartment of the small intestine, whereas the same treatment induces mostly Foxp3$^+$ Tregs in the lamina propria of the colon.[97] What type of inflammatory immune response is preferentially regulated by Foxp3$^+$ Tregs, Tr1 cells or both is currently not clear.

Altogether, immune tolerance in the intestine is orchestrated by a cooperative network of several populations of regulatory CD4$^+$ T cells, including Foxp3$^+$ Tregs and Foxp3^{neg}CD4$^+$ T-cell populations, with a prominent role for Foxp3$^+$ Treg cells in the colon and Foxp3^{neg}CD4$^+$ T cells in the small intestine. Both populations likely use multiple mechanisms to suppress inflammatory immune responses, the best characterized of which involves IL-10 production.

Inflammatory CD4$^+$ T cells: "Yang"

In addition to regulatory CD4$^+$T-cell subsets, the lamina propria harbors diverse populations of pro-inflammatory effector CD4$^+$T cells that are critically required for adequate responses to microbial challenges as occur upon extensive translocation of commensal microbiota, or trespassing pathogenic bacteria, viruses or fungi. Upon activation through TCR signaling in intestinal draining lymph nodes and/or GALT, naive CD4$^+$ T cells can differentiate into different subsets of inflammatory effector CD4$^+$T cells, denoted as CD4$^+$T helper (Th) cells: Th1, Th2 and Th17. The cytokines and transcription factors required for the differentiation of Th1, Th2 and Th17 cells are summarized in Figure 2B. Classically, each Th cell subset is associated with predominance of specific effector cytokines. Th1 cells predominantly produce the inflammatory cytokines interferon gamma (IFNγ), tumor necrosis factor alpha (TNFα) and IL-12 and participate in host defense against intracellular bacteria and viruses; Th2 cells predominantly secrete IL-4, IL-5 and IL-13 and defend against parasitic helminths; and Th17 predominantly produce IL-17, IL-21 and IL-22 and are specialized for responses to extracellular bacteria and fungi.[101, 102] Although this categorization is helpful to study Th-cell responses in complex inflammation, these subsets should not be considered separate cell lineages as functional plasticity is maintained within these cells after their differentiation. This allows cells of one Th subset to transform to another under particular

environmental conditions during antigen recognition in tissues.[103]

As both Th1 as well as Th2 and Th17 cells have been implicated in IBD[104], effector cytokines such as IFNγ and IL-17 are often used to identify pro-inflammatory CD4$^+$ T cells in settings of intestinal inflammation. However, recent evidence suggests that effector CD4$^+$ T-cell responses to commensals can also support intestinal homeostasis by producing barrier-protective cytokines.[105, 106] For example, while the Th17-associated IL-23-IL-17 axis is thought to play a role in many autoimmune and chronic inflammatory diseases[107], Th17 cells can cooperate with Treg to promote the repair of damaged epithelial barrier during colitis.[106, 108]

Thus, in the healthy intestinal lamina propria, inflammatory CD4$^+$ T cells are present that are essential to eliminate invasive mucosal pathogens and control resident commensal microbiota. However, infiltration of the lamina propria by inflammatory CD4$^+$ T-cell populations is a key characteristic of chronic intestinal inflammation.[1] Therefore, a tightly controlled balance between regulatory and inflammatory CD4$^+$ T-cell populations is crucial to prevent uncontrolled CD4$^+$ T-cell responses and subsequent intestinal tissue damage.[104]

Inflammatory Bowel Disease: Disrupted Balance of Intestinal Immune Responses to Commensal Bacteria.

Defects in intestinal immune regulation lead to immunopathology such as IBD. The two most prevalent clinical forms of IBD, Crohn's disease (CD) and ulcerative colitis (UC), can have similar symptoms including abdominal pain, rectal bleeding and diarrhea, but differ with respect to histopathological features. UC affects the colon and is a superficial ulcerative disease, whereas CD is a granulomatous disorder with transmural inflammation that can affect any part of the gastrointestinal tract. IBD impacts every aspect of the affected individual's life, and can result in significant long term morbidity, including the risk for surgery and hospitalization, cancer and mortality. Currently, an estimated 3 million people in Europe are affected by IBD, and 5-15 per 100,000 individuals are diagnosed with IBD per year.[109] Given the continuing increase in IBD incidence worldwide and the lack of a permanent cure, the number of patients suffering from IBD is expected to increase even further over the coming decades.[109] In particular, incidence rates of IBD continue to increase in pediatric and adolescent age-groups[110], who may have a more aggressive and a more complicated clinical course than adult-onset IBD.[111, 112]

Even within the two clinical subgroups of CD and UC, presentation of IBD is heterogeneous and varies in terms of clinical symptoms, location of intestinal inflammation, disease extent and severity, presence of extra-intestinal manifestations and response to therapy. Over the past decades, this heterogeneity was confirmed by immunological studies and genome-wide association studies. A total of 163 IBD susceptibility loci have

been associated with IBD.[113] For CD, these studies have identified associations with defects in the processing of intracellular bacteria, autophagy and innate immunity (i.e. *NOD2, ATG16L1*), whereas genetic evidence in UC has suggested involvement of intestinal barrier function (i.e. *HNF4A, LAMB1*).[113, 114] It has now become clear that complex interactions between the immune system, intestinal microbiota, the environment and host genotype are likely involved the development of IBD.[115] Although the precise etiology may differ per patient, the current theory is that IBD is caused by a dysregulated immune response to antigens of the intestinal bacteria in a genetically susceptible host.

Both insufficient host defense as well as insufficient immune regulation can result in dysregulated immune responses to bacterial antigens. For example, insufficient host defense occurs in chronic granulomatous disease (CGD), a primary immunodeficiency caused by genetic defects in components of the phagocyte nicotinamide adenine dinucleotide phosphate (NAPDH) oxidase complex that is essential for killing phagocytosed bacteria. In CGD patients, impaired killing of bacteria after phagocytosis causes reduced bacterial clearance which leads to IL-1b activation and secondary hyperinflammation. As a result, up to 40% of patients with CGD develop intestinal inflammation.[116] An example of insufficient immune regulation leading to intestinal inflammation is seen in patients with IPEX syndrome,Foxp3 deficiency, who develop early-onset autoimmune enteropathy, insulin-dependent diabetes and eczema due to a primary hyperactivation of $CD4^+$ T cells. Although the histological presentation of the autoimmune enteropathy is variable, a graft-versus-host disease-like pattern associated with positive anti-enterocyte antibodies is the most frequent intestinal presentation of IPEX syndrome.[76] Thus, both insufficient host defense (i.e. CGD) as well as insufficient immune regulation (i.e. IPEX) can result in a destructive inflammatory immune response in the intestine.

Regardless of the specific initiating factor and underlying defective immune mechanism, a common disease denominator in all patients is the infiltration of inflammatory $CD4^+$ T cells in intestinal tissue. Given the unique ability of T cells to exert memory to previously encountered antigens[117], the infiltration of $CD4^+$ T cells is a critical step in the chronicity of the disease. The combination of inflammatory $CD4^+$ T cells together with the persistence of commensal microbiota causes a relapsing-remitting disease course that is characteristic for many T-cell mediated inflammatory diseases, including IBD.

Evidence for the Role of Microbiota in IBD

Several lines of evidence support the hypothesis that microbiota are an essential factor in maintaining intestinal inflammation in IBD. Supporting a role of bacteria in human disease, T cells isolated from the intestine of IBD patients proliferate in response to autologous intestinal bacteria, unlike intestinal T cells from healthy individuals.[4] In addition, the fecal stream is critical for disease development and recurrence, whereas antibiotic therapy can induce disease remission and prevent disease relapse.[118-120] Consequently, a lot of effort has been invested in identifying bacterial antigens that are recognized by infiltrating $CD4^+$ T cells in IBD. Interestingly, experimental studies have suggested a selectivity of the immune response to a relatively small number of dominant bacterial antigens, including bacterial flagellin.[121-123] Understanding which bacterial antigens are recognized by $CD4^+$ T cells in IBD is important to provide insight into the regional distribution of the disease and can possibly identify specific IBD-associated bacteria that can be targeted for therapy. In addition, analysis of bacteria-reactive $CD4^+$ T-cell responses is required to establish whether $CD4^+$ T-cell responses are inappropriate in some patients, but protective in others.

Towards Precision Therapy in IBD: Treating Defective Immune Pathways

Despite the likely importance of microbiota in the pathogenesis of IBD, therapy focusses on suppressing the immune system rather than removing the antigen that might be responsible for the aberrant immune response. Classical IBD treatment have broad, non-specific effects on the immune system, and a disadvantage of these approaches is the suppression of both innate and adaptive immunity. Targeted anti-TNFα medications are now considered the most efficacious therapies available for the management of IBD, but not all patients will respond and many will lose response overtime.[124] Disease heterogeneity likely contributes to the difficulties of achieving high response rates within a heterogeneous patient population.[125] Detailed insight in the immune responses occurring in IBD patients would enable patient stratification based on underlying defective immune mechanisms that drive disease. This strategy is required to select the most appropriate treatment for each patient and is therefore pivotal for the successful development of new therapies.

AIM AND OUTLINE OF THIS THESIS

The aim of this thesis is to identify immune regulatory processes that are pivotal for intestinal homeostasis and to yield parameters that classify immunological disease in IBD patients. In addition, by combining immunological disease profiling with extensive clinical characterization of each patient, the research presented in this thesis aims to identify which clinical and immunological factors can be used to predict disease course and response to therapy.

$CD4^+$ T cells play a key role in the pathogenesis of IBD. The intestinal pathology is characterized by infiltration of $CD4^+$ T cells that secrete large amounts of pro-inflammatory cytokines. However, mechanisms leading to this exaggerated $CD4^+$ T-cell response remain largely obscure. In **Chapter 2**, we describe how emerging clinical observations in human

cancer treatment have provided new insight into the critical role of co-inhibitory receptors in the maintenance of intestinal homeostasis. In addition, we provide insight in how co-inhibitory receptor expression may contribute to IBD patient stratification and how stimulating co-inhibitory pathways may offer new opportunities to treat chronic intestinal inflammation.

Monitoring loss of balance between inflammatory and regulatory intestinal $CD4^+$ T-cell responses in IBD patients is highly desired to classify patients and predict their disease course but is difficult as endoscopy is too invasive to routinely be used. In **Chapter 3**, we determined whether regulatory and inflammatory phenotypes of circulating $CD38^+$ effector T cells ($CD62L^{neg}CD4^+$), a population enriched for cells with mucosal antigen specificity, classify disease course in pediatric-onset IBD patients. Thereto, by using transcriptomics and *in vitro* cultures, we identified novel $CD38^+$ cell-expressed regulatory proteins suitable for disease monitoring. In addition, we performed flow cytometric analysis on peripheral blood of pediatric-onset IBD patients before start of treatment (at disease diagnosis) and during longitudinal follow up.

The results presented in **Chapter 3** suggest that expression of the coinhibitory receptor T cell immunoglobulin and ITIM domain (TIGIT) on circulating $CD38^+$ effector T cells is altered in a subgroup of pediatric-onset IBD patients who are at risk of early disease relapse. As TIGIT has been shown to limit T-cell driven inflammation, a better understanding of the mechanisms involved in establishing and maintaining TIGIT expression on $CD4^+$ T cells would aid in the design of novel immunotherapies with the potential to modify or re-balance the immune system. In **Chapter 4**, we studied factors involved in the induction of TIGIT expression on murine and human $CD4^+$ T cells and investigated the role of $TIGIT^+$ cells in regulating immune responses to intestinal bacteria.

The chronic intestinal inflammation in IBD is thought to be maintained by inflammatory $CD4^+$ effector T cells that have specificity for microbial antigens.[4, 126, 127] However, the microbial antigens recognized by these $CD4^+$ T cells remain unknown. Elevated humoral responses to bacterial flagellin, a bacterial protein expressed by both commensal and pathogenic bacteria, are present in a subgroup of CD patients at risk for aggressive and complicated disease.[122, 128-131] In **Chapter 5**, we tested whether elevated anti-flagellin IgG in CD patients may reflect increased activation of flagellin-specific $CD4^+$ T cells. Using a novel approach to identify flagellin-reactive $CD4^+$ T cells in peripheral blood, we determined the frequencies of circulating flagellin-reactive $CD4^+$ T cells in treatment-naive CD patients with elevated flagellin-specific antibodies. In addition, we aimed to evaluate whether flagellin-reactive $CD4^+$ T cells in CD patients have altered effector cytokine production and coinhibitory receptor expression compared to healthy individuals.

Monogenic defects, such as the *IL10, IL10R* and *Foxp3* loss-of-function mutations causing very early onset IBD, have uncovered pathways that are essential to prevent intestinal inflammation.[75, 82] In **Chapter 6** we investigated the functional consequences of

increased IL-2 signaling on intestinal immune responses. Hereto, we have characterized the immune function of a patient with a *de novo* duplication of the 10p15.1 chromosomal region, including the *IL2RA* gene, who developed therapy-resistant very early onset colitis at 2 years of age.

Recent advances in medical therapy for IBD have led to the use of steroid sparing strategies that entail long-term and even life-long immune suppression. Although immune suppression in IBD may reduce the risk of disease complications, surgery and disease-associated tumors such as bowel adenocarcinoma, it is associated with an increased risk of treatment-associated malignancies and opportunistic infections. Given the rarity of some of these events, more detailed information on pediatric-onset IBD patients who develop cancer or have a fatal outcome is needed to obtain more insight in predictive factors of severe outcomes. In **Chapter 7**, we provide a literature overview of patients with pediatric-onset IBD patients who developed cancer or suffered a fatal outcome at any point later in life. In **Chapter 8**, we report the results of a large prospective multinational collaboration, which aimed to describe the most common causes of mortality, types of cancer and previous or current therapy among children and young adults with pediatric-onset IBD. In addition, we also investigated the relationship between severe outcomes, disease characteristics and treatment exposure.

Available evidence and results presented in **Chapter 8** demonstrate that a concomitant diagnosis of sclerosing cholangitis (SC) is a significant risk factor for cancer-associated mortality in patients with pediatric-onset IBD. SC may be easily overlooked, as symptoms are often nonspecific and intestinal disease is frequently more prominent in patients with concomitant IBD. Prognostic factors for complicated disease would allow physicians to include high-risk IBD patients with concomitant SC in an intensified follow-up program aimed at prevention or early detection of complications. In **Chapter 9**, we therefore aimed to identify factors present at SC diagnosis that are associated with development of early hepatobiliary complications in children with IBD-associated SC.

Finally, **Chapter 10** provides a general discussion of the data described in this thesis and highlights results that could be of specific interest for future research.

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REFERENCES

1. Shale M, Schiering C, Powrie F. CD4(+) T-cell subsets in intestinal inflammation. Immunol Rev 2013;252:164-82.
2. Preza GC, Yang OO, Elliott J, et al. T lymphocyte density and distribution in human colorectal mucosa, and inefficiency of current cell isolation protocols. PLoS One 2015;10:e0122723.
3. Mowat AM. To respond or not to respond - a personal perspective of intestinal tolerance. Nat Rev Immunol 2018;18:405-415.
4. Duchmann R, Kaiser I, Hermann E, et al. Tolerance exists towards resident intestinal flora but is broken in active inflammatory bowel disease (IBD). Clin Exp Immunol 1995;102:448-55.
5. Agace WW, McCoy KD. Regionalized Development and Maintenance of the Intestinal Adaptive Immune Landscape. Immunity 2017;46:532-548.
6. Mowat AM, Agace WW. Regional specialization within the intestinal immune system. Nat Rev Immunol 2014;14:667-85.
7. Mabbott NA, Donaldson DS, Ohno H, et al. Microfold (M) cells: important immunosurveillance posts in the intestinal epithelium. Mucosal Immunol 2013;6:666-77.
8. Gutzeit C, Magri G, Cerutti A. Intestinal IgA production and its role in host-microbe interaction. Immunol Rev 2014;260:76-85.
9. Roy MJ, Varvayanis M. Development of dome epithelium in gut-associated lymphoid tissues: association of IgA with M cells. Cell Tissue Res 1987;248:645-51.
10. Niess JH, Brand S, Gu X, et al. CX3CR1-mediated dendritic cell access to the intestinal lumen and bacterial clearance. Science 2005;307:254-8.
11. Schulz O, Jaensson E, Persson EK, et al. Intestinal CD103+, but not CX3CR1+, antigen sampling cells migrate in lymph and serve classical dendritic cell functions. J Exp Med 2009;206:3101-14.
12. Anjuere F, Luci C, Lebens M, et al. In vivo adjuvant-induced mobilization and maturation of gut dendritic cells after oral administration of cholera toxin. J Immunol 2004;173:5103-11.
13. Johansson-Lindbom B, Svensson M, Pabst O, et al. Functional specialization of gut CD103+ dendritic cells in the regulation of tissue-selective T cell homing. J Exp Med 2005;202:1063-73.
14. Veenbergen S, van Berkel LA, du Pre MF, et al. Colonic tolerance develops in the iliac lymph nodes and can be established independent of CD103(+) dendritic cells. Mucosal Immunol 2016;9:894-906.
15. Scott CL, Bain CC, Wright PB, et al. CCR2(+)CD103(-) intestinal dendritic cells develop from DC-committed precursors and induce interleukin-17 production by T cells. Mucosal Immunol 2015;8:327-39.
16. Mayer JU, Demiri M, Agace WW, et al. Different populations of CD11b(+) dendritic cells drive Th2 responses in the small intestine and colon. Nat Commun 2017;8:15820.
17. Coombes JL, Powrie F. Dendritic cells in intestinal immune regulation. Nat Rev Immunol 2008;8:435-46.
18. Iwasaki A, Kelsall BL. Freshly isolated Peyer's patch, but not spleen, dendritic cells produce interleukin 10 and induce the differentiation of T helper type 2 cells. J Exp Med 1999;190:229-39.
19. Sun CM, Hall JA, Blank RB, et al. Small intestine lamina propria dendritic cells promote de novo generation of Foxp3 T reg cells via retinoic acid. J Exp Med 2007;204:1775-85.
20. Mucida D, Park Y, Kim G, et al. Reciprocal TH17 and regulatory T cell differentiation mediated by retinoic acid. Science 2007;317:256-60.
21. Mottet C, Uhlig HH, Powrie F. Cutting edge: cure of colitis by CD4+CD25+ regulatory T cells. J Immunol 2003;170:3939-43.
22. Khattri R, Cox T, Yasayko SA, et al. An essential role for Scurfin in CD4+CD25+ T regulatory cells. Nat Immunol 2003;4:337-42.
23. Fontenot JD, Gavin MA, Rudensky AY. Foxp3 programs the development and function of CD4+CD25+ regulatory T cells. Nat Immunol 2003;4:330-6.
24. Hori S, Nomura T, Sakaguchi S. Control of regulatory T cell development by the transcription factor Foxp3. Science 2003;299:1057-61.
25. Zigmond E, Varol C, Farache J, et al. Ly6C hi monocytes in the inflamed colon give rise to proinflammatory effector cells and migratory antigen-presenting cells. Immunity 2012;37:1076-90.
26. Warnock RA, Askari S, Butcher EC, et al. Molecular mechanisms of lymphocyte homing to peripheral lymph nodes. J Exp Med 1998;187:205-16.

27. Mueller DL, Jenkins MK, Schwartz RH. An accessory cell-derived costimulatory signal acts independently of protein kinase C activation to allow T cell proliferation and prevent the induction of unresponsiveness. J Immunol 1989;142:2617-28.
28. Kantele A, Zivny J, Hakkinen M, et al. Differential homing commitments of antigen-specific T cells after oral or parenteral immunization in humans. J Immunol 1999;162:5173-7.
29. Johansson-Lindbom B, Svensson M, Wurbel MA, et al. Selective generation of gut tropic T cells in gut-associated lymphoid tissue (GALT): requirement for GALT dendritic cells and adjuvant. J Exp Med 2003;198:963-9.
30. Hamann A, Andrew DP, Jablonski-Westrich D, et al. Role of alpha 4-integrins in lymphocyte homing to mucosal tissues in vivo. J Immunol 1994;152:3282-93.
31. Svensson M, Marsal J, Ericsson A, et al. CCL25 mediates the localization of recently activated CD8alphabeta(+) lymphocytes to the small-intestinal mucosa. J Clin Invest 2002;110:1113-21.
32. Iwata M, Hirakiyama A, Eshima Y, et al. Retinoic acid imprints gut-homing specificity on T cells. Immunity 2004;21:527-38.
33. Mora JR, Bono MR, Manjunath N, et al. Selective imprinting of gut-homing T cells by Peyer's patch dendritic cells. Nature 2003;424:88-93.
34. Berlin C, Berg EL, Briskin MJ, et al. Alpha 4 beta 7 integrin mediates lymphocyte binding to the mucosal vascular addressin MAdCAM-1. Cell 1993;74:185-95.
35. Kunkel EJ, Campbell JJ, Haraldsen G, et al. Lymphocyte CC chemokine receptor 9 and epithelial thymus-expressed chemokine (TECK) expression distinguish the small intestinal immune compartment: Epithelial expression of tissue-specific chemokines as an organizing principle in regional immunity. J Exp Med 2000;192:761-8.
36. Briskin M, Winsor-Hines D, Shyjan A, et al. Human mucosal addressin cell adhesion molecule-1 is preferentially expressed in intestinal tract and associated lymphoid tissue. Am J Pathol 1997;151:97-110.
37. Habtezion A, Nguyen LP, Hadeiba H, et al. Leukocyte Trafficking to the Small Intestine and Colon. Gastroenterology 2016;150:340-54.
38. Wang C, Hanly EK, Wheeler LW, et al. Effect of alpha4beta7 blockade on intestinal lymphocyte subsets and lymphoid tissue development. Inflamm Bowel Dis 2010;16:1751-62.
39. Picarella D, Hurlbut P, Rottman J, et al. Monoclonal antibodies specific for beta 7 integrin and mucosal addressin cell adhesion molecule-1 (MAdCAM-1) reduce inflammation in the colon of scid mice reconstituted with CD45RBhigh CD4+ T cells. J Immunol 1997;158:2099-106.
40. Kim SV, Xiang WV, Kwak C, et al. GPR15-mediated homing controls immune homeostasis in the large intestine mucosa. Science 2013;340:1456-9.
41. Nguyen LP, Pan J, Dinh TT, et al. Role and species-specific expression of colon T cell homing receptor GPR15 in colitis. Nat Immunol 2015;16:207-213.
42. Oyama T, Miura S, Watanabe C, et al. CXCL12 and CCL20 play a significant role in mucosal T-lymphocyte adherence to intestinal microvessels in mice. Microcirculation 2007;14:753-66.
43. du Pre MF, van Berkel LA, Raki M, et al. 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;106:1147-59.
44. Hauet-Broere F, Unger WW, Garssen J, et al. Functional CD25- and CD25+ mucosal regulatory T cells are induced in gut-draining lymphoid tissue within 48 h after oral antigen application. Eur J Immunol 2003;33:2801-10.
45. van Leeuwen MA, du Pre MF, van Wanrooij RL, et al. Changes in natural Foxp3(+)Treg but not mucosally-imprinted CD62L(neg)CD38(+)Foxp3(+)Treg in the circulation of celiac disease patients. PLoS One 2013;8:e68432.
46. Zinselmeyer BH, Dempster J, Gurney AM, et al. In situ characterization of CD4+ T cell behavior in mucosal and systemic lymphoid tissues during the induction of oral priming and tolerance. J Exp Med 2005;201:1815-23.
47. Houston SA, Cerovic V, Thomson C, et al. The lymph nodes draining the small intestine and colon are anatomically separate and immunologically distinct. Mucosal Immunol 2016;9:468-78.
48. Tanoue T, Atarashi K, Honda K. Development and maintenance of intestinal regulatory T cells. Nat Rev Immunol 2016;16:295-309.
49. Veenbergen S, Samsom JN. Maintenance of small intestinal and colonic tolerance by IL-10-producing regulatory T cell subsets. Curr Opin Immunol 2012;24:269-76.

50. Abbas AK, Benoist C, Bluestone JA, et al. Regulatory T cells: recommendations to simplify the nomenclature. Nat Immunol 2013;14:307-8.
51. Yadav M, Louvet C, Davini D, et al. Neuropilin-1 distinguishes natural and inducible regulatory T cells among regulatory T cell subsets in vivo. J Exp Med 2012;209:1713-22, S1-19.
52. Weiss JM, Bilate AM, Gobert M, et al. Neuropilin 1 is expressed on thymus-derived natural regulatory T cells, but not mucosa-generated induced Foxp3+ T reg cells. J Exp Med 2012;209:1723-42, S1.
53. Thornton AM, Korty PE, Tran DQ, et al. Expression of Helios, an Ikaros transcription factor family member, differentiates thymic-derived from peripherally induced Foxp3+ T regulatory cells. J Immunol 2010;184:3433-41.
54. Yang BH, Hagemann S, Mamareli P, et al. Foxp3(+) T cells expressing RORgammat represent a stable regulatory T-cell effector lineage with enhanced suppressive capacity during intestinal inflammation. Mucosal Immunol 2016;9:444-57.
55. Ohnmacht C, Park JH, Cording S, et al. MUCOSAL IMMUNOLOGY. The microbiota regulates type 2 immunity through RORgammat(+) T cells. Science 2015;349:989-93.
56. Sefik E, Geva-Zatorsky N, Oh S, et al. MUCOSAL IMMUNOLOGY. Individual intestinal symbionts induce a distinct population of RORgamma(+) regulatory T cells. Science 2015;349:993-7.
57. Coombes JL, Siddiqui KR, Arancibia-Carcamo CV, et al. A functionally specialized population of mucosal CD103+ DCs induces Foxp3+ regulatory T cells via a TGF-beta and retinoic acid-dependent mechanism. J Exp Med 2007;204:1757-64.
58. Fantini MC, Becker C, Monteleone G, et al. Cutting edge: TGF-beta induces a regulatory phenotype in CD4+CD25- T cells through Foxp3 induction and down-regulation of Smad7. J Immunol 2004;172:5149-53.
59. Tran DQ, Ramsey H, Shevach EM. Induction of Foxp3 expression in naive human CD4+Foxp3 T cells by T-cell receptor stimulation is transforming growth factor-beta dependent but does not confer a regulatory phenotype. Blood 2007;110:2983-90.
60. Atarashi K, Tanoue T, Shima T, et al. Induction of colonic regulatory T cells by indigenous Clostridium species. Science 2011;331:337-41.
61. Geuking MB, Cahenzli J, Lawson MA, et al. Intestinal bacterial colonization induces mutualistic regulatory T cell responses. Immunity 2011;34:794-806.
62. Gaboriau-Routhiau V, Rakotobe S, Lecuyer E, et al. The key role of segmented filamentous bacteria in the coordinated maturation of gut helper T cell responses. Immunity 2009;31:677-89.
63. Lathrop SK, Bloom SM, Rao SM, et al. Peripheral education of the immune system by colonic commensal microbiota. Nature 2011;478:250-4.
64. Powrie F, Mauze S, Coffman RL. CD4+ T-cells in the regulation of inflammatory responses in the intestine. Res Immunol 1997;148:576-81.
65. Powrie F, Leach MW, Mauze S, et al. Phenotypically distinct subsets of CD4+ T cells induce or protect from chronic intestinal inflammation in C. B-17 scid mice. Int Immunol 1993;5:1461-71.
66. Asseman C, Mauze S, Leach MW, et al. An essential role for interleukin 10 in the function of regulatory T cells that inhibit intestinal inflammation. J Exp Med 1999;190:995-1004.
67. Read S, Malmstrom V, Powrie F. Cytotoxic T lymphocyte-associated antigen 4 plays an essential role in the function of CD25(+)CD4(+) regulatory cells that control intestinal inflammation. J Exp Med 2000;192:295-302.
68. Read S, Greenwald R, Izcue A, et al. Blockade of CTLA-4 on CD4+CD25+ regulatory T cells abrogates their function in vivo. J Immunol 2006;177:4376-83.
69. Nakamura K, Kitani A, Fuss I, et al. TGF-beta 1 plays an important role in the mechanism of CD4+CD25+ regulatory T cell activity in both humans and mice. J Immunol 2004;172:834-42.
70. Powrie F, Carlino J, Leach MW, et al. A critical role for transforming growth factor-beta but not interleukin 4 in the suppression of T helper type 1-mediated colitis by CD45RB(low) CD4+ T cells. J Exp Med 1996;183:2669-74.
71. Aranda R, Sydora BC, McAllister PL, et al. Analysis of intestinal lymphocytes in mouse colitis mediated by transfer of CD4+, CD45RBhigh T cells to SCID recipients. J Immunol 1997;158:3464-73.
72. De Winter H, Cheroutre H, Kronenberg M. Mucosal immunity and inflammation. II. The yin and yang of T cells in intestinal inflammation: pathogenic and protective roles in a mouse colitis model. Am J Physiol 1999;276:G1317-21.

73. Wirtz S, Billmeier U, McHedlidze T, et al. Interleukin-35 mediates mucosal immune responses that protect against T-cell-dependent colitis. Gastroenterology 2011;141:1875-86.
74. Chatila TA, Blaeser F, Ho N, et al. JM2, encoding a fork head-related protein, is mutated in X-linked autoimmunity-allergic disregulation syndrome. J Clin Invest 2000;106:R75-81.
75. Bennett CL, Christie J, Ramsdell F, et al. The immune dysregulation, polyendocrinopathy, enteropathy, X-linked syndrome (IPEX) is caused by mutations of Foxp3. Nat Genet 2001;27:20-1.
76. Patey-Mariaud de Serre N, Canioni D, Ganousse S, et al. Digestive histopathological presentation of IPEX syndrome. Mod Pathol 2009;22:95-102.
77. Barzaghi F, Passerini L, Bacchetta R. Immune dysregulation, polyendocrinopathy, enteropathy, x-linked syndrome: a paradigm of immunodeficiency with autoimmunity. Front Immunol 2012;3:211.
78. Kuhn R, Lohler J, Rennick D, et al. Interleukin-10-deficient mice develop chronic enterocolitis. Cell 1993;75:263-74.
79. Kullberg MC, Ward JM, Gorelick PL, et al. Helicobacter hepaticus triggers colitis in specific-pathogen-free interleukin-10 (IL-10)-deficient mice through an IL-12- and gamma interferon-dependent mechanism. Infect Immun 1998;66:5157-66.
80. Berg DJ, Davidson N, Kuhn R, et al. Enterocolitis and colon cancer in interleukin-10-deficient mice are associated with aberrant cytokine production and CD4(+) TH1-like responses. J Clin Invest 1996;98:1010-20.
81. Sellon RK, Tonkonogy S, Schultz M, et al. Resident enteric bacteria are necessary for development of spontaneous colitis and immune system activation in interleukin-10-deficient mice. Infect Immun 1998;66:5224-31.
82. Glocker EO, Kotlarz D, Boztug K, et al. Inflammatory bowel disease and mutations affecting the interleukin-10 receptor. N Engl J Med 2009;361:2033-45.
83. Uhlig HH, Schwerd T, Koletzko S, et al. The diagnostic approach to monogenic very early onset inflammatory bowel disease. Gastroenterology 2014;147:990-1007 e3.
84. Shouval DS, Ouahed J, Biswas A, et al. Interleukin 10 receptor signaling: master regulator of intestinal mucosal homeostasis in mice and humans. Adv Immunol 2014;122:177-210.
85. Veenbergen S, van Leeuwen MA, Driessen GJ, et al. Development and Function of Immune Cells in an Adolescent Patient With a Deficiency in the Interleukin-10 Receptor. J Pediatr Gastroenterol Nutr 2017;65:e5-e15.
86. Rubtsov YP, Rasmussen JP, Chi EY, et al. Regulatory T cell-derived interleukin-10 limits inflammation at environmental interfaces. Immunity 2008;28:546-58.
87. Unutmaz D, Pulendran B. The gut feeling of Treg cells: IL-10 is the silver lining during colitis. Nat Immunol 2009;10:1141-3.
88. Murai M, Turovskaya O, Kim G, et al. Interleukin 10 acts on regulatory T cells to maintain expression of the transcription factor Foxp3 and suppressive function in mice with colitis. Nat Immunol 2009;10:1178-84.
89. Krause P, Morris V, Greenbaum JA, et al. IL-10-producing intestinal macrophages prevent excessive antibacterial innate immunity by limiting IL-23 synthesis. Nat Commun 2015;6:7055.
90. Shouval DS, Biswas A, Goettel JA, et al. Interleukin-10 receptor signaling in innate immune cells regulates mucosal immune tolerance and anti-inflammatory macrophage function. Immunity 2014;40:706-19.
91. Girard-Madoux MJ, Ober-Blobaum JL, Costes LM, et al. IL-10 control of CD11c+ myeloid cells is essential to maintain immune homeostasis in the small and large intestine. Oncotarget 2016;7:32015-30.
92. Zigmond E, Bernshtein B, Friedlander G, et al. Macrophage-restricted interleukin-10 receptor deficiency, but not IL-10 deficiency, causes severe spontaneous colitis. Immunity 2014;40:720-33.
93. Groux H, O'Garra A, Bigler M, et al. A CD4+ T-cell subset inhibits antigen-specific T-cell responses and prevents colitis. Nature 1997;389:737-42.
94. Vieira PL, Christensen JR, Minaee S, et al. IL-10-secreting regulatory T cells do not express Foxp3 but have comparable regulatory function to naturally occurring CD4+CD25+ regulatory T cells. J Immunol 2004;172:5986-93.
95. Huber S, Gagliani N, Esplugues E, et al. Th17 cells express interleukin-10 receptor and are controlled by Foxp3(-) and Foxp3+ regulatory CD4+ T cells in an interleukin-10-dependent manner. Immunity 2011;34:554-65.
96. Uhlig HH, Coombes J, Mottet C, et al. Characterization of Foxp3+CD4+CD25+ and IL-10-secreting CD4+CD25+ T cells during cure of colitis. J Immunol 2006;177:5852-60.
97. Kamanaka M, Kim ST, Wan YY, et al. Expression of interleukin-10 in intestinal lymphocytes detected by an interleukin-10 reporter knockin tiger mouse. Immunity 2006;25:941-52.

98. Maynard CL, Harrington LE, Janowski KM, et al. Regulatory T cells expressing interleukin 10 develop from Foxp3+ and Foxp3- precursor cells in the absence of interleukin 10. Nat Immunol 2007;8:931-41.
99. Foussat A, Cottrez F, Brun V, et al. A comparative study between T regulatory type 1 and CD4+CD25+ T cells in the control of inflammation. J Immunol 2003;171:5018-26.
100. Cong Y, Weaver CT, Lazenby A, et al. Bacterial-reactive T regulatory cells inhibit pathogenic immune responses to the enteric flora. J Immunol 2002;169:6112-9.
101. Murphy KM, Reiner SL. The lineage decisions of helper T cells. Nat Rev Immunol 2002;2:933-44.
102. Weaver CT, Harrington LE, Mangan PR, et al. Th17: an effector CD4 T cell lineage with regulatory T cell ties. Immunity 2006;24:677-88.
103. O'Shea JJ, Paul WE. Mechanisms underlying lineage commitment and plasticity of helper CD4+ T cells. Science 2010;327:1098-102.
104. Maynard CL, Weaver CT. Intestinal effector T cells in health and disease. Immunity 2009;31:389-400.
105. Hegazy AN, West NR, Stubbington MJT, et al. Circulating and Tissue-Resident CD4(+) T Cells With Reactivity to Intestinal Microbiota Are Abundant in Healthy Individuals and Function Is Altered During Inflammation. Gastroenterology 2017;153:1320-1337 e16.
106. Song X, Dai D, He X, et al. Growth Factor FGF2 Cooperates with Interleukin-17 to Repair Intestinal Epithelial Damage. Immunity 2015;43:488-501.
107. Yang J, Sundrud MS, Skepner J, et al. Targeting Th17 cells in autoimmune diseases. Trends Pharmacol Sci 2014;35:493-500.
108. Kinugasa T, Sakaguchi T, Gu X, et al. Claudins regulate the intestinal barrier in response to immune mediators. Gastroenterology 2000;118:1001-11.
109. Burisch J, Jess T, Martinato M, et al. The burden of inflammatory bowel disease in Europe. J Crohns Colitis 2013;7:322-37.
110. Chouraki V, Savoye G, Dauchet L, et al. The changing pattern of Crohn's disease incidence in northern France: a continuing increase in the 10- to 19-year-old age bracket (1988-2007). Aliment Pharmacol Ther 2011;33:1133-42.
111. Van Limbergen J, Russell RK, Drummond HE, et al. Definition of phenotypic characteristics of childhood-onset inflammatory bowel disease. Gastroenterology 2008;135:1114-22.
112. Haller CA, Markowitz J. IBD in children: lessons for adults. Curr Gastroenterol Rep 2007;9:528-32.
113. Jostins L, Ripke S, Weersma RK, et al. Host-microbe interactions have shaped the genetic architecture of inflammatory bowel disease. Nature 2012;491:119-24.
114. Lees CW, Barrett JC, Parkes M, et al. New IBD genetics: common pathways with other diseases. Gut 2011;60:1739-53.
115. Sartor RB. Mechanisms of disease: pathogenesis of Crohn's disease and ulcerative colitis. Nat Clin Pract Gastroenterol Hepatol 2006;3:390-407.
116. Uhlig HH. Monogenic diseases associated with intestinal inflammation: implications for the understanding of inflammatory bowel disease. Gut 2013;62:1795-805.
117. Dutton RW, Bradley LM, Swain SL. T cell memory. Annu Rev Immunol 1998;16:201-23.
118. Rutgeerts P, Goboes K, Peeters M, et al. Effect of faecal stream diversion on recurrence of Crohn's disease in the neoterminal ileum. Lancet 1991;338:771-4.
119. D'Haens GR, Geboes K, Peeters M, et al. Early lesions of recurrent Crohn's disease caused by infusion of intestinal contents in excluded ileum. Gastroenterology 1998;114:262-7.
120. Khan KJ, Ullman TA, Ford AC, et al. Antibiotic therapy in inflammatory bowel disease: a systematic review and meta-analysis. Am J Gastroenterol 2011;106:661-73.
121. Brandwein SL, McCabe RP, Cong Y, et al. Spontaneously colitic C3H/HeJBir mice demonstrate selective antibody reactivity to antigens of the enteric bacterial flora. J Immunol 1997;159:44-52.
122. Lodes MJ, Cong Y, Elson CO, et al. Bacterial flagellin is a dominant antigen in Crohn disease. J Clin Invest 2004;113:1296-306.
123. Rath HC, Herfarth HH, Ikeda JS, et al. Normal luminal bacteria, especially Bacteroides species, mediate chronic colitis, gastritis, and arthritis in HLA-B27/human beta2 microglobulin transgenic rats. J Clin Invest 1996;98:945-53.
124. Joosse ME, Samsom JN, van der Woude CJ, et al. The Role of Therapeutic Drug Monitoring of Anti-Tumor Necrosis Factor Alpha Agents in Children and Adolescents with Inflammatory Bowel Disease. Inflamm Bowel Dis 2015;21:2214-21.

GENERAL INTRODUCTION AND OUTLINE OF THIS THESIS

125. Bilsborough J, Targan SR, Snapper SB. Therapeutic Targets in Inflammatory Bowel Disease: Current and Future. The American Journal Of Gastroenterology Supplements 2016;3:27.
126. Kaser A, Zeissig S, Blumberg RS. Inflammatory bowel disease. Annu Rev Immunol 2010;28:573-621.
127. Sartor RB. Microbial influences in inflammatory bowel diseases. Gastroenterology 2008;134:577-94.
128. Kugathasan S, Denson LA, Walters TD, et al. Prediction of complicated disease course for children newly diagnosed with Crohn's disease: a multicentre inception cohort study. Lancet 2017;389:1710-1718.
129. Dubinsky MC, Lin YC, Dutridge D, et al. Serum immune responses predict rapid disease progression among children with Crohn's disease: immune responses predict disease progression. Am J Gastroenterol 2006;101:360-7.
130. Papadakis KA, Yang H, Ippoliti A, et al. Anti-flagellin (CBir1) phenotypic and genetic Crohn's disease associations. Inflamm Bowel Dis 2007;13:524-30.
131. Targan SR, Landers CJ, Yang H, et al. Antibodies to CBir1 flagellin define a unique response that is associated independently with complicated Crohn's disease. Gastroenterology 2005;128:2020-8.
132. Chen L, Flies DB. Molecular mechanisms of T cell co-stimulation and co-inhibition. Nat Rev Immunol 2013;13:227-42.

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2

TIPPING THE BALANCE: INHIBITORY CHECKPOINTS IN INTESTINAL HOMEOSTASIS

Chapter 2

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2

TIPPING THE BALANCE: INHIBITORY CHECKPOINTS IN INTESTINAL HOMEOSTASIS

ABSTRACT

The small intestinal and colonic lamina propria are populated with Forkhead box P3 (Foxp3)$^{+}$CD4^{+} regulatory T cells (Tregs) and interleukin-10-producing T cells that orchestrate intestinal tolerance to harmless microbial and food antigens. Expression of co-inhibitory receptors such as cytotoxic T-lymphocyte associated protein 4 (CTLA-4) and programmed cell death protein 1 (PD-1) serve as checkpoints to these cells controlling their T-cell receptor (TCR)- and CD28- mediated activation and modulating the phenotype of neighboring antigen presenting cells. Recent discoveries on the diversity of co-inhibitory receptors and their selective cellular expression has shed new light on their tissue-dependent function. In this review, we provide an overview of the co-inhibitory pathways and checkpoints of Treg and effector T cells and their mechanisms of action in intestinal homeostasis. Better understanding of these inhibitory checkpoints is desired as their blockade harbors clinical potential for the treatment of cancer and their stimulation may offer new opportunities to treat chronic intestinal inflammation such as inflammatory bowel disease.

INTRODUCTION

The intestinal mucosa is continuously exposed to exogenous antigens derived from food proteins and microbiota. Upon encounter of foreign antigens, antigen presenting cells (APCs) migrate to draining lymph nodes to present antigens to naive T cells. The interactions between APCs and responding T cells are modulated by additional signals from costimulatory and co-inhibitory receptors. The balance between these signals determines the functional outcome of T-cell receptor (TCR)-mediated activation, including the strength, nature and duration of the T-cell response.[1] In addition to controlling APC-T-cell interactions during the initial activation of naive T cells, costimulatory and co-inhibitory signals also control effector, memory and regulatory T-cell responses.[2] Under homeostatic circumstances, encounter of innocuous antigens such as food proteins and molecular components of commensal bacteria in mucosa draining lymph nodes result in a preferential tolerogenic T-cell response. Inflammatory T-cell responses to food and microbial antigens can result in allergy and chronic inflammation, such as seen in patients with inflammatory bowel disease (IBD) and celiac disease, and might contribute to autoimmune diseases such as systemic lupus erythematosus (SLE) and rheumatoid arthritis (RA).[3-7]

Over the years, studies in both mice and humans have established a central role for Forkhead box P3 (Foxp3)$^{+}$CD4^{+} regulatory T cells (Tregs) and interleukin 10 (IL-10)-producing CD4^{+}Foxp3^{neg} T cells in maintaining intestinal tolerance to harmless microbial and food antigens.[8] Foxp3^{+} Tregs are classified into thymus-derived Treg (tTreg) and peripherally-derived Treg (pTreg). tTreg arise during CD4^{+} T-cell differentiation in the thymus, whereas pTreg differentiate from naive CD4^{+} T cells exposed to antigens under tolerogenic conditions in lymphoid tissue.[9] Special environmental control in intestinal lymphoid tissues favors *de novo* pTreg development in response to TCR-specific recognition of food- and microbiota-derived antigens.[8, 10-12] In particular, soluble factors including transforming growth factor-β (TGF-β) and retinoic acid promote intestinal pTreg differentiation.[13-16] Besides Foxp3-expressing Tregs, the intestine contains IL-10-secreting CD4^{+}Foxp3^{neg} type 1 regulatory T cells (Tr1 cells), differentiation of which is facilitated by the cytokine IL-27.[17, 18] A common feature of both Foxp3^{+} and Foxp3^{neg} regulatory CD4^{+} T-cell populations is the expression of inhibitory receptors.[19] Recently, immunotherapies directed against co-inhibitory receptors such as cytotoxic T-lymphocyte associated protein 4 (CTLA-4) and programmed cell death protein 1 (PD-1), aiming to enhance anti-tumor T-cell responses in cancer, show that co-inhibitory receptor blockade can lead to the development of immune-mediated intestinal inflammation.[20] These findings provide new insight into the critical role of co-inhibitory receptors in the maintenance of intestinal homeostasis, but their involvement in the regulation of mucosal immune responses in the intestine remains poorly understood. In this review, we will discuss the role of

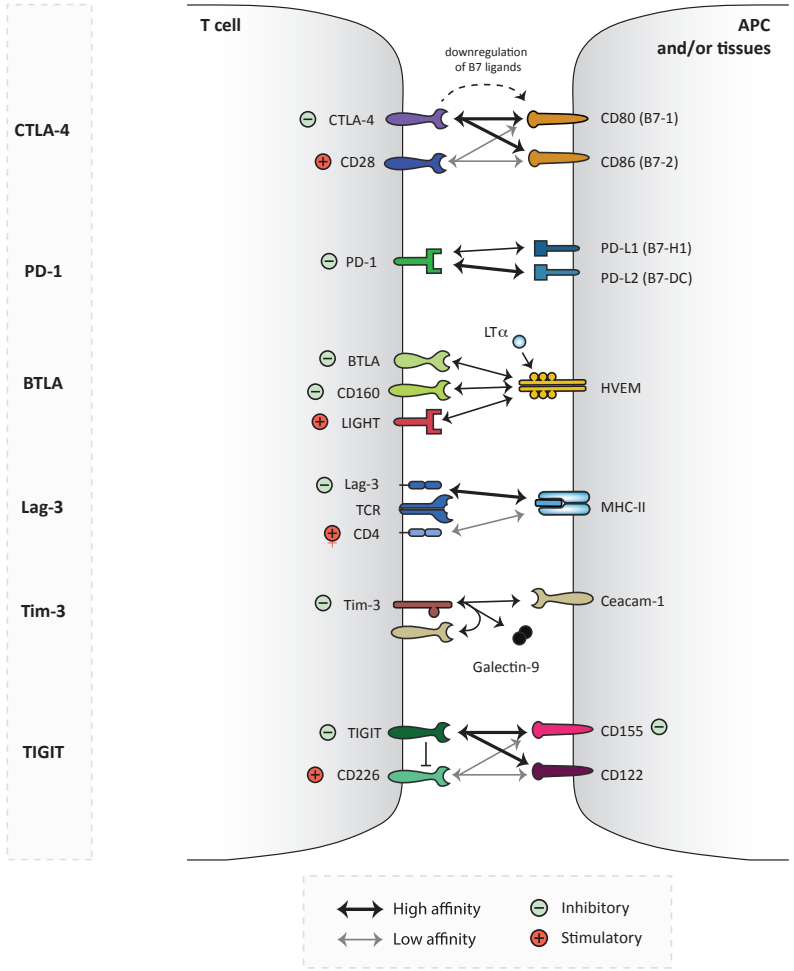

Figure 1. Interactions between co-inhibitory receptors of the immunoglobulin superfamily and their ligands. The majority of T-cell co-inhibitory receptors belong to the immunoglobulin (Ig) superfamily. Many co-inhibitory receptors of the Ig superfamily are expressed on activated T cells, and their specific ligands are expressed in professional antigen presenting cells (APCs), neutrophils, macrophages, or stromal cells. The inhibitory receptor cytotoxic T-lymphocyte associated antigen 4 (CTLA-4) is a structural homolog of the costimulatory receptor CD28, but binds B7 ligands CD80 and CD86 with much higher binding affinity. Programmed cell death 1 (PD-1) is a member of the B7/CD28 family that has two ligands, PD-L1 and PD-L2. B- and T-lymphocyte attenuator (BTLA) is part of a shared receptor-ligand network and binds to the TNF receptor family member herpesvirus entry mediator (HVEM). Lymphocyte activation gene-3 (Lag-3) structurally resembles CD4 and binds to MHC-II with higher affinity. T-cell immunoglobulin and mucin domain-containing protein (Tim-3) has many ligands, including galectin-9 and Ceacam-1. T-cell immunoreceptor with Ig and ITIM domain (TIGIT) and the costimulatory receptor CD226 share the ligands CD112 and CD155. Together they form a pathway reminiscent of the CTLA-4/CD28/B7 pathway, in which TIGIT delivers an inhibitory signal.

co-inhibitory receptors in the maintenance of intestinal homeostasis and how the timing of co-inhibitory receptor upregulation, ligand distributions and specific effects on different cell-types contribute to this regulation. Better mechanistic understanding of how co-inhibitory receptor pathway modulation leads to intestinal inflammation is desired as stimulating co-inhibitory receptor pathways may offer new opportunities to treat chronic intestinal inflammation as observed in IBD.

INHIBITORY RECEPTORS IN THE IG SUPERFAMILY THAT ENGAGE B7 LIGANDS: CTLA-4 AND PD-1

Cytotoxic T-lymphocyte associated antigen 4 (CTLA-4; CD152).

CTLA-4: expression, ligands and function

CTLA-4, a member of the Ig superfamily, is a structural homologue of the costimulatory receptor CD28 and is a ligand for CD80 (B7-1) and CD86 (B7-2) expressed on the surface of APCs.[21, 22] Unlike CD28, CTLA-4 undergoes constitutive clathrin-mediated endocytosis such that it is rapidly internalized after reaching the cell surface.[23-25] CTLA-4 has a higher affinity for CD80 and CD86 than CD28 and can prevent the costimulatory signal normally provided by CD28/B7 interactions (Figure 1).[26] As CD28/B7 costimulation is essential to prime naive T cells, the CTLA-4 pathway is thought to inhibit T-cell proliferation early in the immune response, preferentially in the lymph node.[27, 28] Since CTLA-4 is a target gene of Foxp3[29-31], Tregs have high intracellular stores of CTLA-4 and constitutively traffic high levels of CTLA-4 to their cell surface.[32, 33] Resting naive T cells can be induced to express CTLA-4 in response to TCR ligation, in particular together with CD28 costimulation, reaching a maximum level after 48 to 72 hours.[34-36]

The critical role of CTLA-4 in controlling T-cell activation and tolerance is well-established. *Ctla4*$^{-/-}$ mice develop a lymphoproliferative disorder within 3 weeks of age that is characterized by the infiltration of CD4^{+} T cells into multiple non-lymphoid tissues, leading to organ destruction and death.[37, 38] Specific deletion of CTLA-4 in CD4^{+}Foxp3^{+} Tregs results in a similar lymphoproliferative disease and multi-organ failure as seen in *Ctla4*$^{-/-}$ mice, but with a delayed onset, demonstrating that expression of CTLA-4 by Foxp3^{+} Tregs is essential to prevent autoimmunity *in vivo*.[39] Mice lacking both CTLA-4 and CD28 or their ligands CD80/CD86 show no signs of disease[40-42], demonstrating that the main function of CTLA-4 is to control CD28-dependent T-cell activation by competing for CD80 and CD86. This so-called cell extrinsic regulation, in which CTLA-4^{+} cells inhibit the activation of neighboring CTLA-4^{neg} cells, is exerted by conventional T cells as well as by Tregs.[43-46] In addition to competing for CD80/CD86, CTLA-4 can downregulate expression of these

ligands on APCs.[39, 47, 48] This can occur via transendocytosis, whereby CTLA-4 captures CD80 and CD86 from the surface of APCs and internalizes them for degradation, impairing the capacity of these APCs to provide CD28 costimulation.[49] The role of transendocytosis in CTLA-4 function offers a plausible biological explanation for the unusual endocytic and recycling behavior of the CTLA-4 molecule. Although it has been suggested that the cytoplasmic domain of CTLA-4 transmits an inhibitory signal leading to cell-intrinsic regulation within CTLA-4 expressing cells, this is not supported by experiments with mixed bone marrow chimeric mice containing a mixture of wildtype and CTLA-4-deficient cells.[50-52] Thus while cell intrinsic competition between CTLA-4 and CD28 expressed on the same cell must surely occur, this does not appear to be a major mechanism of CTLA-4 action, at least in conventional T cells.[32, 52] In sum, CTLA-4 regulates T-cell function primarily by restricting T-cell activation early in the immune response through cell extrinsic ligand competition.

Intestinal CTLA-4 in a nutshell. CTLA-4 uses multiple mechanisms to exert a critical inhibitory effect on intestinal T-cell responses. CTLA-4 favors higher frequencies of pTregs in the intestinal lamina propria.[53] Moreover, mouse models of colitis have demonstrated that CTLA-4 expression on Treg is essential to suppress colitogenic $CD4^+$ effector T cells.[32, 45, 54-56] In humans, Tregs of patients with heterozygous mutations in CTLA-4 have impaired suppressive capacity.[57-59] Limited data is available on the functional role of CTLA-4 on intestinal regulatory $CD4^+Foxp3^{neg}$ T-cell populations.

CTLA-4 and intestinal homeostasis

In the intestine, approximately 90% of all $Foxp3^+$ Tregs express CTLA-4.[60] Several murine studies have addressed the role of CTLA-4 in the induction of intestinal $Foxp3^+$ cells, its effect on suppressive capacity and its role in maintaining the $Foxp3^+$ Tregs pool. CTLA-4 is not required for pTreg induction, as in culture with TGF-β, IL-2 and TCR signals, naive *Ctla4*$^{-/-}$ T cells and *Ctla4*$^{+/+}$ T cells are equally efficient in converting into $Foxp3^+$ pTregs.[53] However, CTLA-4 does appear to play a role in maintaining the intestinal Treg pool. The intestinal lamina propria of *Rag2*$^{-/-}$ mice reconstituted with a mixture of *Ctla4*$^{-/-}$ and *Ctla4*-sufficient BALB/c bone marrow cells contains reduced percentages of $Foxp3^+$ T cells derived from *Ctla4*$^{-/-}$ bone marrow when compared to $Foxp3^+$ T cells derived from BALB/c bone marrow.[53] In contrast, in the spleen and mesenteric lymph nodes, CTLA-4-deficient (*Ctla4*$^{-/-}$) and -sufficient (BALB/c) T cells contributed equally to the $Foxp3^+$ and $Foxp3^{neg}$ compartments. This is not because of a general inability of CTLA-4$^{-/-}$ cells to migrate to the gut, as CTLA-4$^{-/-}$ cells in the intestine contributed to the $CD4^+Foxp3^{neg}$ cell pool to a similar percentage as in other organs.[53] These observations thus argue that, *in vivo*, CTLA-4 plays a role in regulating pTreg frequencies in the intestine in particular.[53] In sum, CTLA-4 is not

essential for intestinal pTreg induction but does regulate the accumulation of intestinal pTreg in the intestine.

CTLA-4 plays a fundamental role in Treg suppressive function during intestinal inflammation.[33, 40, 55, 56, 61] The majority of data on the role of CTLA-4 in intestinal inflammation has been generated using the T-cell transfer model of colitis. In this model, naive $CD4^+CD45RB^{high}$ T cells are adoptively transferred into lymphopenic *Scid*$^{-/-}$ or *Rag*$^{-/-}$ mice and cause colitis upon activation in response to intestinal antigens.[62, 63] Co-transfer of $CD4^+CD25^+$ T cells, enriched in Tregs, with naive $CD4^+CD45RB^{high}$ T cells prevents development of colitis.[64] In most studies, CTLA-4 expression on co-transferred $CD4^+CD25^+$ T cells is essential to prevent colitis induced by naive T-cell transfer into *Rag*$^{-/-}$ recipients (Table 1).[32, 45, 54] Similarly, blocking the interaction of CTLA-4 and its ligands through use of anti-CTLA-4 antibodies or Fab fragments, abrogates $CD4^+CD25^+$ cell mediated suppression of colitis.[40, 55, 56] This argues that CTLA-4 expression on Tregs is required for colitis suppression (Figure 2.2). It should be noted that genetic deficiency of CTLA-4 in $CD4^+CD25^+$ Tregs has been reported to result in a compensatory IL-10 production in some settings, allowing the prevention of transfer colitis in an IL-10 dependent fashion (Table 1). It is unclear whether CTLA-4 expression by colitogenic naive $CD4^+CD45RB^{high}$ T cells plays a role in transfer colitis, as anti-CTLA-4 antibody treatment exacerbated colitis in some experimental settings but not others.[40, 56] An overview of the role of CTLA-4 in colitis suppression in the different transfer colitis studies is provided in Table 1. Altogether, data demonstrate a key role for CTLA-4 in $Foxp3^+$ Tregs to prevent T-cell transfer induced colitis.

In addition to their crucial role in preventing intestinal inflammation, $Foxp3^+$ pTregs are required for the induction of oral tolerance.[10, 65, 66] Feeding of soluble antigen induces systemic immunological hyporesponsiveness that is characterized by a suppressed delayed type hypersensitivity (DTH) reaction after antigen injection in the footpad or the auricle.[67, 68] At the cellular level, tolerance is a consequence of $Foxp3^+$ pTreg mediated suppression of the Th1 and Th2 response to the particular antigen. Several studies have demonstrated a role for CTLA-4 function in the development of oral tolerance, but effects on cytokine secretion varied on the dose of the antigen fed while the DTH response was not monitored.[69, 70] Administration of anti-CTLA-4 antibodies during 50 mg ovalbumin (OVA) feeding completely abrogated the suppression of cytokine production by Th1 and Th2 cells.[70] By contrast, the administration of anti-CTLA-4 antibodies during 1 mg OVA feeding only abrogated suppression of Th2 type immune responses but not Th1.[69] Co-administration of IL-12 along with anti-CTLA-4 antibodies was required to abrogate the suppression of Th1 cytokine secretion during tolerance induction 1 mg OVA feed.[69] These results indicate that the CTLA-4 pathway is important for establishing oral tolerance, but suggests that its role is most important in high dose oral tolerance. Whether this relates to different mechanisms mediating oral tolerance to low and high antigen doses, i.e. low

doses favoring the induction of pTregs and higher doses the induction of anergy or clonal deletion, remains to be established. In particular, whether OVA-specific pTregs require CTLA-4 for full suppression of Th1 and Th2 immune responses in models for low dose oral tolerance remains to be further investigated (Figure 2.2).

In some settings, CTLA-4 deficiency may allow for induction of other subsets of $CD4^+$ T cells with the capacity to suppress colitis. Cre-recombinase based deletion of CTLA-4 in adult mice results in significantly increased frequencies of $Foxp3^{neg}$ T cells, that exhibit high levels of IL-10 and increased expression of co-inhibitory receptors Lag-3 and PD-1.[71] High IL-10-producing capacity and the expression of co-inhibitory receptors such as Lag-3 and PD-1 are characteristic of IL-10-secreting $CD4^+Foxp3^{neg}$ Tr1, a cell type that has been shown to inhibit T-cell responses and colitis in an IL-10-dependent manner.[72-74] Along the same lines, it was recently demonstrated that anti-CTLA-4 treatment can induce IL-10-producing $ICOS^+$ regulatory $CD4^+$ T cells with anti-inflammatory properties that inhibit 2,4,6-Trinitrobenzenesulfonic acid solution (TNBS) induced colitis.[75] This is in contrast with the finding that anti-CTLA-4 antibodies abrogated Treg control of colitis in the setting of T-cell transfer experiments (described above), but fits with the observation that IL-10 can compensate for a genetic deficiency of CTLA-4 in some settings (Table 1).[40] The precise circumstances in which blockade or lack of CTLA-4 allows for the differentiation of immunosuppressive IL-10 secreting $CD4^+Foxp3^{neg}$ T cells with the ability suppress colitis requires further investigation.

Quantitative deficiencies in CTLA-4 expression in humans

In humans, heterozygous mutations in CTLA-4 resulting in CTLA-4 haploinsufficiency are associated with lymphoproliferation reminiscent of the mouse model, resulting in severe clinical manifestations of autoimmunity and early-onset Crohn's disease.[57-59] The organs of affected *CTLA4*$^{+/-}$ individuals, including the intestine and lungs, show extensive $CD4^+$ T-cell infiltration.[59] Frequencies of Tregs within the $CD4^+$ T-cell compartment of *CTLA4*$^{+/-}$ individuals are significantly increased compared to healthy *CTLA4*$^{+/+}$ controls and their suppressive function is impaired.[57-59] In addition to impaired Treg function, it has been suggested that the *CTLA4*$^{+/-}$ genotype alters naive T-cell responses, as naive T cells

An overview of the studies using *Ctla4*$^{-/-}$ $CD4^+$ T cells and/or anti-CTLA-4 monoclonal antibodies to investigate the functional effect of CTLA-4 in the T-cell transfer model of colitis. In this model, transfer of naive $CD4^+CD45RB^{high}$ T cells into lymphopenic recipients causes colitis. Co-transfer of $CD4^+CD25^+$ Tregs prevents development of colitis. In most studies depicted in this table, use of *Ctla4*$^{-/-}$ Tregs or anti-CTLA-4 blocking antibodies abrogates colitis prevention. The * indicates a study using *Ctla4*$^{-/-}$ Tregs in which compensatory IL-10 production by *Ctla4*$^{-/-}$ Tregs was shown to prevent colitis. Additional experiments using anti-CTLA-4 antibodies, indicated in the bottom section of the table, demonstrated that even though *Ctla4*$^{-/-}$ Tregs have compensatory mechanisms, suppression by wildtype Treg is dependent on CTLA-4.Abbreviations: KO = knockout; BM = bone marrow; WT = wildtype; mAb = monoclonal antibody; i.p. = intraperitoneally. >>

Table 1. The role of CTLA-4 in preventing intestinal inflammation in transfer colitis models.

	Colitogenic T-cell population	Recipient	Treg-cell population	Colitis
***Ctla4*$^{-/-}$ regulatory cells**				
Sojka et al. 2009 [54]	$CD4^+CD25^{neg}CD62L^{high}$ T cells from WT C57BL/6.CD90.2 mice.	*Rag2*$^{-/-}$	CTLA-4 deficient $CD4^+CD25^+CD62L^{high}$ cells isolated from young *Ctla4*$^{-/-}$ mice.	Yes
Jain et al. 2010 [45]	$CD4^+CD25^{neg}$ cells isolated from WT BALB/c mice.	*Rag1*$^{-/-}$	CTLA-4 deficient $CD4^+CD25^+$ cells isolated from transgenic CD57BL/6J mice with CTLA-4 expression restricted to activated T cells (CT4Act mice, *Ctla4* expression under the control the *Il2* promoter).	Yes
Tai et al. 2012 [32]	$CD4^+CD45RB^{high}$ T cells from WT B6 mice.	*Rag2*$^{-/-}$	CTLA-4 deficient $CD4^+CD25^+$ cells isolated from B6+*Ctla4*$^{-/-}$→B6 mixed BM chimeras.	Yes
Read et al. 2006 [40]	$CD4^+CD45RB^{high}$ T cells from WT BALB/c mice.	*Scid*	B7/CTLA-4 deficient $CD4^+CD25^+$ cells from B7-1/B7-2/CTLA-4 KO mice.	No*
Read et al. 2006 [40]	$CD4^+CD45RB^{high}$ T cells from WT BALB/c mice.	*Rag2*$^{-/-}$	B7-sufficient CTLA-4 deficient $CD4^+CD25^+$ cells isolated from BALB/c.CTLA-4 KO mixed BM chimeras.	No*
Anti-CTLA-4 antibodies				
Read et al. 2006 [40]	*CTLA-4 deficient* $CD4^+CD45RB^{high}$ T cells from B7-1/B7-2/CTLA-4$^{-/-}$ mice.	*Scid*	*CTLA-4 sufficient* Transfer of $CD4^+CD25^+$ T cells, followed by anti-CTLA-4 mAb i.p. (200 mg), the day after T-cell reconstitution and then on alternate days for 6-8 weeks.	Yes
Read et al. 2006 [40]	*CTLA-4 sufficient* $CD4^+CD45RB^{high}$ T cells from WT mice.	*Scid*	*CTLA-4 deficient* Transfer of $CD4^+CD25^+$ T cells from B7-1/B7-2/CTLA-4$^{-/-}$ mice, followed by anti-CTLA-4 mAb i.p. (200 μg), the day after T-cell reconstitution and then on alternate days for 6-8 weeks.	No*
Read et al. 2000 [55]	Mixture of $CD45RB^{high}$ and $CD45RB^{low}$ cells.	*Scid*	Transfer of $CD25^+CD45RB^{low}$ cells, followed by anti–CTLA-4 mAb i.p. (200 μg), the day after T-cell reconstitution and then on alternate days for 6 weeks.	Yes
Liu et al. 2001 [56]	$CD4^+CD45RB^{high}$ T cells from WT mice.	*Scid*	No Tregs transferred. Anti-CTLA-4 mAb i.p. (250 μg), twice a week starting at the beginning of T-cell transfer up to 8 weeks.	Yes (exacerbated colitis)

obtained from a clinically affected *CTLA4*$^{+/-}$ individual showed hyperproliferation after *in vitro* activation in the presence of autologous T-cell depleted feeder cells.[57] However, other studies did not observe hyperproliferation in naive T-cell responses isolated from *CTLA4*$^{+/-}$ individuals suggesting that the variations in experimental set-up may be key.[59, 76] Interestingly, the clinical penetrance of CTLA-4 haploinsufficiency is incomplete, suggesting that additional genetic or environmental factors influence disease susceptibility in individuals with impaired CTLA-4 function. It is currently unknown whether clinically unaffected *CTLA4*$^{+/-}$ individuals are protected from disease by CD4^{+} T cells with suppressive capacity independent of CTLA-4.

Genetic variants in the *CTLA4* gene locus are associated with intestinal inflammation and autoimmune diseases.[77-79] The single nucleotide polymorphism (SNP) CT60 (rs3087243; A/G) located in the 3' UTR of the *CTLA4* gene has been associated with IBD in a Slovenian cohort of adult IBD patients.[80] This variant of the *CTLA4* gene is associated with a functionally altered TCR signaling in CD4^{+} T cells and decreased production of the soluble CTLA-4 isoform.[81, 82] However, the association between the CT60 *CTLA4* allele and IBD was not confirmed in a separate cohort of Spanish patients.[77] Several studies have shown that the CT60 *CTLA4* allele is also weakly associated with celiac disease.[83, 84] Taken together, these data indicate that quantitative deficiencies in CTLA-4 protein expression can predispose selected subgroups of individuals to autoimmunity, including CD4^{+} T-cell mediated inflammation in the gastrointestinal tract.

Programmed cell death protein 1 (PD-1; CD279).

PD-1: expression, ligands and function

PD-1 is a cell-surface molecule belonging to the B7 receptor family that contains an immunoreceptor tyrosine-based inhibitory motif (ITIM) and an immunoreceptor tyrosine-based switch motif (ITSM) in its cytoplasmic tail.[85, 86] PD-1 has two ligands, PD-L1 (B7-H1; also termed CD274) and PD-L2 (B7-DC; also termed CD273). Most of the inhibitory roles of PD-1 have been attributed to its interaction with PD-L1 (Figure 1).[87] Upon PD-L1 or PD-L2 binding, the ITIM or ITSM tyrosine motifs in the cytoplasmic tail of PD-1 are phosphorylated.[88] This leads to recruitment of Src-homology 2 domain-containing phosphatase 2 (SHP-2) and augments phosphatase and tensin homolog (PTEN) expression, inhibiting phosphatidylinositol 3-kinase (PI3K) and Akt activation.[89-91] CD28, not the TCR or its associated components, has been reported to be the most sensitive target of PD-1, as shown by the strong degree of CD28-dephosphorylation compared to dephosphorylation of TCR-signaling components after PD-1 activation.[90, 92] In line with its inhibitory function, PD-1 deficient mice (*Pdcd1*$^{-/-}$) develop spontaneous autoimmune disease although the incidence of disease depends on the genetic background and symptoms only manifest later in life.[93-95]

Moreover, the disease is often tissue-specific which is in contrast to the rapid multi-organ autoimmune disease observed in CTLA-4$^{-/-}$ mice in the first few weeks of life.[37, 38]

PD-1 is expressed on diverse hematopoietic cells, including T and B cells[96], natural killer (NK) cells and other innate immune cells.[97] PD-1 expression is absent on naive T cells but is rapidly upregulated after antigen encounter and can be detected after only 6 hours, with a peak in expression at 48 hours after antigen encounter.[96, 98-100] Of the two PD-1 ligands, PD-L2 is mainly expressed by DCs and macrophages, whereas PD-L1 is more widely expressed by both hematopoietic and non-hematopoietic cells[101-103] and can be induced by inflammatory cytokines such as interferons.[104, 105] The broad expression of PD-1 and PD-L1 is in contrast to CTLA-4 and its ligands that are mainly expressed by hematopoietic cells present in the lymph node environment. Therefore, it is often hypothesized that while CTLA-4 impacts T-cell activation primarily during the priming phase in secondary lymphoid organs, PD-1 plays a dominant role during the maintenance of tolerance in peripheral tissues.[27, 28] Many murine models for autoimmune disease and cancer have established a role for PD-1/PD-L1 interactions in maintaining tissue tolerance by controlling the effector T-cell responses in non-lymphoid tissues.[94, 106-110] However, the rapid upregulation of PD-1 after activation suggests that PD-1 can control T-cell activation at the time of initial antigen encounter in the lymph node in addition to modulating effector responses in target tissues.

INTESTINAL PD-1 IN A NUTSHELL. PD-1/PD-L1 interactions preserve the local homeostasis of the gastrointestinal tract by acting on a wide variety of immune cell types, mostly through interaction with its ligand PD-L1. PD-1 mainly promotes peripheral induction and survival of Foxp3^{+} Tregs[111-114], but does not seem to determine Treg function.[114] PD-1 expression on intestinal CD4^{+}Foxp3^{neg} Tr1 cells enriches for IL-10-producing capacities.[115] Moreover, PD-1 has a non-redundant role in preventing excessive CD8^{+} effector T cell responses to intestinal self-antigens.[116, 117] Lastly, PD-1 expression on T-follicular helper (Tfh) cells controls Tfh-cell numbers in Peyer's patches, regulating microbial-host interaction through modulating secretory IgA.[118, 119]

PD-1 and intestinal homeostasis

Although Foxp3^{+} Tregs highly express PD-1[111], the role of PD-1 on Tregs is only beginning to be fully understood. PD-1 regulates the generation of pTreg and PD-L1 synergizes with TGF-β to promote pTreg induction[111, 112], suggesting that PD-1/PD-L1-mediated pTreg induction might play a role in TGF-β-rich environments such as the intestinal mucosa-draining lymphoid tissue (Figure 2.1). A role for PD-1 in the maintenance of Tregs has been suggested by several studies. PD-1 maintains the suppressive phenotype of Tregs through inducing PTEN expression.[120] In line with this finding, PD-1 prevents the conversion of Foxp3^{+} Tregs into pro-inflammatory effector/memory CD4^{+} T cells .[113] Recently, it was shown

that low dose IL-2 therapy, used as a therapy to induce expansion of pTregs, increases PD-1 expression on Tregs of patients treated for graft-versus-host disease.[114] Examination of Tregs from IL-2-treated mice demonstrated that PD-1 reduced IL-2-induced Treg proliferation but prevented their terminal differentiation, rendering Tregs less susceptible for apoptosis and promoting their survival and subsequent regulation.[114] IL-2-induced Tregs isolated from PD-1$^{-/-}$ mice exhibited normal levels of suppressive activity, indicating that PD-1 does not directly affect Treg function.[114] In addition to Foxp3^{+} Tregs, PD-1 expression has recently been described on intestinal Tr1 cells (defined as CD3^{+}CD4^{+}CD25^{-}IL-7R^{+} cells) in both mice and humans.[115] Expression of CCR5 and PD-1 allowed enrichment for IL-10^{+} Tr1 cells in the human intestine. In mice, co-transfer of IL-10-producing CCR5^{+}PD-1^{+} Tr1 cells strongly inhibited colitis induced by transfer of Th17 cells, whereas IL-10-producing control T cells that lacked CCR5 and PD-1 were less efficient.[115] Taken together, PD-1 is expressed on both Foxp3^{+} Treg and CD4^{+}Foxp3^{neg} Tr1 cells, and can take part in regulating the induction, survival and IL-10 production of these cells in the intestinal environment.

In addition to PD-1 on regulatory CD4^{+} T-cell populations, PD-1 expression on CD8^{+} effector T cells is involved in preventing responses to intestinal self-antigens (Figure 2.3).[116, 117] In a transfer model of OVA-specific OT-I CD8^{+} T cells into iFABP-OVA mice that express OVA as a self-antigen on intestinal epithelial cells, blocking PD-1/PD-L1 interaction at the time of OT-I cell transfer prevents tolerance induction and resulted in small intestinal inflammtion.[116, 117] The intestinal pathology was characterized by apoptosis of epithelial cells, villous atrophy and leukocytic infiltration, reminiscent of the pathology in human celiac disease. Crucially, when PD-L1 is blocked 30 days after OT-I cell transfer, mice do not develop intestinal pathology, indicating that PD-1/PD-L1 interaction is crucial for the induction, but not the maintenance of mucosal CD8^{+} T-cell tolerance.[117] PD-1 upregulation on CD8^{+} effector T cells upon antigen encounter *in vivo* is highly dependent on the type of antigen recognized. Transfer of CD8^{+} effector T cells specific for influenza hemagglutinin (HA) into mice expressing HA as a self-antigen induces robust PD-1 expression on HA-specific CD8^{+} T cells.[87] In contrast, when HA-specific CD8^{+} T cells were transferred into mice infected with an HA-expressing *Listeria monocytogenes*, there was virtually no PD-1 expression on HA-specific CD8^{+} T cells, demonstrating that PD-1 is differentially induced on CD8^{+} effector T cells responding to self-antigen versus microbial antigen.[87] Of note, the recipient mice in this model expressed HA under the control of the C3 promoter, which directs HA expression on a variety of parenchymal tissues. Whether HA expressed as a self-antigen specifically on intestinal cells induces PD-1 expression on HA-specific T cells remains unknown. Overall, these data demonstrate that the role of PD-1/PD-L1 interactions in CD8^{+} T-cell tolerance to self-antigens and microbial antigens is likely determined by multiple factors, including the timing of the immune response, type of antigen and signals from the tissue microenvironment.

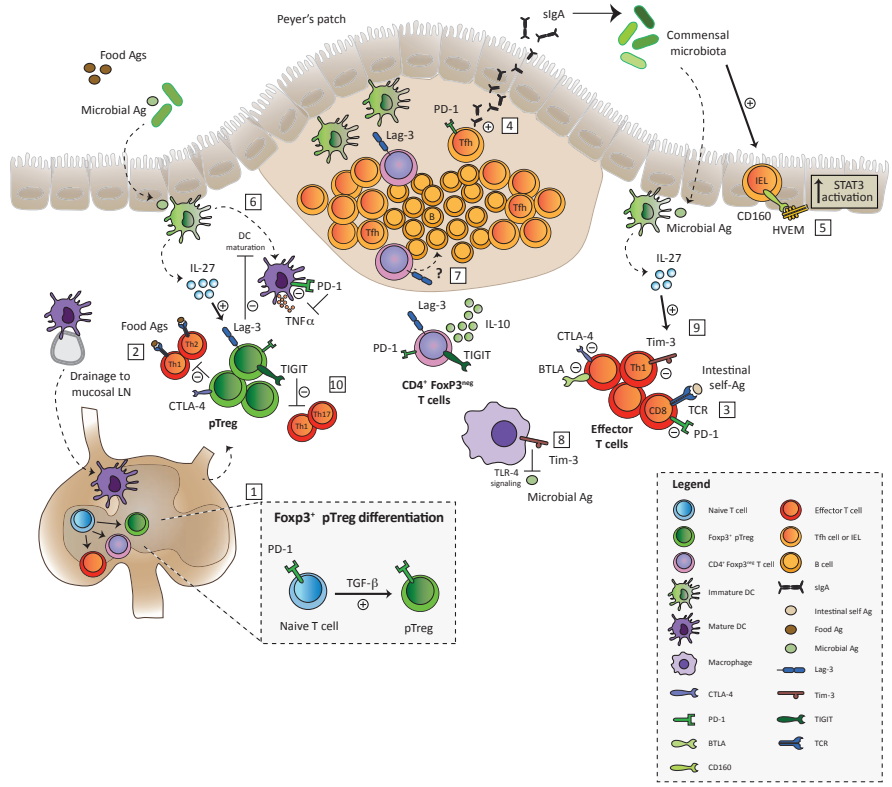

Figure 2. Functional effects of co-inhibitory receptors in the intestine. (1) PD-1 promotes peripheral Foxp3 induction in naive CD4^{+} T cells, especially in TGF-β rich environments. **(2)** CTLA-4 enhances the suppressive function of Tregs to suppress colitogenic CD4^{+} effector T cells. In addition, the CTLA-4 pathway is important in establishing oral tolerance through suppression of Th1 and/or Th2 responses. **(3)** PD-1 expression on CD8^{+} effector T cells is involved in preventing responses to intestinal self-antigens. **(4)** PD-1 expression on Tfh cells regulates Tfh-cell numbers in Peyer's patches, promoting intestinal tolerance to microbiota through secretory IgA. **(5)** HVEM-CD160 interactions at the mucosal surface enhance IL-22R mediated STAT-3 activation in epithelial cells. **(6)** IL-27 is produced by APCs upon activation by microbial products. IL-27 induces Lag-3 expression on Tregs and inhibiting effector T-cell responses through enhancing Treg-suppressive function. Additionally, Lag-3 can prevent T-cell activation through inhibition of DC maturation. **(7)** Frequencies of CD4^{+}CD25^{neg}Lag-3^{+} T cells are high in Peyer's patches, but their role in regulating intestinal humoral immune responses remains unknown. **(8)** Tim-3 inhibits polarization of pathogenic pro-inflammatory M1 macrophages. **(9)** IL-27 enhances TCR-mediated induction of Tim-3 on naive CD4^{+} T cells and can directly suppress Th1 cell-mediated colitis via the induction of Tim-3. **(10)** TIGIT identifies a Treg subset that specifically suppresses pro-inflammatory Th1 and Th17 but not Th2 responses. Abbreviatons: Ag, antgen; LN, lymph node; sIgA, secretory IgA.

Besides CD8^{+} effector T cells, PD-1-expressing Tfh cells are involved in regulating microbial-host interactions through secretory IgA (Figure 2.4). PD-1 is highly expressed on cells in the germinal centers of Peyer's patches[121], the major sites for induction of mucosal

secretory IgA antibody responses.[122] Secretory IgA plays a key role in regulating microbial-host interactions at the mucosal surface by maintaining a balanced and highly diverse microbial communities in the gut.[123] In humans, the protective role of IgA is illustrated by the fact that IgA-deficiency is associated with increased susceptibility to autoimmunity and gastrointestinal infections.[124-126] It has been demonstrated that PD-1 deficient mice (*Pdcd1*$^{-/-}$) have significantly more Tfh cells in Peyer's patches compared to wildtype mice.[118] The production of IL-21, the cytokine that promotes proliferation and differentiation of IgA^{+} B cells into plasma cells[127, 128], is reduced in Tfh cells from *Pdcd1*$^{-/-}$ mice causing an impaired ability to support the generation of IgA plasma cells in gut.[118, 119] As a result, *Pdcd1*$^{-/-}$ mice have lower proportions of bacteria coated with IgA and altered microbial communities in the intestine[118], which resemble alterations of the microbiome observed in several pathological conditions including human IBD.[129] Serum from *Pdcd1*$^{-/-}$ mice contains increased titers of commensal-specific IgG[118], indicating a breach of normal host-microbe interaction in the absence of PD-L1/PD-1 interactions.

Besides a role for the PD-1/PD-L1 pathway in adaptive immune cells, PD-L1 serves as an essential ligand for innate immune cells in the intestine to prevent intestinal inflammation.[130] Experiments with bone marrow chimeras have demonstrated that PD-L1 expression on intestinal epithelial cells reduces dextran sulfate sodium (DSS)-induced intestinal inflammation.[130] This protective effect was mediated through inhibition of TNFα secretion of CD11c^{+}CD11b^{+} lamina propria cells and was independent of adaptive immunity, as PD-L1-deficient *Rag1*$^{-/-}$ mice exhibited a significantly higher morbidity and mortality than *Rag1*$^{-/-}$ mice after DSS administration.[130] In humans, PD-L1 is expressed by intestinal epithelial cells of IBD patients but not of healthy controls[131], and PD-1 expression is increased on lamina propria mononuclear cells[132], which might reflect an effort to promote protective intestinal immune responses through PD-1.

Lessons learned from CTLA-4 and PD-1 blockade in cancer.

Over the past decade, a role for co-inhibitory receptors in the maintenance of intestinal homeostasis in humans has been widely appreciated following the implementation of anti-CTLA-4 and anti-PD-1/PD-L1 immunotherapies to promote anti-tumor T-cell responses and tumor regression in cancer patients. Blockade of CTLA-4 and PD-1/PD-L1 is thought to activate a wide repertoire of T cells, not only tumor-specific T cells. In consequence, these therapies have a broad range of adverse effects, of which diarrhea and colitis are very frequently observed.[133, 134] The incidence of diarrhea and colitis is 35.4% and 8.8%, respectively with CTLA-4 inhibitors and 13.7% and 1.6%, respectively for PD-1 inhibitors.[135, 136] Combining CTLA-4 and PD-1 inhibitors may increase the risk of diarrhea but not colitis.[135, 136] Colonic bowel perforation is the most common cause of fatal immune-related adverse events in patients who develop immunotherapy-induced colitis, but the

incidence of life-threatening colon perforation is low (<1% of patients).[20] Thus, although blockade of co-inhibitory receptors is a promising new approach to improve tumor control, it can cause severe life-threatening immune-related adverse events, most often through a dysregulation of intestinal homeostasis.

In contrast to the chronic colitis-associated histological alterations observed in IBD, anti-CTLA-4 and anti-PD-1 colitis are characterized by neutrophilic inflammation or a mononuclear expansion in the lamina propria and increased intraepithelial lymphocytes (IEL).[137-139] In most patients with immunotherapy-induced colitis, disease remission is achieved by discontinuing the drug[134], but patients are likely to relapse when restarting the same drug.[137, 140] Development of recurrent colitis has been observed in patients without a past medical history of intestinal inflammation who had stopped anti-CTLA-4 or anti-PD-1 therapy for multiple months.[137, 140] The incidence of recurrent colitis is unknown. Colonic biopsies in these patients often demonstrated features of chronicity such as crypt architectural irregularity, suggesting that immunotherapy-induced colitis may progress to chronic intestinal inflammation as seen in IBD. Further studies are needed to understand how short-term co-inhibitory receptor blockade can result in long-term effects.

The exact mechanisms of immunotherapy-induced colitis are still elusive. There is evidence that Treg-cell depletion contributes to the pathogenesis of CTLA-4 induced colitis in some studies[141, 142], but not in others.[143, 144] More investigation is required to establish a role for Tregs in immunotherapy-induced colitis. Other possible mechanisms of immunotherapy-induced colitis include the priming of naive lymphocytes with reactivity to intestinal bacteria, self-antigens or cross-reactivity with tumor antigens, and the worsening of a pre-existing colitogenic immune response upon co-inhibitory receptor blockade. Interestingly, the risk of immunotherapy-related colitis is increased in patients with lower abundance of intestinal bacteria belonging to the Bacteroidetes phylum before start of anti-CTLA-4 therapy, which is reminiscent of the dysbiosis observed in IBD patients.[145-147] It has been suggested that the risk of immunotherapy-related colitis may be depend on a patient's co-inhibitory receptor allele polymorphisms,[148] although in general SNPs associated with autoimmune disease do not appear useful in predicting the side-effects of immunotherapy.[149] Moreover, the majority of individuals experience no immunotherapy-related adverse events on anti-CTLA-4 or anti-PD-(L)1 therapy. Therefore, it is likely that multiple factors contribute to intestinal disease development during co-inhibitory receptor blockade.

OTHER MEMBERS OF THE IG SUPERFAMILY: BTLA, LAG-3, TIM-3 AND TIGIT

The success of immunotherapies directed against CTLA-4 and PD-1 in enhancing anti-tumor activity has prompted intense investigation into the targeting of other co-inhibitory receptors in order to broaden the therapeutic repertoire. A next generation immune checkpoint inhibitors directed against co-inhibitory receptors BTLA, Lag-3, Tim-3 and TIGIT, are currently being explored in clinical trials and may emerge soon.[150, 151] It is expected that many of these new checkpoint inhibitors will be used in combination with the already approved checkpoint inhibitors against CTLA-4 and PD-1.[152] In order to maximize success of novel combination therapies while minimizing gastro-intestinal adverse effects, it is crucial to increase our current knowledge on the basic molecular mechanisms of these co-inhibitory molecules and their tissue-specific functions. In the paragraphs below, we highlight the unique tissue-specific functions in the intestine of four newly emerging immune checkpoints.

HVEM ligands: B- and T-lymphocyte attenuator (BTLA; also known as CD272) and CD160. BTLA is a co-inhibitory receptor that is expressed on a wide range of hematopoietic cells, including $CD4^+$ and $CD8^+$ T cells, γδ T cells, B cells, innate lymphoid cells (ILCs), NK cells and DCs.[153, 154] In contrast to other co-inhibitory receptors that are induced upon TCR ligation, BTLA is constitutively expressed on naive $CD4^+$ T cells and its expression increases upon T-cell activation, with the highest level of BTLA observed 2-3 days after TCR stimulation.[155] Unlike CTLA-4 and PD-1, BTLA expression is not enriched on Tregs compared to naive $CD4^+$ T cells.[156] However, BTLA is expressed most highly on $CD4^+$ T cells after antigen-specific anergy induction *in vivo.*[156] *In vitro* ligation of BTLA with an agonistic antibody during $CD4^+$ T-cell stimulation reduces IL-2 and CD25 induction. BTLA is part of a shared receptor-ligand network and binds to the TNF receptor family member Herpesvirus entry mediator (HVEM), a receptor that is constitutively expressed on the surface of various cell types, including hematopoietic cells and non-hematopoietic cells.[154] HVEM also binds to the co-inhibitory receptor CD160, an Ig superfamily member that is co-expressed by a small percentage of unstimulated BTLA-expressing $CD4^+$ T cells and can be upregulated during $CD4^+$ T-cell activation (Figure 1).[157] In BTLA and CD160 co-expressing $CD4^+$ T cells both co-inhibitory receptors may act coordinately in HVEM-mediated inhibition and use different intracellular pathways to exert their suppression.[157] Additionally, HVEM also triggers costimulatory signals by ligating tumor necrosis factor (TNF) superfamily members, including the TNF superfamily members LIGHT and LTa.[158, 159] As a result, the functional outcome of HVEM engagement can be opposing with negative regulation of T-cell responses through BTLA and CD160-derived inhibitory signals but stimulatory signals when binding LIGHT and LTa.[1, 154] Overall, *in vivo* the net outcome of HVEM-signaling via its stimulatory and inhibitory ligands on $CD4^+$ T cells appears a more dominant inhibitory function as HVEM-knockout mice have higher T-cell activation[160], indicating that inhibition via HVEM is the essential, non-redundant function of HVEM. This dominant inhibitory function of HVEM-ligand interaction in T cells is also seen in mouse and human $CD4^+$ T cells stimulated with APCs transfected with mouse or human HVEM, respectively.[157, 161]

Antagonistic anti-human BTLA antibodies are currently in clinical development for cancer treatment and urge us to further understand the dual effects of HVEM-LIGHT-LTa-BTLA-CD160 interactions in intestinal immune function. In line with the pro-inflammatory capacities of HVEM-ligand interaction, *Hvem*$^{-/-}$ mice are resistant to DSS colitis.[162, 163] As LIGHT deficiency only partially reduces DSS colitis, both LIGHT and other stimulatory ligands may convey HVEM signaling, a process attributed to innate immune cells.[162] Deletion of HVEM expression in transferred naive $CD4^+CD45RB^{high}$ T cells was reported to contribute to the induction of T-cell mediated colitis in recipient *Rag*$^{-/-}$ mice in some studies but not others, suggesting that the role of HVEM in T cells is not crucial in intestinal homeostasis.[155, 162] Conversely, absence of HVEM expression on irradiation resistant structural cells in the recipient *Rag*$^{-/-}$ mice (*Hvem*$^{-/-}$*Rag*$^{-/-}$ mice) results in a dramatic acceleration of colitis development compared to *Rag*$^{-/-}$ recipients. This suggested that recipient HVEM expression on structural cells binds inhibitory ligands leading to an anti-inflammatory HVEM-mediated regulation of T-cell transfer colitis.[155] As treatment of *Hvem*$^{-/-}$*Rag*$^{-/-}$ recipients with an agonistic anti-BTLA antibody during T-cell transfer rescued disease, it was postulated that inhibitory BTLA signaling on T cells mediated by HVEM is crucial to prevent colitis acceleration in the transfer colitis model. However, a possible role for ligation of HVEM to the other co-inhibitory ligand CD160 was not experimentally addressed in this study.[155] Using the *Citrobacter rodentium*-induced murine colitis model, the Kronenberg group demonstrated that CD160 is the non-redundant ligand engaging HVEM in the mucosa during intestinal anti-bacterial responses.[164] HVEM-CD160 interactions at the mucosal surface enhanced IL-22R mediated STAT-3 activation in epithelial cells and promoted their innate response to acute bacterial infection (Figure 2.5).[164] In both uninfected and infected mice, CD160 was expressed by several intraepithelial lymphocyte subpopulations, particularly CD8αα-expressing IEL. These $CD160^+CD8\alpha\alpha^+$ IEL rapidly increased during the early stages of *C. Rodentium* infection.[164] Given their close contact with intestinal epithelial cells, $CD160^+CD8\alpha\alpha^+$ IEL seem likely candidates to interact with HVEM, which is known to be highly expressed on intestinal epithelial cells.[155, 164] Whether, reciprocally, epithelial ligation of HVEM to CD160 expressed on IEL elicits functional changes in the IEL is not clear. Human CD160 expression on intestinal IEL has been described[165], but further studies are needed to decipher whether it functions to prevent intestinal inflammation as shown in mice. Taken together, BTLA-HVEM and CD160-HVEM interactions help maintain mucosal

immune homeostasis and protect against mucosal infections. Caution is warranted when blocking BTLA-HVEM interactions due to the potential interaction of HVEM with costimulatory ligands present in the intestine.

Lymphocyte activation gene-3 (Lag-3; also known as CD223).

Lag-3 is a co-inhibitory receptor that is expressed on activated $CD4^+$ and $CD8^+$ T cells and subsets of NK cells.[166, 167] Lag-3 structurally resembles CD4 and binds to MHC-II with higher affinity than CD4 (Figure 1)[168], resulting in downregulation of antigen-specific $CD4^+$ T-cell responses.[169-171] Lag-3 also interferes with $CD8^+$ T-cell function[172], suggesting that Lag-3 has other unidentified ligands in addition to MHC-II.[173] In addition to activated T cells[174], Lag-3 is expressed on Tregs and is often used as a marker for IL-10-secreting $CD4^+Foxp3^{neg}$ Tr1 cells.[175]

Multiple companies have developed Lag-3 specific antagonist antibodies that are currently being tested in phase I clinical trials, but it remains to be seen what the effects on gastro-intestinal homeostasis will be. Lag-3 expression in Tregs is critical for Treg-mediated suppression of colitogenic T-cell responses.[176] Lag-3 expression on Tregs is induced by IL-27, a cytokine that is mainly produced by APCs upon activation by microbial products.[177] IL-27 stimulation has been shown to enhance Treg-suppressive function through a Lag-3-dependent mechanism (Figure 2.6).[176] In contrast to wildtype Tregs, *Lag3*$^{-/-}$ Tregs failed to suppress effector T-cell expansion and cytokine expression in mesenteric lymph nodes, even after IL-27 stimulation.[176] In addition to inhibiting effector T-cell responses through enhancing Treg-suppressive function, Lag-3 can prevent T-cell activation through inhibition of DC maturation (Figure 2.6).[176, 178]

Lag-3 on $CD4^+Foxp3^{neg}$ T cells also has a role in regulating or suppressing other cells in the intestine. $CD4^+CD25^{neg}$Lag-3^+ T cells prevent colitis induced in *Rag1*$^{-/-}$ recipients by the transfer of naive $CD4^+CD25^{neg}CD45RB^{high}$ T cells in a Foxp3-independent, IL-10-dependent manner.[179] In a murine model for SLE, $CD4^+CD25^{neg}$Lag-3^+ T cells suppressed B-cell activation and antibody production through TGF-β3.[180] As frequencies of $CD4^+CD25^{neg}$Lag-3^+ T cells are high in Peyer's patches[179], it can be expected that intestinal Lag-3^+ T-cells regulate intestinal humoral immune responses, but further studies are needed to prove this hypothesis (Figure 2.7). In sum, Lag-3-dependent mechanisms contribute to intestinal homeostasis influencing the function of both intestinal Tregs and suppressive $CD4^+Foxp3^{neg}$ T-cell subsets. However, whether Lag-3-driven regulation also modulates effector T cells or B-cell responses in the intestine has not yet been determined.

T-cell immunoglobulin and mucin domain-containing protein-3 (Tim-3).

Tim-3 is a co-inhibitory receptor initially identified on interferon-gamma (IFNγ)-producing $CD4^+$ T cells and $CD8^+$ T cells[181], but is also expressed on innate immune cells including DCs,

NK cells and monocytes.[182-184] TCR ligation induces Tim-3 expression on conventional $CD4^+$ T cells and Tregs, although with different kinetics.[185] Activated human $CD4^+$ T cells that express Tim-3 secrete reduced levels of IFNγ and IL-17A[186], and Tim-3^+ Tregs have been shown to specifically suppress Th17 cells.[185] Over the past decade, Tim-3 expression on $CD4^+$ and $CD8^+$ T cells has especially been associated with exhausted and dysfunctional T-cell phenotypes[187-189], suggesting that Tim-3 negatively regulates T cells that have previously undergone activation. Tim-3 has many ligands, including galectin-9[190] and Ceacam-1[191], and can bind to its ligands both *in cis* and *in trans* (Figure 1).[186, 191] Therefore, Tim-3 has the capacity to function via both autocrine and paracrine mechanisms to inhibit T-cell responses.

Several Tim-3 antagonists are currently being tested in phase I clinical trials. Data available from animal models support a role for Tim-3 in inhibiting intestinal inflammation. Tim-3 inhibits polarization of pathogenic pro-inflammatory M1 macrophages by preventing phosphorylation of IRF3, a transcriptional factor downstream of TLR-4 that regulates macrophage polarization.[183] In consequence, blockade of Tim-3 exacerbates DSS-induced colitis in wildtype mice, but not in *Tlr4*$^{-/-}$ mice (Figure 2.8).[192] In addition to a role in macrophages, IL-27 greatly enhances TCR-mediated induction of Tim-3 on naive $CD4^+$ T cells[190] but not on Tregs.[176] IL-27-conditioned Th1 cells induce less severe colitis in *Rag1*$^{-/-}$ recipients compared to unconditioned Th1 cells, suggesting that IL-27 can directly suppress Th1 cell-mediated colitis via the induction of Tim-3 (Figure 2.9).[183, 190]

In humans, Tim-3 expression is strongly enriched on $CD4^+$ T cells isolated from the intestinal mucosa compared to $CD4^+$ T cells in peripheral blood.[193] In contrast, IBD patients had significantly lower frequencies of Tim-3^+ cells in $CD4^+$ T-cell populations isolated from peripheral blood and intestinal biopsies when compared to healthy individuals, possibly suggesting that decreased Tim-3 expression or blockade of Tim-3 may contribute to intestinal inflammation.[193, 194] Whether restoration of Tim-3 expression in patients with IBD harbors potential clinical benefit needs to be further investigated.

T-cell immunoreceptor with Ig and ITIM domain (TIGIT).

TIGIT is a co-inhibitory receptor specifically expressed by immune cells, including NK cells, memory $CD4^+$ T cells and subsets of Tregs.[195, 196] TIGIT binds to CD155 and CD112, which are expressed on the surface of APCs, T cells and non-hematopoietic cells.[195, 197] The costimulatory receptor CD226 also binds to the same ligands[198] and together with TIGIT forms a pathway in which CD226 enhances the activation of T cells while TIGIT inhibits their activation[199], which is reminiscent of the CTLA-4/CD28/B7 pathway (Figure 1). On effector T cells, TIGIT directly targets molecules in the TCR signaling pathway, dampening effector T-cell activation, proliferation and inflammatory cytokine secretion.[200] Moreover, TIGIT modifies DC function via bi-directional co-signaling through CD155, promoting

Table 2. Role of co-inhibitory receptors in intestinal homeostasis.

	Treg differentiation	Treg suppressive capacity	Lineage stability	Regulatory CD4$^+$Foxp3^{neg} T cells	Inflammatory effector T cells	Other effects	Human intestinal disease
CTLA-4	CTLA-4 is not required for intestinal pTreg induction but does regulate the accumulation of intestinal pTreg *in vivo*.[53]	CTLA-4 is essential for Treg function; Ctla4$^{-/-}$ CD4$^+$CD25$^+$ T cells fail to prevent colitis induced by naive T-cell transfer into Rag$^{-/-}$ recipients in most studies.[32, 40, 45, 54-56]		Limited data available on the functional role of CTLA-4 on intestinal CD4$^+$Foxp3^{neg} T cells. Blockade or lack of CTLA-4 in adulthood might allow for the differentiation of immunosuppressive CD4$^+$Foxp3^{neg} T cells.[40, 71, 75]	CTLA-4 prevents hyperactivation of colitogenic naive CD4$^+$CD45RB^{high} T cells[40]; CTLA-4 is essential to suppress systemic Th1 and Th2 responses in high-dose oral tolerance.[70] In low dose oral tolerance, other factors than CTLA-4 are required for the suppression of Th1 cell responses.[69]	CTLA-4 limits Treg expansion[47, 71, 72]; CTLA-4 regulates pTreg frequencies in the intestinal lamina propria.[53]	Patients with CTLA-4 haploinsufficiency have impaired Treg function[57-59]; The CTLA4/CT60 polymorphism that increases production of a soluble CTLA-4 isoform, has been associated with IBD.
PD-1	Synergizes with TGF-β to promote pTreg differentiation.[111, 112]	PD-1 does not directly affect pTreg function, as Tregs from PD-1$^{-/-}$ mice exhibited normal levels of suppressive activity.[114]	PD-1 prevents terminal differentiation of Tregs, rendering Tregs less susceptible for apoptosis and promoting their survival.[113, 114, 120]	PD-1 on intestinal CD4$^+$Foxp3^{neg} T cells enriches for IL-10-producing cells.[115]	PD-1 expression on CD8$^+$ effector T cells is involved in preventing responses to intestinal self-antigens.[87, 116, 117]	PD-1$^+$ Tfh cells in Peyers patches promote secretory IgA production[118, 119]; PD-1 inhibits TFNα secretion by CD11c$^+$CD11b$^+$ lamina propria cells in the setting of DSS colitis.[130]	Increased PD-L1 expression on intestinal epithelial cells of IBD patients.[131]
BTLA					HVEM/BTLA interactions are required to prevent colitis acceleration in T-cell transfer colitis.[155]	HVEM/CD160 interactions promote innate responses to bacterial infection.[164]	
Lag-3		Lag-3 expression in Tregs is critical to mediate Treg suppression of colitogenic responses.[176]		CD4$^+$CD25$^-$Lag-3$^+$ T cells prevent colitis induced in Rag1$^{-/-}$ recipients by the transfer of naive CD4$^+$CD25$^-$ CD45RB^{high} T cells in a Foxp3-independent, IL-10-dependent manner.[179]	Prevents effector T-cell activation through inhibition of DC maturation.[176, 178]		
Tim-3				Tim-3 expression correlates with IL-10 production by Tr1-like human T cells generated in vitro from CD4$^+$ memory T cells.[225]	Induction of Tim-3 on effector T cells via IL-27 directly suppress Th1 cell-mediated colitis.[183, 190]	Inhibits DC maturation and polarization of pathogenic pro-inflammatory M1 macrophages.[183, 192]	IBD patients have lower frequencies of intestinal Tim-3$^+$CD4$^+$ T cells when compared to healthy individuals.[193, 194]
TIGIT		TIGIT identifies a Treg subset that specifically suppresses pro-inflammatory Th1 and Th17 but not Th2 responses through effector molecule Fgl-2.[202]	TIGIT$^+$ Tregs show enhanced demethylation of Treg-specific demethylated regions (TSDR) ensuring stable Foxp3 expression.[202, 203]	TIGIT is expressed by approximately 30% of intestinal CD4$^+$Foxp3^{neg} T cells.[60]	TIGIT inhibits IFNγ production by CD4$^+$ and CD8$^+$ effector T cells.[199]		

the generation of immunoregulatory DCs with decreased IL-12 and increased IL-10 production.[195]

In addition to effector cells, TIGIT promotes the suppressive function of both Foxp3$^+$ Treg and regulatory CD4$^+$Foxp3^{neg} T cells. TIGIT expression on CD4$^+$Foxp3^{neg} T cells discriminates IL-10-secreting CD4$^+$ T cells induced by immunotherapy.[201] On Tregs, TIGIT ligation induces the expression of IL-10 and other effector molecules, such as fibrinogen-like protein 2 (Fgl2).[202] TIGIT identifies a Treg subset that specifically suppresses pro-inflammatory Th1 and Th17 but not Th2 responses (Figure 2.10).[202] The *TIGIT* gene is a direct target of Foxp3 and TIGIT expression on Foxp3$^+$ Tregs results in higher levels of Treg signature genes.[202, 203] Moreover, TIGIT$^+$ Tregs show enhanced demethylation of Treg-specific demethylated regions (TSDR) ensuring stable Foxp3 expression.[202] Combined, these data show that TIGIT can promote the stability and function of various subsets of regulatory T cells.

In line with its inhibitory functions, TIGIT deficiency or blockade exacerbates disease in models for autoimmune disease.[200, 204] However, little is known regarding the role of TIGIT in intestinal homeostasis. One study has investigated TIGIT expression in human colonic tissue, and demonstrated that TIGIT is expressed by approximately 30% of intestinal CD4$^+$Foxp3^{neg} T cells and virtually all Tregs.[60] Recently, our group has identified TIGIT as a key regulatory molecule in circulating CD38$^+$ effector T cells, a population enriched for T-cells with specificity for mucosal antigens.[205] Frequencies of TIGIT$^+$CD38$^+$ effector T cells were decreased in a subgroup of pediatric IBD patients before start of treatment and TIGIT percentages below 25% identified patients with a shorter duration of clinical remission.[205] Mechanistic studies are needed to establish whether TIGIT contributes to regulatory T-cell function in the intestine and whether reduced functioning of the TIGIT pathway accelerates or exacerbates intestinal inflammation.

FUTURE PERSPECTIVES: TARGETING CO-INHIBITORY RECEPTORS IN INTESTINAL INFLAMMATION

Over the past decades, the concepts of T-cell costimulation and co-inhibition have substantially increased our understanding of the mechanisms controlling the development and maintenance of intestinal homeostasis (Table 2, Figure 2). In autoimmune diseases such as SLE and RA, mechanistic analysis of co-inhibitory pathways has already led to the identification of potential therapeutic targets and initiation of clinical trials.[3] As such, co-inhibitory receptors may represent potential novel therapeutic targets to treat chronic intestinal inflammation as seen in patients with IBD or intestinal graft-versus-host disease. Moreover, studies have only now begun to identify altered expression of co-inhibitory

receptors in peripheral blood and intestinal tissue of IBD patients, raising the possibility that their expression could serve as predictive biomarkers to identify patients that are most likely benefit from targeted therapies.

Increasing the expression levels of co-inhibitory receptors is one possible mechanism to reduce intestinal inflammation. In peripheral blood of IBD patients, expression of Tim-3 is significantly decreased compared to healthy controls and preliminary data indicate that Tim-3 expression increases after anti-TNFα therapy.[206] Similar results have been obtained in patients with multiple sclerosis (MS).[207, 208] Tim-3 expression is decreased on T cells in peripheral blood of MS patients during active disease and is induced specifically in responders to IFN-β therapy[209, 210], a potent inducer of IL-27.[211] This suggests that IL-27 administration might be a promising therapy to treat chronic inflammatory disease, possibly by overcoming intrinsic defects in the upregulation co-inhibitory receptor expression during inflammation.[207, 212] In humans, IL-27 polymorphisms are associated with susceptibility to IBD.[213] Individuals homozygous for the IBD risk allele near the *IL27* gene express less colonic IL-27 than individuals homozygous for the protective allele.[214] Although multiple studies in mice have identified IL-27 as a suppressor of intestinal inflammation[176, 215-218], investigations are ongoing to establish whether IL-27 administration is effective in IBD. In this context, it will be interesting to see whether decreased co-inhibitory receptor expression in intestinal tissue or peripheral blood are predictive biomarkers to identify patients that are most likely benefit from IL-27-directed therapy, or from other cytokines involved in inducing co-inhibitory receptor expression.

In addition to restoration of co-inhibitory receptor expression, co-inhibitory receptor stimulation or mimicking co-inhibitory receptor function could be beneficial to treat intestinal inflammation. An example of such an approach is abatacept, a chimeric CTLA-4 and IgG-Fc fusion protein that mimics the function of CTLA-4 by blocking CD28 costimulation.[219] CTLA-4-Fc results in downregulation of T-cell activation and proliferation and has demonstrated efficacy in the treatment of autoimmune diseases such as RA and psoriatic arthritis.[220] However, abatacept was not effective treating intestinal inflammation in IBD patients[221], which could reflect the relative lack of dependence of CD28 costimulation in memory T cells in the intestine. Moreover, as costimulation through CD28 is also required for the maintenance of Tregs in the periphery[222], abatacept may impede intestinal Treg survival by inhibiting essential costimulation mediated by CD28.[223] Co-inhibitory receptor fusion proteins other than CTLA-4-Fc might be more successful to treat intestinal inflammation. As an example, the therapeutic potential of targeting the TIGIT/CD226/CD155 pathway by using a TIGIT-Fc fusion protein has been demonstrated *in vivo* in collagen-induced arthritis[204], but its efficacy in models of IBD remains to be tested. Co-inhibitory molecules often have synergistic effects to dampen effector T-cell responses and enhance regulatory T-cell function. This is illustrated by models for T-cell exhaustion,

where co-inhibitory receptors, such as TIGIT and PD-1, are often co-expressed on exhausted virus-specific $CD8^+$ T cells.[224] Expression of PD-1, Lag-3, Tim-3 and TIGIT all correlate with IL-10 production by human $CD4^+$ T cells, although none of these inhibitory receptors are exclusively expressed on IL-10-producing T cells.[201] This suggests that cooperative function of co-inhibitory receptor molecules is needed to achieve optimal T-cell regulation.[201] In consequence, targeting multiple co-inhibitory receptors simultaneously might be needed for effective treatment of intestinal inflammation. Bi-specific antibodies can be used to target more than one co-inhibitory receptor and could co-ligate co-inhibitory receptors and stimulatory receptors simultaneously. As an example of the latter, crosslinking of Lag-3 and CD3 inhibits T-cell proliferation[172], suggesting that Lag-3-CD3 bispecific antibodies could harbor potential clinical efficacy in T-cell mediated inflammatory diseases, including inflammatory disease of the intestine.

Taken together, accumulating evidence suggests that targeting co-inhibitory receptors is a promising approach to treat intestinal inflammation that warrants further investigation. To define co-inhibitory targets for successful treatment of intestinal inflammation, it is essential to acknowledge their tissue-dependent functions and distinct responses based on cell type and associated kinetics.

CONCLUSION

This review highlights the emerging role of co-inhibitory receptors in intestinal homeostasis and elucidates many potential prospects for translation to human disease, such as IBD. Despite the substantial insights into the cell-specific expression and function of co-inhibitory receptors in the intestine over the past decades, there is still much to be learned. Key issues remaining to be resolved include the mechanisms of co-inhibitory molecule induction in the intestinal environment, how co-inhibitory signaling pathways integrate to achieve intestinal homeostasis, and whether memory T cells that reside in the intestinal mucosa require specific co-inhibitory signals. In addition, there is a need for a better mechanistic understanding of why inhibitory receptor pathway blockade leads to intestinal inflammation in some individuals but not in others. This should help us to develop more effective therapies while guaranteeing their safety profiles and obtain a better understanding of chronic intestinal inflammation.

REFERENCES

1. Chen L, Flies DB. Molecular mechanisms of T cell co-stimulation and co-inhibition. Nat Rev Immunol 2013;13:227-42.
2. Schildberg FA, Klein SR, Freeman GJ, et al. Coinhibitory Pathways in the B7-CD28 Ligand-Receptor Family. Immunity 2016;44:955-72.
3. Zhang Q, Vignali DA. Co-stimulatory and Co-inhibitory Pathways in Autoimmunity. Immunity 2016;44:1034-51.
4. Brusca SB, Abramson SB, Scher JU. Microbiome and mucosal inflammation as extra-articular triggers for rheumatoid arthritis and autoimmunity. Curr Opin Rheumatol 2014;26:101-7.
5. Wu HJ, Ivanov, II, Darce J, et al. Gut-residing segmented filamentous bacteria drive autoimmune arthritis via T helper 17 cells. Immunity 2010;32:815-27.
6. Lee YK, Menezes JS, Umesaki Y, et al. Proinflammatory T-cell responses to gut microbiota promote experimental autoimmune encephalomyelitis. Proc Natl Acad Sci U S A 2011;108 Suppl 1:4615-22.
7. Pianta A, Arvikar SL, Strle K, et al. Two rheumatoid arthritis-specific autoantigens correlate microbial immunity with autoimmune responses in joints. J Clin Invest 2017;127:2946-2956.
8. Tanoue T, Atarashi K, Honda K. Development and maintenance of intestinal regulatory T cells. Nat Rev Immunol 2016;16:295-309.
9. Abbas AK, Benoist C, Bluestone JA, et al. Regulatory T cells: recommendations to simplify the nomenclature. Nat Immunol 2013;14:307-8.
10. Hauet-Broere F, Unger WW, Garssen J, et al. Functional CD25- and CD25+ mucosal regulatory T cells are induced in gut-draining lymphoid tissue within 48 h after oral antigen application. Eur J Immunol 2003;33:2801-10.
11. Veenbergen S, van Berkel LA, du Pre MF, et al. Colonic tolerance develops in the iliac lymph nodes and can be established independent of CD103(+) dendritic cells. Mucosal Immunol 2016;9:894-906.
12. Mowat AM. To respond or not to respond - a personal perspective of intestinal tolerance. Nat Rev Immunol 2018;18:405-415.
13. Chen W, Jin W, Hardegen N, et al. Conversion of peripheral CD4+CD25- naive T cells to CD4+CD25+ regulatory T cells by TGF-beta induction of transcription factor Foxp3. J Exp Med 2003;198:1875-86.
14. Coombes JL, Siddiqui KR, Arancibia-Carcamo CV, et al. A functionally specialized population of mucosal CD103+ DCs induces Foxp3+ regulatory T cells via a TGF-beta and retinoic acid-dependent mechanism. J Exp Med 2007;204:1757-64.
15. Mucida D, Park Y, Kim G, et al. Reciprocal TH17 and regulatory T cell differentiation mediated by retinoic acid. Science 2007;317:256-60.
16. Sun CM, Hall JA, Blank RB, et al. Small intestine lamina propria dendritic cells promote de novo generation of Foxp3 T reg cells via retinoic acid. J Exp Med 2007;204:1775-85.
17. Awasthi A, Carrier Y, Peron JP, et al. A dominant function for interleukin 27 in generating interleukin 10-producing anti-inflammatory T cells. Nat Immunol 2007;8:1380-9.
18. Zhao H, Liao X, Kang Y. Tregs: Where We Are and What Comes Next? Front Immunol 2017;8:1578.
19. Akdis M, Verhagen J, Taylor A, et al. Immune responses in healthy and allergic individuals are characterized by a fine balance between allergen-specific T regulatory 1 and T helper 2 cells. J Exp Med 2004;199:1567-75.
20. De Velasco G, Je Y, Bosse D, et al. Comprehensive Meta-analysis of Key Immune-Related Adverse Events from CTLA-4 and PD-1/PD-L1 Inhibitors in Cancer Patients. Cancer Immunol Res 2017;5:312-318.
21. Carreno BM, Collins M. The B7 family of ligands and its receptors: new pathways for costimulation and inhibition of immune responses. Annu Rev Immunol 2002;20:29-53.
22. Collins AV, Brodie DW, Gilbert RJ, et al. The interaction properties of costimulatory molecules revisited. Immunity 2002;17:201-10.
23. Qureshi OS, Kaur S, Hou TZ, et al. Constitutive clathrin-mediated endocytosis of CTLA-4 persists during T cell activation. J Biol Chem 2012;287:9429-40.
24. Chuang E, Alegre ML, Duckett CS, et al. Interaction of CTLA-4 with the clathrin-associated protein AP50 results in ligand-independent endocytosis that limits cell surface expression. J Immunol 1997;159:144-51.
25. Leung HT, Bradshaw J, Cleaveland JS, et al. Cytotoxic T lymphocyte-associated molecule-4, a high-avidity receptor for CD80 and CD86, contains an intracellular localization motif in its cytoplasmic tail. J Biol Chem 1995;270:25107-14.
26. Sansom DM. CD28, CTLA-4 and their ligands: who does what and to whom? Immunology 2000;101:169-77.

27. Fife BT, Bluestone JA. Control of peripheral T-cell tolerance and autoimmunity via the CTLA-4 and PD-1 pathways. Immunol Rev 2008;224:166-82.
28. Buchbinder EI, Desai A. CTLA-4 and PD-1 Pathways: Similarities, Differences, and Implications of Their Inhibition. Am J Clin Oncol 2016;39:98-106.
29. Zheng Y, Josefowicz SZ, Kas A, et al. Genome-wide analysis of Foxp3 target genes in developing and mature regulatory T cells. Nature 2007;445:936-40.
30. Wu Y, Borde M, Heissmeyer V, et al. Foxp3 controls regulatory T cell function through cooperation with NFAT. Cell 2006;126:375-87.
31. Hori S, Nomura T, Sakaguchi S. Control of regulatory T cell development by the transcription factor Foxp3. Science 2003;299:1057-61.
32. Tai X, Van Laethem F, Pobezinsky L, et al. Basis of CTLA-4 function in regulatory and conventional CD4(+) T cells. Blood 2012;119:5155-63.
33. Takahashi T, Tagami T, Yamazaki S, et al. Immunologic self-tolerance maintained by CD25(+)CD4(+) regulatory T cells constitutively expressing cytotoxic T lymphocyte-associated antigen 4. J Exp Med 2000;192:303-10.
34. Jago CB, Yates J, Camara NO, et al. Differential expression of CTLA-4 among T cell subsets. Clin Exp Immunol 2004;136:463-71.
35. Perez-Garcia A, Osca G, Bosch-Vizcaya A, et al. Kinetics of the CTLA-4 isoforms expression after T-lymphocyte activation and role of the promoter polymorphisms on CTLA-4 gene transcription. Hum Immunol 2013;74:1219-24.
36. Alegre ML, Noel PJ, Eisfelder BJ, et al. Regulation of surface and intracellular expression of CTLA4 on mouse T cells. J Immunol 1996;157:4762-70.
37. Tivol EA, Borriello F, Schweitzer AN, et al. Loss of CTLA-4 leads to massive lymphoproliferation and fatal multiorgan tissue destruction, revealing a critical negative regulatory role of CTLA-4. Immunity 1995;3:541-7.
38. Waterhouse P, Penninger JM, Timms E, et al. Lymphoproliferative disorders with early lethality in mice deficient in Ctla-4. Science 1995;270:985-8.
39. Wing K, Onishi Y, Prieto-Martin P, et al. CTLA-4 control over Foxp3+ regulatory T cell function. Science 2008;322:271-5.
40. Read S, Greenwald R, Izcue A, et al. Blockade of CTLA-4 on CD4+CD25+ regulatory T cells abrogates their function in vivo. J Immunol 2006;177:4376-83.
41. Mandelbrot DA, McAdam AJ, Sharpe AH. B7-1 or B7-2 is required to produce the lymphoproliferative phenotype in mice lacking cytotoxic T lymphocyte-associated antigen 4 (CTLA-4). J Exp Med 1999;189:435-40.
42. Tai X, Van Laethem F, Sharpe AH, et al. Induction of autoimmune disease in CTLA-4-/- mice depends on a specific CD28 motif that is required for in vivo costimulation. Proc Natl Acad Sci U S A 2007;104:13756-61.
43. Corse E, Allison JP. Cutting edge: CTLA-4 on effector T cells inhibits in trans. J Immunol 2012;189:1123-7.
44. Wang CJ, Kenefeck R, Wardzinski L, et al. Cutting edge: cell-extrinsic immune regulation by CTLA-4 expressed on conventional T cells. J Immunol 2012;189:1118-22.
45. Jain N, Nguyen H, Chambers C, et al. Dual function of CTLA-4 in regulatory T cells and conventional T cells to prevent multiorgan autoimmunity. Proc Natl Acad Sci U S A 2010;107:1524-8.
46. Walker LS, Sansom DM. Confusing signals: recent progress in CTLA-4 biology. Trends Immunol 2015;36:63-70.
47. Schmidt EM, Wang CJ, Ryan GA, et al. Ctla-4 controls regulatory T cell peripheral homeostasis and is required for suppression of pancreatic islet autoimmunity. J Immunol 2009;182:274-82.
48. Oderup C, Cederbom L, Makowska A, et al. Cytotoxic T lymphocyte antigen-4-dependent down-modulation of costimulatory molecules on dendritic cells in CD4+ CD25+ regulatory T-cell-mediated suppression. Immunology 2006;118:240-9.
49. Qureshi OS, Zheng Y, Nakamura K, et al. Trans-endocytosis of CD80 and CD86: a molecular basis for the cell-extrinsic function of CTLA-4. Science 2011;332:600-3.
50. Walker LS, Sansom DM. The emerging role of CTLA4 as a cell-extrinsic regulator of T cell responses. Nat Rev Immunol 2011;11:852-63.
51. Bachmann MF, Kohler G, Ecabert B, et al. Cutting edge: lymphoproliferative disease in the absence of CTLA-4 is not T cell autonomous. J Immunol 1999;163:1128-31.
52. Homann D, Dummer W, Wolfe T, et al. Lack of intrinsic CTLA-4 expression has minimal effect on regulation of antiviral T-cell immunity. J Virol 2006;80:270-80.

53. Barnes MJ, Griseri T, Johnson AM, et al. CTLA-4 promotes Foxp3 induction and regulatory T cell accumulation in the intestinal lamina propria. Mucosal Immunol 2013;6:324-34.
54. Sojka DK, Hughson A, Fowell DJ. CTLA-4 is required by CD4+CD25+ Treg to control CD4+ T-cell lymphopenia-induced proliferation. Eur J Immunol 2009;39:1544-51.
55. Read S, Malmstrom V, Powrie F. Cytotoxic T lymphocyte-associated antigen 4 plays an essential role in the function of CD25(+)CD4(+) regulatory cells that control intestinal inflammation. J Exp Med 2000;192:295-302.
56. Liu Z, Geboes K, Hellings P, et al. B7 interactions with CD28 and CTLA-4 control tolerance or induction of mucosal inflammation in chronic experimental colitis. J Immunol 2001;167:1830-8.
57. Zeissig S, Petersen BS, Tomczak M, et al. Early-onset Crohn's disease and autoimmunity associated with a variant in CTLA-4. Gut 2015;64:1889-97.
58. Kuehn HS, Ouyang W, Lo B, et al. Immune dysregulation in human subjects with heterozygous germline mutations in CTLA4. Science 2014;345:1623-1627.
59. Schubert D, Bode C, Kenefeck R, et al. Autosomal dominant immune dysregulation syndrome in humans with CTLA4 mutations. Nat Med 2014;20:1410-6.
60. Lord JD, Shows DM, Chen J, et al. Human Blood and Mucosal Regulatory T Cells Express Activation Markers and Inhibitory Receptors in Inflammatory Bowel Disease. PLoS One 2015;10:e0136485.
61. Walker LS. Treg and CTLA-4: two intertwining pathways to immune tolerance. J Autoimmun 2013;45:49-57.
62. Powrie F, Mason D. OX-22high CD4+ T cells induce wasting disease with multiple organ pathology: prevention by the OX-22low subset. J Exp Med 1990;172:1701-8.
63. Powrie F, Leach MW, Mauze S, et al. Phenotypically distinct subsets of CD4+ T cells induce or protect from chronic intestinal inflammation in C. B-17 scid mice. Int Immunol 1993;5:1461-71.
64. Mottet C, Uhlig HH, Powrie F. Cutting edge: cure of colitis by CD4+CD25+ regulatory T cells. J Immunol 2003;170:3939-43.
65. Hadis U, Wahl B, Schulz O, et al. Intestinal tolerance requires gut homing and expansion of Foxp3+ regulatory T cells in the lamina propria. Immunity 2011;34:237-46.
66. Curotto de Lafaille MA, Kutchukhidze N, Shen S, et al. Adaptive Foxp3+ regulatory T cell-dependent and -independent control of allergic inflammation. Immunity 2008;29:114-26.
67. Husby S, Mestecky J, Moldoveanu Z, et al. Oral tolerance in humans. T cell but not B cell tolerance after antigen feeding. J Immunol 1994;152:4663-70.
68. Mowat AM, Strobel S, Drummond HE, et al. Immunological responses to fed protein antigens in mice. I. Reversal of oral tolerance to ovalbumin by cyclophosphamide. Immunology 1982;45:105-13.
69. Barone KS, Herms B, Karlosky L, et al. Effect of in vivo administration of anti-CTLA-4 monoclonal antibody and IL-12 on the induction of low-dose oral tolerance. Clin Exp Immunol 2002;130:196-203.
70. Samoilova EB, Horton JL, Zhang H, et al. CTLA-4 is required for the induction of high dose oral tolerance. Int Immunol 1998;10:491-8.
71. Paterson AM, Lovitch SB, Sage PT, et al. Deletion of CTLA-4 on regulatory T cells during adulthood leads to resistance to autoimmunity. J Exp Med 2015;212:1603-21.
72. Vieira PL, Christensen JR, Minaee S, et al. IL-10-secreting regulatory T cells do not express Foxp3 but have comparable regulatory function to naturally occurring CD4+CD25+ regulatory T cells. J Immunol 2004;172:5986-93.
73. Groux H, O'Garra A, Bigler M, et al. A CD4+ T-cell subset inhibits antigen-specific T-cell responses and prevents colitis. Nature 1997;389:737-42.
74. Huber S, Gagliani N, Esplugues E, et al. Th17 cells express interleukin-10 receptor and are controlled by Foxp3(-) and Foxp3+ regulatory CD4+ T cells in an interleukin-10-dependent manner. Immunity 2011;34:554-65.
75. Coquerelle C, Oldenhove G, Acolty V, et al. Anti-CTLA-4 treatment induces IL-10-producing ICOS+ regulatory T cells displaying IDO-dependent anti-inflammatory properties in a mouse model of colitis. Gut 2009;58:1363-73.
76. Verma N, Burns SO, Walker LSK, et al. Immune deficiency and autoimmunity in patients with CTLA-4 (CD152) mutations. Clin Exp Immunol 2017;190:1-7.
77. Rueda B, Zhernakova A, Lopez-Nevot MA, et al. CTLA4/CT60 polymorphism is not relevant in susceptibility to autoimmune inflammatory intestinal disorders. Hum Immunol 2005;66:321-5.

78. Howson JM, Dunger DB, Nutland S, et al. A type 1 diabetes subgroup with a female bias is characterised by failure in tolerance to thyroid peroxidase at an early age and a strong association with the cytotoxic T-lymphocyte-associated antigen-4 gene. Diabetologia 2007;50:741-6.
79. Blomhoff A, Lie BA, Myhre AG, et al. Polymorphisms in the cytotoxic T lymphocyte antigen-4 gene region confer susceptibility to Addison's disease. J Clin Endocrinol Metab 2004;89:3474-6.
80. Repnik K, Potocnik U. CTLA4 CT60 single-nucleotide polymorphism is associated with Slovenian inflammatory bowel disease patients and regulates expression of CTLA4 isoforms. DNA Cell Biol 2010;29:603-10.
81. Maier LM, Anderson DE, De Jager PL, et al. Allelic variant in CTLA4 alters T cell phosphorylation patterns. Proc Natl Acad Sci U S A 2007;104:18607-12.
82. Ueda H, Howson JM, Esposito L, et al. Association of the T-cell regulatory gene CTLA4 with susceptibility to autoimmune disease. Nature 2003;423:506-11.
83. Hunt KA, McGovern DP, Kumar PJ, et al. A common CTLA4 haplotype associated with coeliac disease. Eur J Hum Genet 2005;13:440-4.
84. van Belzen MJ, Vrolijk MM, Meijer JW, et al. A genomewide screen in a four-generation Dutch family with celiac disease: evidence for linkage to chromosomes 6 and 9. Am J Gastroenterol 2004;99:466-71.
85. Greenwald RJ, Freeman GJ, Sharpe AH. The B7 family revisited. Annu Rev Immunol 2005;23:515-48.
86. Ishida Y, Agata Y, Shibahara K, et al. Induced expression of PD-1, a novel member of the immunoglobulin gene superfamily, upon programmed cell death. Embo J 1992;11:3887-95.
87. Goldberg MV, Maris CH, Hipkiss EL, et al. Role of PD-1 and its ligand, B7-H1, in early fate decisions of CD8 T cells. Blood 2007;110:186-92.
88. Okazaki T, Maeda A, Nishimura H, et al. PD-1 immunoreceptor inhibits B cell receptor-mediated signaling by recruiting src homology 2-domain-containing tyrosine phosphatase 2 to phosphotyrosine. Proc Natl Acad Sci U S A 2001;98:13866-71.
89. Patsoukis N, Li L, Sari D, et al. PD-1 increases PTEN phosphatase activity while decreasing PTEN protein stability by inhibiting casein kinase 2. Mol Cell Biol 2013;33:3091-8.
90. Hui E, Cheung J, Zhu J, et al. T cell costimulatory receptor CD28 is a primary target for PD-1-mediated inhibition. Science 2017;355:1428-1433.
91. Parry RV, Chemnitz JM, Frauwirth KA, et al. CTLA-4 and PD-1 receptors inhibit T-cell activation by distinct mechanisms. Mol Cell Biol 2005;25:9543-53.
92. Kamphorst AO, Wieland A, Nasti T, et al. Rescue of exhausted CD8 T cells by PD-1-targeted therapies is CD28-dependent. Science 2017;355:1423-1427.
93. Nishimura H, Nose M, Hiai H, et al. Development of lupus-like autoimmune diseases by disruption of the PD-1 gene encoding an ITIM motif-carrying immunoreceptor. Immunity 1999;11:141-51.
94. Ansari MJ, Salama AD, Chitnis T, et al. The programmed death-1 (PD-1) pathway regulates autoimmune diabetes in nonobese diabetic (NOD) mice. J Exp Med 2003;198:63-9.
95. Nishimura H, Okazaki T, Tanaka Y, et al. Autoimmune dilated cardiomyopathy in PD-1 receptor-deficient mice. Science 2001;291:319-22.
96. Agata Y, Kawasaki A, Nishimura H, et al. Expression of the PD-1 antigen on the surface of stimulated mouse T and B lymphocytes. Int Immunol 1996;8:765-72.
97. Yao S, Wang S, Zhu Y, et al. PD-1 on dendritic cells impedes innate immunity against bacterial infection. Blood 2009;113:5811-8.
98. Vibhakar R, Juan G, Traganos F, et al. Activation-induced expression of human programmed death-1 gene in T-lymphocytes. Exp Cell Res 1997;232:25-8.
99. Yamazaki T, Akiba H, Iwai H, et al. Expression of programmed death 1 ligands by murine T cells and APC. J Immunol 2002;169:5538-45.
100. Terawaki S, Chikuma S, Shibayama S, et al. IFN-alpha directly promotes programmed cell death-1 transcription and limits the duration of T cell-mediated immunity. J Immunol 2011;186:2772-9.
101. Dong H, Strome SE, Salomao DR, et al. Tumor-associated B7-H1 promotes T-cell apoptosis: a potential mechanism of immune evasion. Nat Med 2002;8:793-800.
102. Nomi T, Sho M, Akahori T, et al. Clinical significance and therapeutic potential of the programmed death-1 ligand/programmed death-1 pathway in human pancreatic cancer. Clin Cancer Res 2007;13:2151-7.
103. Hamanishi J, Mandai M, Iwasaki M, et al. Programmed cell death 1 ligand 1 and tumor-infiltrating CD8+ T lymphocytes are prognostic factors of human ovarian cancer. Proc Natl Acad Sci U S A 2007;104:3360-5.
104. Freeman GJ, Long AJ, Iwai Y, et al. Engagement of the PD-1 immunoinhibitory receptor by a novel B7 family member leads to negative regulation of lymphocyte activation. J Exp Med 2000;192:1027-34.

105. Taube JM, Anders RA, Young GD, et al. Colocalization of inflammatory response with B7-h1 expression in human melanocytic lesions supports an adaptive resistance mechanism of immune escape. Sci Transl Med 2012;4:127ra37.
106. Salama AD, Chitnis T, Imitola J, et al. Critical role of the programmed death-1 (PD-1) pathway in regulation of experimental autoimmune encephalomyelitis. J Exp Med 2003;198:71-8.
107. Blank C, Brown I, Peterson AC, et al. PD-L1/B7H-1 inhibits the effector phase of tumor rejection by T cell receptor (TCR) transgenic CD8+ T cells. Cancer Res 2004;64:1140-5.
108. Keir ME, Liang SC, Guleria I, et al. Tissue expression of PD-L1 mediates peripheral T cell tolerance. J Exp Med 2006;203:883-95.
109. Spranger S, Spaapen RM, Zha Y, et al. Up-regulation of PD-L1, IDO, and T(regs) in the melanoma tumor microenvironment is driven by CD8(+) T cells. Sci Transl Med 2013;5:200ra116.
110. Gubin MM, Zhang X, Schuster H, et al. Checkpoint blockade cancer immunotherapy targets tumour-specific mutant antigens. Nature 2014;515:577-81.
111. Francisco LM, Salinas VH, Brown KE, et al. PD-L1 regulates the development, maintenance, and function of induced regulatory T cells. J Exp Med 2009;206:3015-29.
112. Wang L, Pino-Lagos K, de Vries VC, et al. Programmed death 1 ligand signaling regulates the generation of adaptive Foxp3+CD4+ regulatory T cells. Proc Natl Acad Sci U S A 2008;105:9331-6.
113. Zhang B, Chikuma S, Hori S, et al. Nonoverlapping roles of PD-1 and Foxp3 in maintaining immune tolerance in a novel autoimmune pancreatitis mouse model. Proc Natl Acad Sci U S A 2016;113:8490-5.
114. Asano T, Meguri Y, Yoshioka T, et al. PD-1 modulates regulatory T-cell homeostasis during low-dose interleukin-2 therapy. Blood 2017;129:2186-2197.
115. Alfen JS, Larghi P, Facciotti F, et al. Intestinal IFN-gamma-producing type 1 regulatory T cells coexpress CCR5 and programmed cell death protein 1 and downregulate IL-10 in the inflamed guts of patients with inflammatory bowel disease. J Allergy Clin Immunol 2018.
116. Reynoso ED, Elpek KG, Francisco L, et al. Intestinal tolerance is converted to autoimmune enteritis upon PD-1 ligand blockade. J Immunol 2009;182:2102-12.
117. Pauken KE, Nelson CE, Martinov T, et al. Cutting edge: identification of autoreactive CD4+ and CD8+ T cell subsets resistant to PD-1 pathway blockade. J Immunol 2015;194:3551-3555.
118. Kawamoto S, Tran TH, Maruya M, et al. The inhibitory receptor PD-1 regulates IgA selection and bacterial composition in the gut. Science 2012;336:485-9.
119. Good-Jacobson KL, Szumilas CG, Chen L, et al. PD-1 regulates germinal center B cell survival and the formation and affinity of long-lived plasma cells. Nat Immunol 2010;11:535-42.
120. Sharma MD, Shinde R, McGaha TL, et al. The PTEN pathway in Tregs is a critical driver of the suppressive tumor microenvironment. Sci Adv 2015;1:e1500845.
121. Maruya M, Kawamoto S, Kato LM, et al. Impaired selection of IgA and intestinal dysbiosis associated with PD-1-deficiency. Gut Microbes 2013;4:165-71.
122. Gutzeit C, Magri G, Cerutti A. Intestinal IgA production and its role in host-microbe interaction. Immunol Rev 2014;260:76-85.
123. Johansen FE, Pekna M, Norderhaug IN, et al. Absence of epithelial immunoglobulin A transport, with increased mucosal leakiness, in polymeric immunoglobulin receptor/secretory component-deficient mice. J Exp Med 1999;190:915-22.
124. Ludvigsson JF, Neovius M, Hammarstrom L. Association between IgA deficiency & other autoimmune conditions: a population-based matched cohort study. J Clin Immunol 2014;34:444-51.
125. Latiff AH, Kerr MA. The clinical significance of immunoglobulin A deficiency. Ann Clin Biochem 2007;44:131-9.
126. Zinneman HH, Kaplan AP. The association of giardiasis with reduced intestinal secretory immunoglobulin A. Am J Dig Dis 1972;17:793-7.
127. Kwon H, Thierry-Mieg D, Thierry-Mieg J, et al. Analysis of interleukin-21-induced Prdm1 gene regulation reveals functional cooperation of STAT3 and IRF4 transcription factors. Immunity 2009;31:941-52.
128. Vinuesa CG, Tangye SG, Moser B, et al. Follicular B helper T cells in antibody responses and autoimmunity. Nat Rev Immunol 2005;5:853-65.
129. Arthur JC, Perez-Chanona E, Muhlbauer M, et al. Intestinal inflammation targets cancer-inducing activity of the microbiota. Science 2012;338:120-3.
130. Scandiuzzi L, Ghosh K, Hofmeyer KA, et al. Tissue-expressed B7-H1 critically controls intestinal inflammation. Cell Rep 2014;6:625-32.

131. Nakazawa A, Dotan I, Brimnes J, et al. The expression and function of costimulatory molecules B7H and B7-H1 on colonic epithelial cells. Gastroenterology 2004;126:1347-57.
132. Kanai T, Totsuka T, Uraushihara K, et al. Blockade of B7-H1 suppresses the development of chronic intestinal inflammation. J Immunol 2003;171:4156-63.
133. Bertrand A, Kostine M, Barnetche T, et al. Immune related adverse events associated with anti-CTLA-4 antibodies: systematic review and meta-analysis. BMC Med 2015;13:211.
134. Gupta A, De Felice KM, Loftus EV, Jr., et al. Systematic review: colitis associated with anti-CTLA-4 therapy. Aliment Pharmacol Ther 2015;42:406-17.
135. Tandon P, Bourassa-Blanchette S, Bishay K, et al. The Risk of Diarrhea and Colitis in Patients With Advanced Melanoma Undergoing Immune Checkpoint Inhibitor Therapy: A Systematic Review and Meta-Analysis. J Immunother 2018.
136. Wolchok JD, Chiarion-Sileni V, Gonzalez R, et al. Overall Survival with Combined Nivolumab and Ipilimumab in Advanced Melanoma. N Engl J Med 2017;377:1345-1356.
137. Chen JH, Pezhouh MK, Lauwers GY, et al. Histopathologic Features of Colitis Due to Immunotherapy With Anti-PD-1 Antibodies. Am J Surg Pathol 2017;41:643-654.
138. Oble DA, Mino-Kenudson M, Goldsmith J, et al. Alpha-CTLA-4 mAb-associated panenteritis: a histologic and immunohistochemical analysis. Am J Surg Pathol 2008;32:1130-7.
139. Garcia-Varona A, Odze RD, Makrauer F. Lymphocytic colitis secondary to ipilimumab treatment. Inflamm Bowel Dis 2013;19:E15-6.
140. Marthey L, Mateus C, Mussini C, et al. Cancer Immunotherapy with Anti-CTLA-4 Monoclonal Antibodies Induces an Inflammatory Bowel Disease. J Crohns Colitis 2016;10:395-401.
141. Nancey S, Boschetti G, Cotte E, et al. Blockade of cytotoxic T-lymphocyte antigen-4 by ipilimumab is associated with a profound long-lasting depletion of Foxp3+ regulatory T cells: a mechanistic explanation for ipilimumab-induced severe enterocolitis? Inflamm Bowel Dis 2012;18:E1598-600.
142. Simpson TR, Li F, Montalvo-Ortiz W, et al. Fc-dependent depletion of tumor-infiltrating regulatory T cells co-defines the efficacy of anti-CTLA-4 therapy against melanoma. J Exp Med 2013;210:1695-710.
143. Maker AV, Attia P, Rosenberg SA. Analysis of the cellular mechanism of antitumor responses and autoimmunity in patients treated with CTLA-4 blockade. J Immunol 2005;175:7746-54.
144. Lord JD, Hackman RC, Moklebust A, et al. Refractory colitis following anti-CTLA4 antibody therapy: analysis of mucosal Foxp3+ T cells. Dig Dis Sci 2010;55:1396-405.
145. Zhou Y, Zhi F. Lower Level of Bacteroides in the Gut Microbiota Is Associated with Inflammatory Bowel Disease: A Meta-Analysis. Biomed Res Int 2016;2016:5828959.
146. Swidsinski A, Ladhoff A, Pernthaler A, et al. Mucosal flora in inflammatory bowel disease. Gastroenterology 2002;122:44-54.
147. Andoh A, Kuzuoka H, Tsujikawa T, et al. Multicenter analysis of fecal microbiota profiles in Japanese patients with Crohn's disease. J Gastroenterol 2012;47:1298-307.
148. Sanderson K, Scotland R, Lee P, et al. Autoimmunity in a phase I trial of a fully human anti-cytotoxic T-lymphocyte antigen-4 monoclonal antibody with multiple melanoma peptides and Montanide ISA 51 for patients with resected stages III and IV melanoma. J Clin Oncol 2005;23:741-50.
149. June CH, Warshauer JT, Bluestone JA. Is autoimmunity the Achilles' heel of cancer immunotherapy? Nat Med 2017;23:540-547.
150. Torphy RJ, Schulick RD, Zhu Y. Newly Emerging Immune Checkpoints: Promises for Future Cancer Therapy. Int J Mol Sci 2017;18.
151. Marin-Acevedo JA, Dholaria B, Soyano AE, et al. Next generation of immune checkpoint therapy in cancer: new developments and challenges. J Hematol Oncol 2018;11:39.
152. Collin M. Immune checkpoint inhibitors: a patent review (2010-2015). Expert Opin Ther Pat 2016;26:555-64.
153. Bekiaris V, Sedy JR, Macauley MG, et al. The inhibitory receptor BTLA controls gammadelta T cell homeostasis and inflammatory responses. Immunity 2013;39:1082-1094.
154. Murphy TL, Murphy KM. Slow down and survive: Enigmatic immunoregulation by BTLA and HVEM. Annu Rev Immunol 2010;28:389-411.
155. Steinberg MW, Turovskaya O, Shaikh RB, et al. A crucial role for HVEM and BTLA in preventing intestinal inflammation. J Exp Med 2008;205:1463-76.
156. Hurchla MA, Sedy JR, Gavrieli M, et al. B and T lymphocyte attenuator exhibits structural and expression polymorphisms and is highly Induced in anergic CD4+ T cells. J Immunol 2005;174:3377-85.

157. Cai G, Anumanthan A, Brown JA, et al. CD160 inhibits activation of human CD4+ T cells through interaction with herpesvirus entry mediator. Nat Immunol 2008;9:176-85.
158. Mauri DN, Ebner R, Montgomery RI, et al. LIGHT, a new member of the TNF superfamily, and lymphotoxin alpha are ligands for herpesvirus entry mediator. Immunity 1998;8:21-30.
159. Montgomery RI, Warner MS, Lum BJ, et al. Herpes simplex virus-1 entry into cells mediated by a novel member of the TNF/NGF receptor family. Cell 1996;87:427-36.
160. Wang Y, Subudhi SK, Anders RA, et al. The role of herpesvirus entry mediator as a negative regulator of T cell-mediated responses. J Clin Invest 2005;115:711-7.
161. Sedy JR, Gavrieli M, Potter KG, et al. B and T lymphocyte attenuator regulates T cell activation through interaction with herpesvirus entry mediator. Nat Immunol 2005;6:90-8.
162. Schaer C, Hiltbrunner S, Ernst B, et al. HVEM signalling promotes colitis. PLoS One 2011;6:e18495.
163. Kim WK, Park JS, Sul OJ, et al. Role of TNFR-related 2 mediated immune responses in dextran sulfate sodium-induced inflammatory bowel disease. Mol Cells 2011;31:99-104.
164. Shui JW, Larange A, Kim G, et al. HVEM signalling at mucosal barriers provides host defence against pathogenic bacteria. Nature 2012;488:222-5.
165. Anumanthan A, Bensussan A, Boumsell L, et al. Cloning of BY55, a novel Ig superfamily member expressed on NK cells, CTL, and intestinal intraepithelial lymphocytes. J Immunol 1998;161:2780-90.
166. Triebel F, Jitsukawa S, Baixeras E, et al. LAG-3, a novel lymphocyte activation gene closely related to CD4. J Exp Med 1990;171:1393-405.
167. Bruniquel D, Borie N, Hannier S, et al. Regulation of expression of the human lymphocyte activation gene-3 (LAG-3) molecule, a ligand for MHC class II. Immunogenetics 1998;48:116-24.
168. Huard B, Prigent P, Tournier M, et al. CD4/major histocompatibility complex class II interaction analyzed with CD4- and lymphocyte activation gene-3 (LAG-3)-Ig fusion proteins. Eur J Immunol 1995;25:2718-21.
169. Huard B, Prigent P, Pages F, et al. T cell major histocompatibility complex class II molecules down-regulate CD4+ T cell clone responses following LAG-3 binding. Eur J Immunol 1996;26:1180-6.
170. Workman CJ, Dugger KJ, Vignali DA. Cutting edge: molecular analysis of the negative regulatory function of lymphocyte activation gene-3. J Immunol 2002;169:5392-5.
171. Workman CJ, Vignali DA. The CD4-related molecule, LAG-3 (CD223), regulates the expansion of activated T cells. Eur J Immunol 2003;33:970-9.
172. Hannier S, Tournier M, Bismuth G, et al. CD3/TCR complex-associated lymphocyte activation gene-3 molecules inhibit CD3/TCR signaling. J Immunol 1998;161:4058-65.
173. Anderson AC, Joller N, Kuchroo VK. Lag-3, Tim-3, and TIGIT: Co-inhibitory Receptors with Specialized Functions in Immune Regulation. Immunity 2016;44:989-1004.
174. Durham NM, Nirschl CJ, Jackson CM, et al. Lymphocyte Activation Gene 3 (LAG-3) modulates the ability of CD4 T-cells to be suppressed in vivo. PLoS One 2014;9:e109080.
175. Gagliani N, Magnani CF, Huber S, et al. Coexpression of CD49b and LAG-3 identifies human and mouse T regulatory type 1 cells. Nat Med 2013;19:739-46.
176. Do JS, Visperas A, Sanogo YO, et al. An IL-27/Lag3 axis enhances Foxp3+ regulatory T cell-suppressive function and therapeutic efficacy. Mucosal Immunol 2016;9:137-45.
177. Hall AO, Silver JS, Hunter CA. The immunobiology of IL-27. Adv Immunol 2012;115:1-44.
178. Liang B, Workman C, Lee J, et al. Regulatory T cells inhibit dendritic cells by lymphocyte activation gene-3 engagement of MHC class II. J Immunol 2008;180:5916-26.
179. Okamura T, Fujio K, Shibuya M, et al. CD4+CD25-LAG3+ regulatory T cells controlled by the transcription factor Egr-2. Proc Natl Acad Sci U S A 2009;106:13974-9.
180. Okamura T, Sumitomo S, Morita K, et al. TGF-beta3-expressing CD4+CD25(-)LAG3+ regulatory T cells control humoral immune responses. Nat Commun 2015;6:6329.
181. Monney L, Sabatos CA, Gaglia JL, et al. Th1-specific cell surface protein Tim-3 regulates macrophage activation and severity of an autoimmune disease. Nature 2002;415:536-41.
182. Chiba S, Baghdadi M, Akiba H, et al. Tumor-infiltrating DCs suppress nucleic acid-mediated innate immune responses through interactions between the receptor TIM-3 and the alarmin HMGB1. Nat Immunol 2012;13:832-42.
183. Jiang X, Yu J, Shi Q, et al. Tim-3 promotes intestinal homeostasis in DSS colitis by inhibiting M1 polarization of macrophages. Clin Immunol 2015;160:328-35.
184. Patel J, Bozeman EN, Selvaraj P. Taming dendritic cells with TIM-3: another immunosuppressive strategy used by tumors. Immunotherapy 2012;4:1795-8.

185. Gautron AS, Dominguez-Villar M, de Marcken M, et al. Enhanced suppressor function of TIM-3+ Foxp3+ regulatory T cells. Eur J Immunol 2014;44:2703-2711.
186. Hastings WD, Anderson DE, Kassam N, et al. TIM-3 is expressed on activated human CD4+ T cells and regulates Th1 and Th17 cytokines. Eur J Immunol 2009;39:2492-501.
187. Golden-Mason L, Palmer BE, Kassam N, et al. Negative immune regulator Tim-3 is overexpressed on T cells in hepatitis C virus infection and its blockade rescues dysfunctional CD4+ and CD8+ T cells. J Virol 2009;83:9122-30.
188. Jones RB, Ndhlovu LC, Barbour JD, et al. Tim-3 expression defines a novel population of dysfunctional T cells with highly elevated frequencies in progressive HIV-1 infection. J Exp Med 2008;205:2763-79.
189. Jin HT, Anderson AC, Tan WG, et al. Cooperation of Tim-3 and PD-1 in CD8 T-cell exhaustion during chronic viral infection. Proc Natl Acad Sci U S A 2010;107:14733-8.
190. Zhu C, Sakuishi K, Xiao S, et al. An IL-27/NFIL3 signalling axis drives Tim-3 and IL-10 expression and T-cell dysfunction. Nat Commun 2015;6:6072.
191. Huang YH, Zhu C, Kondo Y, et al. CEACAM1 regulates TIM-3-mediated tolerance and exhaustion. Nature 2015;517:386-90.
192. Li X, Chen G, Li Y, et al. Involvement of T cell Ig Mucin-3 (Tim-3) in the negative regulation of inflammatory bowel disease. Clin Immunol 2010;134:169-77.
193. Morimoto K, Hosomi S, Yamagami H, et al. Dysregulated upregulation of T-cell immunoglobulin and mucin domain-3 on mucosal T helper 1 cells in patients with Crohn's disease. Scand J Gastroenterol 2011;46:701-9.
194. Shi F, Guo X, Jiang X, et al. Dysregulated Tim-3 expression and its correlation with imbalanced CD4 helper T cell function in ulcerative colitis. Clin Immunol 2012;145:230-40.
195. Yu X, Harden K, Gonzalez LC, et al. The surface protein TIGIT suppresses T cell activation by promoting the generation of mature immunoregulatory dendritic cells. Nat Immunol 2009;10:48-57.
196. Wang F, Hou H, Wu S, et al. TIGIT expression levels on human NK cells correlate with functional heterogeneity among healthy individuals. Eur J Immunol 2015;45:2886-97.
197. Casado JG, Pawelec G, Morgado S, et al. Expression of adhesion molecules and ligands for activating and costimulatory receptors involved in cell-mediated cytotoxicity in a large panel of human melanoma cell lines. Cancer Immunol Immunother 2009;58:1517-26.
198. Bottino C, Castriconi R, Pende D, et al. Identification of PVR (CD155) and Nectin-2 (CD112) as cell surface ligands for the human DNAM-1 (CD226) activating molecule. J Exp Med 2003;198:557-67.
199. Lozano E, Dominguez-Villar M, Kuchroo V, et al. The TIGIT/CD226 axis regulates human T cell function. J Immunol 2012;188:3869-75.
200. Joller N, Hafler JP, Brynedal B, et al. Cutting edge: TIGIT has T cell-intrinsic inhibitory functions. J Immunol 2011;186:1338-42.
201. Burton BR, Britton GJ, Fang H, et al. Sequential transcriptional changes dictate safe and effective antigen-specific immunotherapy. Nat Commun 2014;5:4741.
202. Joller N, Lozano E, Burkett PR, et al. Treg cells expressing the coinhibitory molecule TIGIT selectively inhibit proinflammatory Th1 and Th17 cell responses. Immunity 2014;40:569-81.
203. Fuhrman CA, Yeh WI, Seay HR, et al. Divergent Phenotypes of Human Regulatory T Cells Expressing the Receptors TIGIT and CD226. J Immunol 2015;195:145-55.
204. Levin SD, Taft DW, Brandt CS, et al. Vstm3 is a member of the CD28 family and an important modulator of T-cell function. Eur J Immunol 2011;41:902-15.
205. Joosse ME, Menckeberg CL, de Ruiter LF, et al. Frequencies of circulating regulatory TIGIT(+)CD38(+) effector T cells correlate with the course of inflammatory bowel disease. Mucosal Immunol 2018.
206. Kim MJ, Lee WY, Choe YH. Expression of TIM-3, Human beta-defensin-2, and Foxp3 and Correlation with Disease Activity in Pediatric Crohn's Disease with Infliximab Therapy. Gut Liver 2015;9:370-80.
207. Koguchi K, Anderson DE, Yang L, et al. Dysregulated T cell expression of TIM3 in multiple sclerosis. J Exp Med 2006;203:1413-8.
208. Yang L, Anderson DE, Kuchroo J, et al. Lack of TIM-3 immunoregulation in multiple sclerosis. J Immunol 2008;180:4409-14.
209. Ottoboni L, Keenan BT, Tamayo P, et al. An RNA profile identifies two subsets of multiple sclerosis patients differing in disease activity. Sci Transl Med 2012;4:153ra131.
210. Boivin N, Baillargeon J, Doss PM, et al. Interferon-beta suppresses murine Th1 cell function in the absence of antigen-presenting cells. PLoS One 2015;10:e0124802.

211. Sweeney CM, Lonergan R, Basdeo SA, et al. IL-27 mediates the response to IFN-beta therapy in multiple sclerosis patients by inhibiting Th17 cells. Brain Behav Immun 2011;25:1170-81.
212. Andrews C, McLean MH, Durum SK. Interleukin-27 as a Novel Therapy for Inflammatory Bowel Disease: A Critical Review of the Literature. Inflamm Bowel Dis 2016;22:2255-64.
213. Li CS, Zhang Q, Lee KJ, et al. Interleukin-27 polymorphisms are associated with inflammatory bowel diseases in a Korean population. J Gastroenterol Hepatol 2009;24:1692-6.
214. Imielinski M, Baldassano RN, Griffiths A, et al. Common variants at five new loci associated with early-onset inflammatory bowel disease. Nat Genet 2009;41:1335-40.
215. Dann SM, Le C, Choudhury BK, et al. Attenuation of intestinal inflammation in interleukin-10-deficient mice infected with Citrobacter rodentium. Infect Immun 2014;82:1949-58.
216. Hanson ML, Hixon JA, Li W, et al. Oral delivery of IL-27 recombinant bacteria attenuates immune colitis in mice. Gastroenterology 2014;146:210-221 e13.
217. Sasaoka T, Ito M, Yamashita J, et al. Treatment with IL-27 attenuates experimental colitis through the suppression of the development of IL-17-producing T helper cells. Am J Physiol Gastrointest Liver Physiol 2011;300:G568-76.
218. Troy AE, Zaph C, Du Y, et al. IL-27 regulates homeostasis of the intestinal CD4+ effector T cell pool and limits intestinal inflammation in a murine model of colitis. J Immunol 2009;183:2037-44.
219. Dall'Era M, Davis J. CTLA4Ig: a novel inhibitor of costimulation. Lupus 2004;13:372-6.
220. Genovese MC, Becker JC, Schiff M, et al. Abatacept for rheumatoid arthritis refractory to tumor necrosis factor alpha inhibition. N Engl J Med 2005;353:1114-23.
221. Sandborn WJ, Colombel JF, Sands BE, et al. Abatacept for Crohn's disease and ulcerative colitis. Gastroenterology 2012;143:62-69 e4.
222. Bour-Jordan H, Bluestone JA. Regulating the regulators: costimulatory signals control the homeostasis and function of regulatory T cells. Immunol Rev 2009;229:41-66.
223. Mayer L, Kaser A, Blumberg RS. Dead on arrival: understanding the failure of CTLA4-immunoglobulin therapy in inflammatory bowel disease. Gastroenterology 2012;143:13-7.
224. Johnston RJ, Comps-Agrar L, Hackney J, et al. The immunoreceptor TIGIT regulates antitumor and antiviral CD8(+) T cell effector function. Cancer Cell 2014;26:923-37.
225. White AM, Wraith DC. Tr1-Like T Cells - An Enigmatic Regulatory T Cell Lineage. Front Immunol 2016;7:355.

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3

FREQUENCIES OF CIRCULATING REGULATORY TIGIT⁺CD38⁺ EFFECTOR T CELLS CORRELATE WITH THE COURSE OF INFLAMMATORY BOWEL DISEASE

Chapter 3

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FREQUENCIES OF CIRCULATING REGULATORY TIGIT⁺CD38⁺ EFFECTOR T CELLS CORRELATE WITH THE COURSE OF INFLAMMATORY BOWEL DISEASE

ABSTRACT

Disease heterogeneity hampers achieving long-term disease remission in inflammatory bowel disease (IBD). Monitoring ongoing tissue-localized regulatory and inflammatory T-cell responses in peripheral blood would empower disease classification. We determined whether regulatory and inflammatory phenotypes of circulating CD38⁺ effector ($CD62L^{neg}CD4^{+}$) T cells, a population enriched for cells with mucosal antigen specificity, classify disease course in pediatric IBD patients. In healthy individuals, circulating CD38⁺ effector T cells had a predominant regulatory component with lower frequencies of IFNγ-secreting T cells, higher frequencies of IL-10-secreting T cells and higher frequencies of inhibitory molecule T-cell immunoglobulin and ITIM domain⁺ (TIGIT) cells than $CD38^{neg}$ effector T cells. TIGIT expression was stable upon stimulation and marked CD38⁺ T cells with inhibitory properties. In IBD patients with active intestinal inflammation this predominant regulatory component was lost: circulating CD38⁺ effector T cells had increased activated $CD25^{+}CD45RA^{neg}$ and decreased TIGIT⁺ cell frequencies. TIGIT percentages below 25% before treatment associated with shorter duration of clinical remission. In conclusion, phenotypic changes in circulating CD38⁺ effector T cells, in particular the frequency of TIGIT⁺ cells, classify pediatric IBD patients and predict severity of disease course. These findings have relevance for IBD and can be exploited in graft-versus-host-disease and checkpoint inhibitor-induced inflammation in cancer.

INTRODUCTION

Being continuously exposed to a large variety of foreign antigens, the intestinal immune system tailors its response to mount balanced inflammatory responses against pathogenic antigens and regulatory responses to harmless antigens thus avoiding excessive tissue damage. Such balancing of T-cell responses is lost in inflammatory bowel disease (IBD) patients who suffer from chronic inflammatory immune responses to commensal microbial antigens characterized by infiltration of interferon gamma (IFNγ) and interleukin-17 (IL-17) secreting effector T cells in the intestinal mucosa.[1] The disease is heterogeneous in terms of severity, disease course and anatomical location. As a result, current therapeutic strategies which aim at suppressing these inflammatory effector memory T-cell responses have variable therapeutic effect. Monitoring loss of balance between inflammatory and regulatory tissue-localized T-cell responses in pediatric IBD patients is highly desired to classify patients and predict their disease course but is difficult as endoscopy is too invasive to routinely be used. Moreover, T cells isolated from the intestinal mucosa cannot be used for accurate quantification and enumeration of cell populations as biopsies are not representative of the entire mucosal surface. Instead, peripheral blood is accessible for enumeration of inflammatory versus regulatory T-cell populations and would enable longitudinal follow-up. However, until now, monitoring the phenotype of the total CD4⁺ T-cell population in peripheral blood has not yielded consistent changes in inflammatory and regulatory populations in IBD. For example, reduced frequencies of circulating CD4⁺ T cells expressing Forkhead box P3 (Foxp3) have been reported in some IBD studies [2-5] but not in others.[6, 7] Monitoring frequencies of cells in the total circulating CD4⁺ T-cell population may simply not be sensitive enough to reliably detect transient changes in inflammatory or regulatory intestinal T-cell responses.

Recently, we demonstrated that CD38 expression on peripheral blood CD4⁺ effector ($CD4^{+}CD62L^{neg}$) T cells enriches for T cells with specificity for mucosal antigens.[8] Circulating CD38⁺ effector T cells, comprising 4-10% of the total CD4⁺ T-cell pool, are enriched in cells expressing the gut-homing chemokine receptor C-C chemokine receptor type 9 (CCR9) and β7-integrin compared to $CD38^{neg}$ effector T cells. Conversely, cells expressing the skin-homing receptor cutaneous leukocyte-associated antigen are almost absent in the CD38⁺ effector T-cell population but enriched in the $CD38^{neg}$ effector T-cell population.[8] After oral gluten challenge all gluten-specific CD4⁺ T cells in peripheral blood of celiac disease patients have the CD38⁺ effector phenotype demonstrating specificity for intestinal luminal antigen is contained within this population.[8] In intestinal tissue virtually all CD4⁺ T cells have the $CD62L^{neg}CD38^{+}$ phenotype.[8-10] Crucially, imprinting of CD38 expression on differentiating T cells occurs irrespectively of the inflammatory or regulatory nature of the differentiating T cell.[8]

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FREQUENCIES OF CIRCULATING REGULATORY TIGIT⁺CD38⁺ EFFECTOR T CELLS CORRELATE WITH THE COURSE OF INFLAMMATORY BOWEL DISEASE

Having established that the circulating CD38⁺ effector T-cell population is enriched in cells with mucosal antigen specificity, we hypothesized that the composition of inflammatory versus regulatory cells in the circulating CD38⁺ effector T-cell population detects ongoing disease activity and predicts disease course and therapy response in pediatric IBD.

METHODS

Patients.

Two cohorts of pediatric IBD patients were investigated. Cohort I consisted of pediatric IBD patients in clinical remission who visited the outpatient clinic (n=26) and pediatric patients who underwent a colonoscopy with suspicion of IBD. After diagnosis, those with biopsy-proven active IBD were included in the analyses and termed "inflammation" (n=22). In cohort I, remission was defined by physician global assessment (PGA). Cohort II consisted of treatment-naive patients with biopsy-proven active IBD termed "inflammation" (n=18). Of this group, peripheral blood of 9 patients was also analyzed during follow up after initiation of treatment. In cohort II, clinical remission and exacerbation were defined using PGA and clinical disease scores. Age-matched controls without any inflammatory or intestinal disease who underwent orthopedic surgery at the time of blood withdrawal (n=22) were included in the control group. Peripheral blood was also obtained from adult healthy controls (n=25). The Medical Ethical Committee of the Erasmus University Medical Centre-Sophia Children's Hospital Rotterdam approved this study (METC 2007-335). Written informed consent was obtained from every patient and parents before study inclusion. Additional patient characteristics are shown in Figure S1 and Figure S6.

T-cell isolation and T-cell/DC co-cultures.

Venous blood was collected in EDTA tubes and peripheral blood mononuclear cells (PBMCs) were isolated using a Ficoll-Hypaque gradient according to standard protocol (Axis-Shield). PBMCs were stained for flow cytometry and $CD3^+CD4^+CD62L^{neg}$ T cells were sorted into $CD38^{neg}$ and $CD38^+$ effector T-cell populations by flow cytometric cell sorting on a BD FACS Aria (BD Biosciences). Purified T-cell populations were stimulated with anti-CD3/CD28 stimulator beads or co-cultured with allogeneic LPS-stimulated monocyte-derived dendritic cells (DCs) for 72 hours.

$CD14^+$ monocytes were isolated using CD14 MicroBeads (Miltenyi Biotec, Bergisch Gladbach, Germany) with a positive selection method according to the manufacturer's instructions. Purified monocytes (>90%) were differentiated into monocyte-derived DCs during a 6-day culture with 800 U/ml GM-CSF (Novartis, Basel, Switzerland) and 400 U/ml IL-4 (R&D). After harvesting on day 6, $1x10^4$ monocyte-derived DCs were matured with LPS (100 ng/ml) for 24 hours. On day 7, supernatants were removed and the matured monocyte-derived DCs were co-cultured with $1x10^5$ purified T cells. Cells were cultured 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.

In some experiments, $CD4^+$ T cells were isolated from PBMCs by negative selection using the Dynabeads Untouched Human $CD4^+$ T cell kit (11346D, Thermo Fisher). The unlabeled $CD4^+$ T cells were separated into $CD62L^{neg}$ and a $CD62L^+$ cell fractions by using CD62L MicroBeads (Miltenyi Biotec). $2x10^5$ $CD62L^{neg}$ T cells were stimulated for 4.5 hours with phorbol 12-myristate 13-acetate (PMA, 0.02 µg/ml, Sigma-Aldrich) and ionomycin (0.5 µg/ml, Sigma-Aldrich) in the presence of Brefeldin A (3 µg/ml eBiosciences) for the last 3.5 hours and subsequently analyzed for intracellular cytokine expression.

Flow cytometry.

After erythrocyte lysis, whole blood samples were stained for flow cytometry using monoclonal antibodies against CD3 (UCHT1, BD, or HIT3a, Biolegend), CD4 (SK3, BD), CD38 (HIT2, BD), CD62L (DREG-56, Biolegend), CD25 (2A3, BD), CD45RA (HI100, BD), CD45RO/RPE (UCHL1, Dako), TIGIT (MBSA43, eBiosciences), ICOS (C398.4A, Biolegend), CD127 (HIL-7R-M21, BD), PD1 (MIH4, eBiosciences) and HLA-DR (L243, BD). Intracellular staining was performed with the Foxp3 fixation and permeabilization staining buffer kit, according to manufacturer's protocol (eBiosciences), followed by staining with anti-Foxp3 (236A/E7 or PCH101, eBiosciences), anti-Helios (22F6, Biolegend), anti-Ki67 (20Raj1, eBiosciences) or anti-CTLA-4 (BNI3, BD) and the appropriate isotype controls.

For intracellular cytokine staining after culture, cells were stimulated for 4.5 hours with PMA and ionomycin in the presence of Brefeldin A as described above. After incubation with antibodies directed to surface proteins, cells were fixed in 2% formaldehyde and permeabilized with saponin (Sigma-Aldrich), and labeled with antibodies to IL-10 (JES3-19F1, BD), IL-17A (eBio64DEC17, eBiosciences), IFNγ (4S.B3, BD), or appropriate isotype controls (eBiosciences). Flow cytometric analysis was performed on a FACSCanto™II (BD Biosciences).

Cytokine analysis.

Cytokine concentrations in cell supernatants were analyzed using an enzyme-linked immunosorbent assay set for IFNγ (eBiosciences), IL-10 (eBiosciences) and IL-17 (R&D Systems) according to the manufacturer's instructions.

RNA extraction and Illumina library preparation.

Total cellular RNA from purified T cells was extracted using the NucleoSpin® RNA-XS

extraction kit for the isolation of RNA (Macherey-Nagel) according to manufacturer instructions. RNA levels, quality and purity were assessed with the RNA 6000 Nano assay on the Agilent 2100 Bioanalyzer.

cDNA was synthesized using a mix of random hexamers (2.5 mM), oligo(dT) primers (20 nM), dNTP (0.2 mM), M-MLV (200 units, Promega) and RNAsin (25 units, Promega). The samples used for transcriptome analysis were amplified using SMARTer Ultra Low RNA kit (Clontech Laboratories) following the manufacturers protocol. Amplified cDNA was further processed according to TruSeq Sample Preparation v2 Guide (Illumina) and paired end sequenced on the HiSeq 2500 (Illumina, San Diego, California; 76 cycles, paired end sequencing, rapid run, 3 samples per lane). Human transcripts were aligned to the RefSeq transcriptome and analyzed with DESeq2. Fragments per kilobase of transcript per million mapped reads (FPKM) values were calculated using Cufflinks.

Quantitative PCR.

A maximum of 1000 ng mRNA was used for the synthesis of cDNA. Real-time quantitative PCR was performed using SYBR Green on an AbiPrismR 7900 Sequence Detection system (PE Applied Biosystems, Foster City, CA). The relative expression to *GAPDH* for each gene was measured as $2^{(-\Delta Ct)}$. Primer sets used were:

Gene	Forward primer	Reverse primer
GAPDH	5′-GTCGGAGTCAACGGATT-3′	5′-AAGCTTCCCGTTCTCAG-3′
TIGIT	5′-TTGGGGTGGCACATCT-3′	5′-CGACCACCACGATGACT-3′
IFNG	5′-CCAGGACCCATATGTAAAAG-3′	5-TGGCTCTGCATTATTTTTC-3′
IL10	5′-CCCCAAGCTGAGAACC-3′	5′-ACGGCCTTGCTCTTGT-3′
CTLA4	5′-GCTTGCCTTGGATTTCA-3′	5′-GCCGCACAGACTTCAGT-3′
ICOS	5′-CTGGCAAACATGAAGTCAG-3′	5′-CACCTCCGTTGTGAAATATAA-3′
CD38	5′-GGCCCATCAGTTCACAC-3′	5′-GAAACCGTTTTCCAGAATACT-3′
IL6	5′-CCCCCAGGAGAAGATTC-3′	5′-GCTGCTTTCACACATGTTACT-3′
IL17A	5′-GAAGGCAGGAATCACAATC-3′	5′-GCCTCCCAGATCACAGA-3′
TNFA	5′-CGCTCCCCAAGAAGAC-3′	5′-GGTTCGAGAAGATGATCTGA-3′
IL12P3	5′-CTGGCCTCCAGAAAGAC-3′	5′-GTGGCACAGTCTCACTGTT-3′
IL23P19	5′-CAGGGACAACAGTCAGTTCT-3′	5′-CTGCGAAGGATTTTGAAG-3′
CD80	5′-TGGGCCATTACCTTAATCT-3′	5′-TCTGCGGACACTGTTATACA-3′
CD86	5′-TGGGGTCATTTCCAGATA-3′	5′-GTGCGGCCCATATACTT-3′
CD40	5′-TTGGGGTCAAGCAGATT-3′	5′-CCTGGGGACCACAGAC-3′

Statistics.

Baseline demographic and disease characteristics were evaluated for the entire cohort using descriptive statistics, including means and standard deviations (SD) or median and interquartile ranges (IQ range) for continuous variables, and frequencies and percentages for categorical outcomes. Significance between two groups was determined using Student's t-test or Mann-Whitney U-test and differences between multiple groups by using the Kruskal-Wallis H test. The Wilcoxon signed-rank test was performed when samples were paired. p Values of <0.05 were considered statistically significant. Prism software (GraphPad Software, Version 5.0, La Jolla, CA) was used for all statistical analysis.

RESULTS

Increased frequencies of activated T cells in the circulating CD38⁺ effector T-cell pool during active IBD.

We have recently established that percentages of peripheral blood $CD38^+$ effector ($CD4^+CD62L^{neg}$) T cells do not differ between patients with chronic intestinal disease and controls.[8] We therefore hypothesize that the composition of inflammatory versus regulatory cells within this circulating $CD38^+$ effector T-cell population detects active inflammatory disease in pediatric IBD. Thereto, we examined changes in T-cell activation in circulating $CD38^+$ effector T cells in pediatric IBD patients at time of disease diagnosis when patients have active biopsy-proven intestinal inflammation and are not yet receiving treatment, and during clinical remission while treated (Figure S1A). During active disease ("inflammation") but not during clinical remission, pediatric IBD patients exhibited increased frequencies of antigen-experienced $CD45RA^{neg}$ cells in the $CD38^+$ effector T-cell population when compared to age-matched healthy controls (Figure 1A). Upon subdividing IBD patients in Crohn's disease (CD) and ulcerative colitis (UC), the increased frequencies of $CD45RA^{neg}$ cells were most prominent in CD (Figure S2A). These changes were not detectable in the total $CD4^+$ T-cell population (Figure 1A). To detect cellular activation, we determined frequencies of cells expressing the IL-2 receptor a chain (CD25), that is induced on naive T cells after activation via T-cell receptor signaling. Frequencies of activated $CD25^+Foxp3^{neg}$ T cells were strongly increased in the $CD38^+$ effector T-cell population, but much less in the total $CD4^+$ T-cell population of pediatric IBD patients with active disease compared to controls (Figure 1B). Patients in clinical remission did not exhibit this increased frequency of $CD25^+Foxp3^{neg}$ $CD38^+$ effector T cells. The frequency of $CD25^+$ cells in the $CD38^+$ effector T-cell population revealed a marked heterogeneity in patients with active disease (Figure 1B). Increased frequencies of activated $CD25^+Foxp3^{neg}$ T cells amongst $CD38^+$ effector T cells were observed in both CD and UC patients (Figure

S2B). Of note, there were no differences in white blood cell (WBC) counts or percentage of CD38⁺ effector T cells expressing gut-homing receptors CCR9 and α4β7 between patient groups (Figure S1B and S3). Medication use is summarized in Figure S1C and gating strategy is shown in Figure S1D. Upon scheduled treatment follow up (median treatment duration 223 days) and despite a wide range of immunosuppressive treatments (Figure S1F), frequencies of CD25⁺ cells decreased in the CD38⁺ effector T-cell pool, but not total CD4⁺ T cells, of all patients achieving clinical remission (Figure 1C). These data demonstrate that monitoring activated CD45RA^{neg} and CD25⁺ T cells in the circulating CD38⁺ effector T-cell population detects active intestinal disease in pediatric IBD while this cannot be achieved by monitoring all CD4⁺ T cells.

As in IBD patients the balance between activated inflammatory T-cell responses and regulatory responses is lost, we next sought the reduced regulatory component that would reflect inflammatory disease. Thereto, we assessed the frequencies of circulating Foxp3⁺ cells in the CD38⁺ effector T-cell population in peripheral blood of IBD patients and controls. However, we detected no differences in the frequencies of Foxp3⁺ cells in total CD4⁺ T cells or CD38⁺ effector T cells between patients and controls (Figure 1D). In humans, Foxp3⁺ T cells are functionally heterogeneous with regulatory T cells (Tregs) and a subpopulation of activated effector CD4⁺ T cells expressing Foxp3.[11] To further investigate Foxp3 expression in regulatory versus activated CD4⁺ T cells, we combined analysis of Foxp3 and CD45RA to distinguish naive Tregs (CD45RA⁺Foxp3^{int}), activated Tregs (CD45RA^{neg}Foxp3^{hi}) and activated effector T cells (CD45RA^{neg}Foxp3^{int}, as depicted in the gating strategy in Figure S4A).[12] Frequencies of activated effector T cells were increased in both cohorts of IBD patients compared to controls, whereas no differences were found in the percentage of naive and activated Tregs between IBD patients with active disease and controls (Figure S4B). In addition, no differences were found in the frequency of CD4⁺CD25^{hi}CD127^{neg}Foxp3⁺ T cells between IBD patients and controls (Figure S4C and S4D). Overall, these data demonstrate that enrichment for CD38⁺ effector T cells instead of total CD4⁺ T cells in peripheral blood detects increased frequencies of activated T cells in IBD patients with intestinal inflammation. This increased frequency normalizes during disease remission. However, frequencies of potentially regulatory populations in the CD38⁺ effector T-cell population are difficult to monitor.

Circulating CD38⁺ effector T cells of adult healthy controls have a predominant regulatory component with decreased inflammatory cytokine production and increased IL-10 production.

We next aimed to identify the main regulatory component in the circulating CD38⁺ effector T-cell pool. Previously, preliminary data suggested that CD38⁺ effector T cells from adult healthy individuals are enriched in *IL10* mRNA expression when compared to

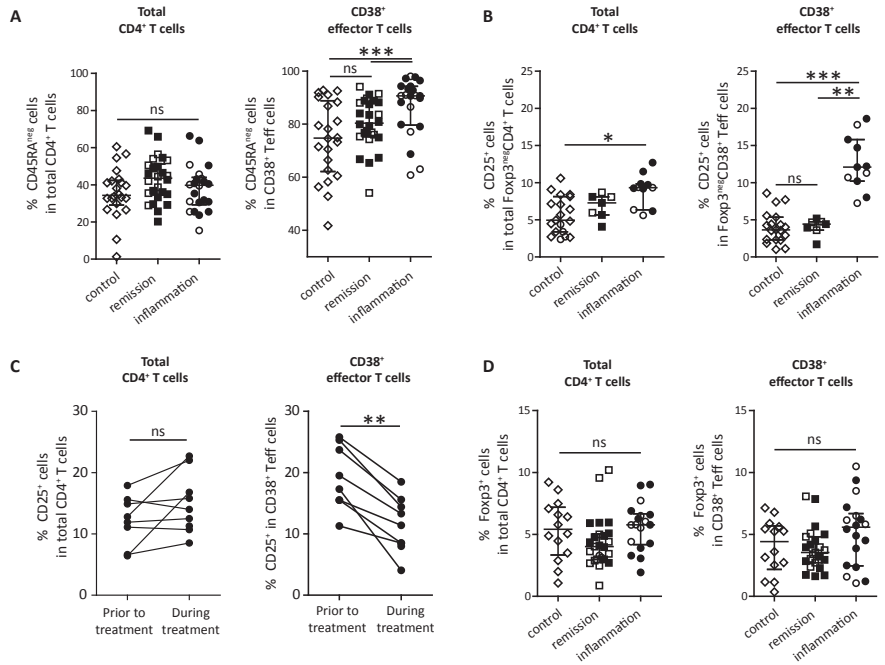

Figure 1. Increased frequencies of activated T cells in the circulating effector CD38⁺ T-cell pool during active IBD. Flow cytometric analysis for CD3, CD4, CD62L, CD38, CD45RA, CD25 and Foxp3 was performed on peripheral blood from pediatric IBD patients (cohort I) with biopsy-proven intestinal inflammation at diagnostic endoscopy and prior to treatment (denoted as "inflammation"), pediatric IBD patients in clinical remission during treatment (denoted as "remission") and age-matched healthy controls. Open and closed symbols depict UC and CD patients, respectively. Cells were gated on single cells, CD3⁺, CD4⁺, and CD62L^{neg}CD38⁺ cells (denoted as CD38⁺ effector T cells). **(A)** Frequency of CD45RA^{neg} cells in the total CD4⁺ T-cell population and CD38⁺ effector T cells within the CD4 gate (cohort I; age-matched healthy controls, n=22, IBD patients with intestinal inflammation, n=22, IBD patients in remission, n=26). **(B)** Frequency of CD25⁺ cells gated on Foxp3^{neg} cells in the total CD4⁺ T-cell population and CD38⁺ effector T cells within the CD4 gate (cohort I; age-matched healthy controls, n=19, IBD patients with intestinal inflammation, n=11, IBD patients in remission, n=7; CD25 analysis was included in a second phase of the study). **(C)** Frequency of CD25⁺ cells in the total CD4⁺ T-cell pool and CD38⁺ effector T-cell population in peripheral blood of pediatric IBD patients at disease diagnosis (prior to treatment) and during immunosuppressive treatment (follow up in n=9 patients from cohort II consisting of a total of n=18; median treatment duration 223 days, IQ range 169-309). p values were calculated using a Wilcoxon signed rank test. **(D)** Frequency of Foxp3⁺ cells in the total CD4⁺ T-cell population and CD38⁺ effector T cells within the CD4 gate. Data are expressed as median +/- interquartile (IQ) range. p values were calculated using a Kruskal-Wallis *H* analysis followed by the Dunn's Multiple Comparison Test. Teff, effector T cell; NS, not significant, *p<0.05, **p<0.01, ***p<0.001.

CD38^{neg} effector T cells homing to other sites of the body.[8] This appears in line with the preferential tolerogenic response to harmless exogenous antigens at the mucosal surface. To corroborate enrichment in regulation, purified CD38^{neg} and CD38⁺ effector T cells from adult healthy individuals were cultured with allogeneic monocyte-derived dendritic cells (DCs) in a mixed lymphocyte reaction (MLR). CD38⁺ effector T cells from healthy individuals

contained lower frequencies of IFNγ- and IL-17-secreting T cells, and higher frequencies of IL-10-secreting T cells when compared to CD38^{neg} effector T cells (Figure 2A and 2B). Both the frequencies and mean fluorescence intensity of HLA-DR and CD25 did not differ between cultured CD38^{neg} and CD38^{+} effector T cells (Figure S5A and S5B), excluding the possibility that the different cytokine profiles were due to overall differences in T-cell activation. Using a second method of activation, i.e. anti-CD3/CD28 stimulation, CD38^{+} effector T cells also produced lower levels of IFNγ and IL-17 compared to CD38^{neg} effector T cells (Figure 2C). Overall, frequencies of CD45RO^{+} (memory) or CD45RA^{+} (naive) cells (Figure S5C), Foxp3^{+}CD25^{hi} (Figure S5D) and Foxp3^{+}CD127^{neg} T cells (data not shown) did not differ between CD38^{neg} and CD38^{+} effector T-cell populations. In addition, CD38^{neg} and CD38^{+} effector T-cell populations had comparable frequencies of Helios^{+} cells in Foxp3^{+}

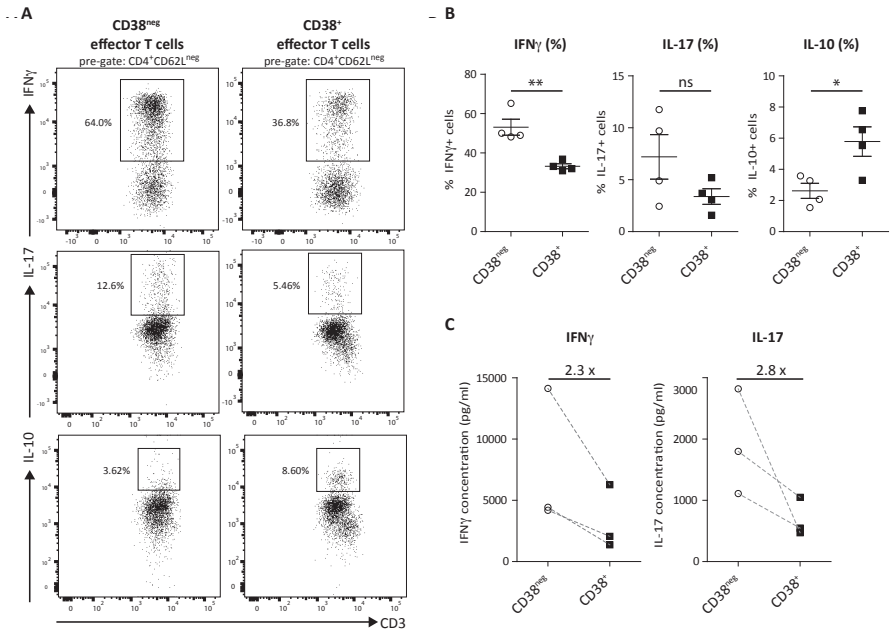

Figure 2. Circulating CD38^{+} T cells of adult healthy controls have a predominant regulatory component with decreased inflammatory cytokine production and increased IL-10 production. (A-B) Purified adult healthy control CD38^{neg} and CD38^{+} effector T cells were cultured for 72 hours with mature monocyte-derived DCs (ratio 10:1). **(A)** Representative dot-plots of the percentage of IFNγ, IL-17 and IL-10 positive cells in CD38^{neg} and CD38^{+} effector T cells at 72 hours are shown. **(B)** Percentages of IFNγ, IL-17 and IL-10 positive cells in CD38^{neg} and CD38^{+} effector T cells after 72 hours of culture. Results are pooled from 4 independent experiments. Data are expressed as means +/- SEM. p values were calculated using an unpaired Student's t test. **(C)** Purified adult healthy control CD38^{neg} and CD38^{+} effector T cells were stimulated for 72 hours with anti-CD3/CD28 stimulation beads (0.5 bead per T cell). IFNγ and IL-17 production in supernatants was measured by ELISA. p values were calculated using a Student's t test. NS, not significant, *p<0.05, **p<0.01.

FREQUENCIES OF CIRCULATING REGULATORY TIGIT^{+}CD38^{+} EFFECTOR T CELLS CORRELATE WITH THE COURSE OF INFLAMMATORY BOWEL DISEASE

Tregs frequencies suggesting similar frequencies of natural Tregs (Figure S5D).

Taken together, in adult healthy individuals, circulating CD38^{+} effector T cells which are enriched in cells with mucosal antigen specificity, have a stronger immunoregulatory component associated with decreased inflammatory cytokine production and increased IL-10 production when compared to CD38^{neg} effector T cells homing to sites such as the skin.

Healthy control CD38^{+} effector T cells are enriched for transcripts associated with immune regulation and gut homing when compared to CD38^{neg} effector T cells.

Next, we set out to define cellular markers to identify this regulatory subpopulation of CD38^{+} effector T cells. Thereto, we performed transcriptome analysis of CD38^{neg} and CD38^{+}effector T cells of adult healthy individuals (n=3) to find differentially expressed proteins reflecting regulatory function of CD38^{+} effector T cells. Overall, a total of 145 and 167 genes were significantly over- and underexpressed in CD38^{+} effector T cells relative to their CD38^{neg} counterparts (with an adjusted p value < 0.05; Figure 3A). Hierarchical clustering performed with this gene set clearly distinguished CD38^{neg} and CD38^{+} effector T cells (Figure 3B). As expected, expression of genes encoding the gut-homing chemokine receptors *CCR9, CXCR6* and *CX3CR1*, as well as integrin α4 *(ITGA4)* were up-regulated in CD38^{+} effector T cells compared to CD38^{neg} effector T cells (Figure 3C). In addition, CD38^{+} effector T cells had increased expression of a number of genes encoding coinhibitory receptors, such as T-cell immunoglobulin and ITIM domain *(TIGIT)*, cytotoxic T lymphocyte antigen-4 *(CTLA-4)*, Fc receptor-like protein 3 *(FCRL3)*, Hepatitis A virus cellular receptor 2 (*HAVCR2*, encoding T cell Immunoglobulin and Mucin 3; *TIM-3*) and the costimulatory molecule inducible T-cell costimulator (*ICOS*, Figure 3C). CD38^{+} effector T cells also showed increased expression of the gene encoding the regulatory cytokine IL-10, although significance was lost after correction for multiple testing (p value = 0.003, adjusted p value = 0.09). Based on the RNA-seq data, we selected immune regulatory genes that were enriched in the CD38^{+} effector T-cell population and validated their differential expression with qRT-PCR analysis (Figure 3D). Overall, these analyses provide us with candidate molecules TIGIT, CTLA-4, ICOS and TIM-3 as putative markers to monitor the IL-10-producing regulatory subpopulation within the CD38^{+} effector T-cell pool.

The inhibitory receptor TIGIT is strongly enriched in the circulating healthy control CD38^{+} effector T-cell pool and its expression is maintained upon cellular activation.

We next analyzed whether this distinctive mRNA profile translated into cellular protein expression of TIGIT, CTLA-4, ICOS and TIM-3 in CD38^{+} effector T cells from peripheral blood of adult healthy individuals using flow cytometry. Frequencies of TIGIT^{+}, CTLA-4^{+}, and ICOS^{+} cells were significantly increased in CD38^{+} effector cells compared to CD38^{neg} effector T

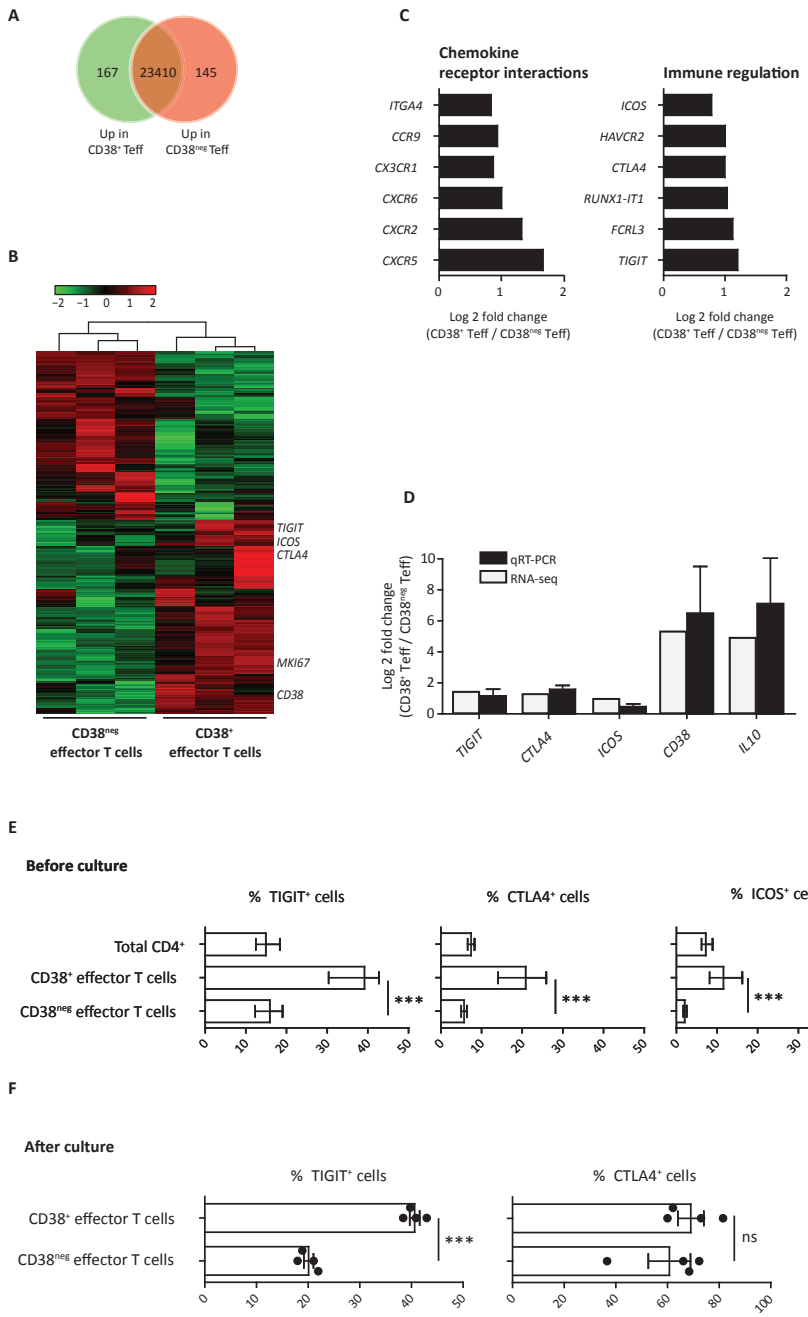

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Figure 3. The inhibitory receptor TIGIT is strongly enriched on circulating healthy control CD38⁺ effector T cells and its expression is maintained upon cellular activation. (A-D) RNA was isolated from adult healthy control CD38^{neg} and CD38⁺ effector T cells (pre-gated on single CD3⁺CD4⁺CD62L^{neg} cells) purified by flow cytometric cell sorting populations from blood of three adult healthy individuals and differentially expressed genes were identified by RNA sequencing. **(A)** Number of differentially expressed genes. **(B)** Heatmap of all genes that were differentially expressed in CD38^{neg} and CD38⁺ effector T cells (adjusted p value < 0.05). **(C)** Log 2 fold change difference for gene subsets significantly upregulated in the CD38⁺ effector T-cell population. **(D)** Validation of RNA-seq results with quantitative RT-PCR analysis. Log 2 fold change of known human regulatory genes in RNA-seq (white) and qRT-PCR (black). Data are represented as means + SEM. **(E-F)** Peripheral blood from adult healthy individuals (n=15) was analyzed for expression of CD3, CD4, CD62L, CD38, TIGIT, CTLA-4 and ICOS by flow cytometry. **(E)** Frequencies of TIGIT⁺, CTLA-4⁺ and ICOS⁺ cells in the total CD4⁺ T-cell population, CD38^{neg} and CD38⁺ T cells (pre-gated on CD4⁺CD62L^{neg} cells). Data are expressed as median +/- IQ range. p values calculated using a Mann-Whitney analysis. **(F)** Purified CD38^{neg} and CD38⁺ T cells (pre-gated on CD4⁺CD62L^{neg} cells) were cultured for 72 hours with mature monocyte-derived DCs (ratio 10:1). Frequencies of CTLA-4⁺ and TIGIT⁺ cells at 72 hours. Results are pooled from 4 independent experiments. Data are expressed as mean +/- SEM. p values were calculated using a Student's t test. NS, not significant, ***p<0.001.

cells. The CD38⁺ effector T-cell population contained 40% TIGIT⁺ cells and 20% CTLA-4⁺ cells versus only 20% and 10% respectively in the CD38^{neg} T-cell population (Figure 3E). TIM-3 expression on CD4⁺ T cells or CD38⁺ effector T cells in peripheral blood was very low when analyzed by flow cytometry and therefore excluded from further analyses.

We next pursued to investigate whether this differential inhibitory profile was stable allowing it to be used for immune monitoring of intestinal tissue-localized responses. Thereto, we cultured purified CD38^{neg} and CD38⁺ effector T cells from adult healthy individuals with allogeneic monocyte-derived DCs in an MLR and determined whether the differential profile was maintained during cellular activation. As has been reported previously, CTLA-4 was upregulated upon activation on both CD38^{neg} and CD38⁺effector T cells causing a loss of differential expression between these two populations (Figure 3F). In contrast, MLR culture did not enhance TIGIT expression in the CD38^{neg} population, while the TIGIT expression was steadily maintained on the CD38⁺ effector cells resulting in a stable differential frequency of TIGIT expressing cells between CD38^{neg} and CD38⁺ T-cell populations after activation (Figure 3F). The same results were obtained when using anti-CD3/CD28 ligation as a mode of activation with no significant changes in frequencies of TIGIT on CD4⁺ T cells after activation (Figure S6A). Virtually all TIGIT⁺ cells in the CD38^{neg} and CD38⁺ effector T-cell populations expressed CD45RO (Figure S6B). Thus, during mucosal homeostasis in adult healthy individuals circulating CD38⁺ effector T cells contain a large population of cells stably expressing the inhibitory receptor TIGIT when compared to the CD38^{neg} effector T-cell population.

Inhibitory receptor TIGIT expression identifies circulating CD38⁺ effector T cells with immunoregulatory properties.

The stable TIGIT expression after *in vitro* activation suggested that it could be a putative marker for monitoring the IL-10-producing regulatory component of the CD38⁺ effector

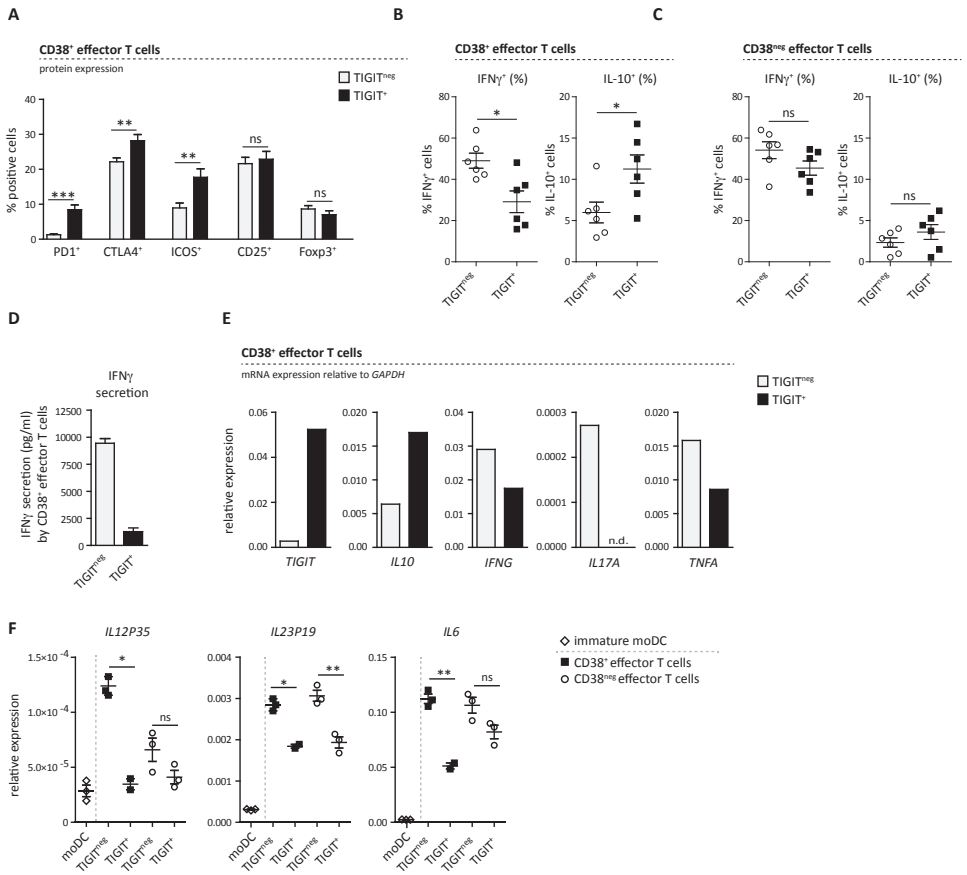

Figure 4. Inhibitory receptor TIGIT expression identifies circulating CD38⁺ effector T cells with immunoregulatory properties. (A) Flow cytometric analysis of TIGIT^{neg} and TIGIT⁺ in CD38⁺ effector T-cell population (pre-gated on CD4⁺CD62L^{neg} cells) of adult healthy individuals (n=15). Frequencies of cells positive for PD-1, CTLA-4, ICOS, Ki67, CD25 and Foxp3 are shown. **(B-C)** CD4⁺CD62L^{neg} T cells were obtained from the blood of adult healthy individuals (n=6) and stimulated for 4.5 hours with PMA plus ionomycin in the presence of Brefeldin A. IFNγ⁺ cells and IL-10⁺ cells were determined in TIGIT^{neg} and TIGIT⁺ cells within CD38⁺TIGIT^{neg}, CD38⁺TIGIT⁺, CD38^{neg}TIGIT^{neg} and CD38^{neg}TIGIT⁺ T-cell populations. **(D)** TIGIT^{neg} and TIGIT⁺ cells were purified from the CD38⁺ effector T-cell population (pre-gated on CD4⁺CD62L^{neg} cells) from the peripheral blood of adult healthy individuals and stimulated for 14 hours with anti-CD3/CD28 stimulation beads (0.5 bead per T cell). IFNγ release in supernatants was measured by ELISA. Representative of two independent experiments. **(E)** Purified adult healthy control TIGIT^{neg}CD38⁺ and TIGIT⁺CD38⁺ T cells analyzed for *TIGIT, IL10, IFNG, IL17* and *TNFA* mRNA expression relative to *GAPDH* by quantitative PCR. Representative of three independent experiments. **(F)** Autologous immature monocyte-derived DCs were cultured together with purified CD38⁺TIGIT^{neg}, CD38⁺TIGIT⁺, CD38^{neg}TIGIT^{neg} or CD38^{neg}TIGIT⁺ effector T cells for 24 hours in the presence of anti-CD3 (1 µg/ml) and LPS (10 ng/mL) in a ratio 2:1 (T cell : monocyte-derived DC). Analysis of inflammatory cytokines *IL12p35, IL23p19* and *IL6* mRNA expression relative to *GAPDH* by quantitative PCR. One representative experiment shown out of two independent experiments. Data are represented as means +/- SEM. p values were calculated using a Kruskal-Wallis H analysis. NS, not significant, *p<0.05, **p<0.01, ***p<0.001.

T-cell population. We therefore further analyzed expression of inhibitory receptors and cytokine profiles by CD38⁺TIGIT⁺ effector T cells. In adult healthy individuals, TIGIT⁺ cells within the CD38⁺ effector T-cell population more often co-expressed a second inhibitory receptor such as programmed death 1 (PD-1) or CTLA-4 when compared to the CD38⁺TIGIT^{neg} cells but did not preferentially co-express Foxp3 or CD25 (Figure 4A). Cells co-expressing the costimulatory molecule ICOS were more frequent in CD38⁺TIGIT⁺ effector T cells when compared to CD38⁺TIGIT^{neg} effector T cells (Figure 4A).

In agreement with the inhibitory function of TIGIT, CD38⁺TIGIT⁺ cells contained higher frequencies of IL-10⁺ cells after PMA-ionomycin stimulation compared to CD38⁺TIGIT^{neg} cells (Figure 4B). The association between TIGIT and IL-10 was specific for the CD38⁺ effector T-cell population, as CD38^{neg}TIGIT⁺ cells did not have higher frequencies of IL-10⁺ cells compared to CD38^{neg}TIGIT^{neg} cells (Figure 4C). CD38⁺TIGIT⁺ cells also contained lower frequencies of IFNγ secreting cells with a lower mean fluorescence intensity after PMA-ionomycin stimulation (Figure 4B) and secreted lower amounts of IFNγ after anti-CD3/CD28 stimulation compared to CD38⁺TIGIT^{neg} effector T cells (Figure 4D). This agreed with high baseline levels of *IL10* mRNA expression and lower *IFNG*, *TNFA* and *IL17* mRNA expression in CD38⁺TIGIT⁺ cells (Figure 4E). Importantly, the frequency of IL-10⁺ cells in CD38⁺TIGIT⁺ cells was significantly higher than the frequency of IL-10⁺ cells in CD38^{neg}TIGIT⁺ cells (p=0.0027, Student's T test). This demonstrates that TIGIT⁺ cells in the CD38⁺ effector T-cell population preferentially produce IL-10 when compared to TIGIT⁺ cells in the CD38^{neg} effector T-cell population.

To test functional regulatory capacity of CD38⁺TIGIT⁺ effector T cells such as modulating DC activation, we cultured immature monocyte-derived DCs together with purified peripheral blood CD38⁺TIGIT⁺ or CD38⁺TIGIT^{neg} effector T cells in the presence of anti-CD3 and lipopolysaccharide (LPS). Co-culture with CD38⁺TIGIT⁺ T cells yielded lower *IL12p35, IL23p19* and *IL6* mRNA expression in DCs when compared to co-cultures with CD38⁺TIGIT^{neg} T cells (Figure 4F). This effect was more pronounced for TIGIT⁺ cells in the CD38⁺ effector T-cell population compared to TIGIT⁺ cells in the CD38^{neg} effector T-cell population (Figure 4F). In contrast, TIGIT expression on CD38⁺ effector T cells did not affect the maturation status of monocyte-derived DCs, as defined by surface expression of CD40, CD80 and CD86 (Figure S7). These data indicate that TIGIT expressing CD38⁺ effector T cells from adult healthy individuals have the capacity to regulate inflammatory cytokine expression by monocyte-derived DCs. Overall, these demonstrate that TIGIT expression identifies a regulatory subpopulation of T cells within the CD38⁺ effector T-cell pool.

Low frequencies of circulating CD38⁺TIGIT⁺ effector T cells identify pediatric IBD patients with reduced duration of clinical remission during follow up.

We next assessed whether TIGIT expression on circulating CD38⁺ effector T cells identified

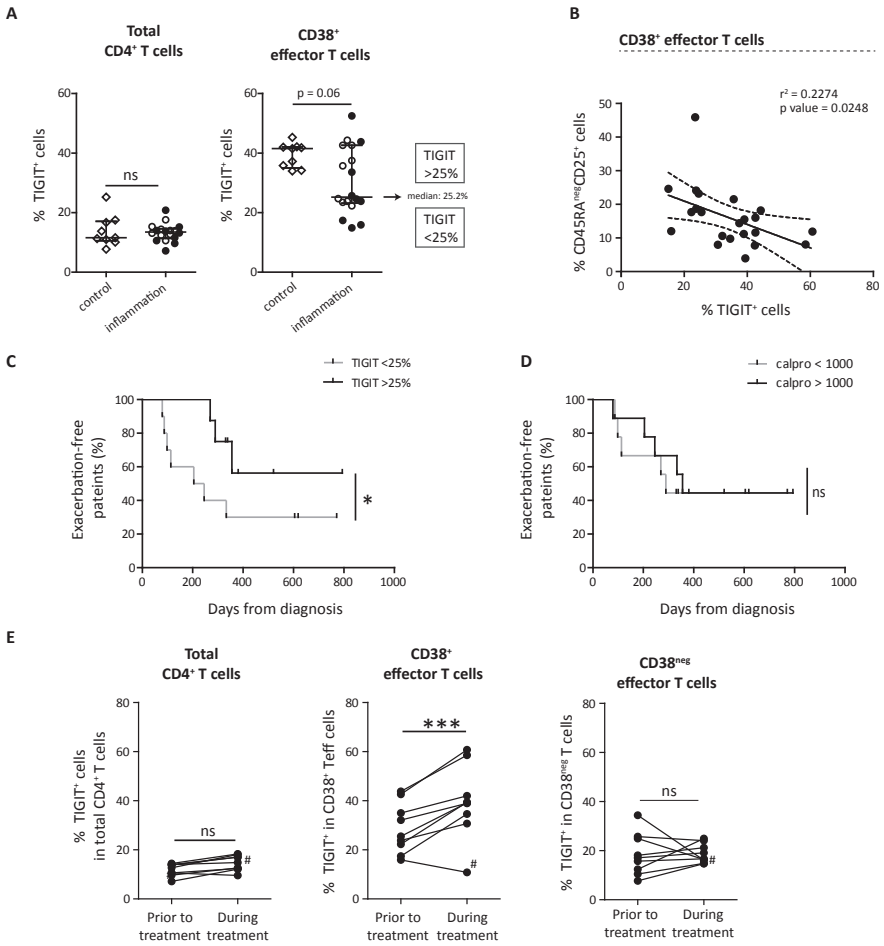

Figure 5. Low frequencies of circulating CD38⁺TIGIT⁺ effector T cells identify pediatric IBD patients with reduced duration of clinical remission during follow-up. Peripheral blood from pediatric IBD patients with active disease (n=18, cohort II) and age-matched healthy controls (n=9) was analyzed by flow cytometry. Open and closed symbols depict UC and CD patients, respectively. **(A)** Frequency of TIGIT⁺ cells in the total CD4⁺ T-cell pool and the CD38⁺ effector T-cell population. Data are expressed as median +/- IQ range. p values were calculated using a Mann-Whitney analysis. **(B)** Correlation of the frequency of activated CD45RA^{neg}CD25⁺ cells and TIGIT⁺ cells in the CD38⁺ effector T-cell population of pediatric IBD patients. **(C)** Kaplan-Meier overall exacerbation-free disease course for low versus high TIGIT frequencies on CD38⁺ effector T cells. **(D)** Kaplan-Meier overall exacerbation-free disease course for fecal calprotectin levels (TIGIT <25%, n=10, median follow up of 618 days; TIGIT >25%, n=8, median follow up of 509 days; NS). **(E)** Frequency of TIGIT⁺ cells in the total CD4⁺ T-cell pool, CD38⁺ effector T-cell population and CD38^{neg} effector T-cell population in peripheral blood of pediatric IBD patients at disease diagnosis (prior to treatment) and during immunosuppressive treatment (median treatment duration 223 days; IQ range 169-309). # indicates a patient not in remission during the follow up analysis and who was subsequently started on a 4 week steroid regimen. p values were calculated using a Wilcoxon signed rank test. NS, not significant, *p<0.05, ***p<0.001.

changes in regulatory responses in peripheral blood of pediatric IBD patients (Figure S8A). Strikingly, the frequency of TIGIT⁺ cells in the CD38⁺ effector T-cell population of patients with active IBD was drastically reduced in a subgroup of patients when compared to age-matched controls, with half of the patients having a TIGIT⁺ cell frequency below 25% (Figure 5A). Frequencies of TIGIT⁺ cells in the total CD4⁺ T-cell pool were not different compared to controls (Figure 5A). Degree of TIGIT expression per cell (MFI) by CD38⁺ effector T cells was not different between IBD patients and controls, nor between IBD patients with low versus high frequencies of TIGIT. Increased frequencies of activated CD45RA^{neg}CD25⁺ cells inversely correlated with reduced frequencies of TIGIT⁺ cells in the CD38⁺ effector T-cell population (Figure 5B), indicating that the regulatory component that is normally present in CD38⁺ effector T cell population is decreased in a subgroup of IBD patients that has a high inflammatory component.

This raised the question whether TIGIT frequency classifies IBD heterogeneity prior to treatment. Crucially, a TIGIT percentage below 25% prior to treatment associated with a reduced duration of clinical remission (Figure 5C and S8B). For comparison attempting to achieve such classification on the basis of fecal calprotectin levels above or below 100, 250 or 1000 µg/g at time of diagnosis were not predictive of duration of remission (Figure 5D, Figure S8C and S8D).

To investigate whether TIGIT frequency in the CD38⁺ effector T-cell population is restored during therapy, we performed a follow up analysis of peripheral blood of pediatric IBD patients. Despite a wide range of immunosuppressive treatments, frequencies of TIGIT⁺ cells significantly increased during therapy except in one patient, who did not achieve clinical remission at time of analysis (Figure 5E). The on-treatment changes in TIGIT frequencies were exclusively confined to CD38⁺ effector T cells, as such changes could not be observed in total CD4⁺ T cells or CD38^{neg} effector T cells (Figure 5E).

Taken together, TIGIT frequencies in CD38⁺ effector T cells are reduced in a subgroup of pediatric IBD patients that are at risk for early disease relapse.

DISCUSSION

We demonstrate that analysis of circulating CD38⁺ effector T cells, a population that is enriched for cells with mucosal antigen specificity, instead of total CD4⁺ T cells, lowers the threshold for detection of changes in regulatory versus inflammatory T-cell responses. We observed that circulating CD38⁺ effector T cells of healthy individuals have a predominant regulatory TIGIT⁺CD38⁺ effector T-cell subpopulation strongly enriched in expression of inhibitory molecules and IL-10. In peripheral blood of pediatric IBD patients with active disease, the phenotype of circulating CD38⁺ effector T cells, but not total CD4⁺ T cells,

was altered, with increased frequencies of activated inflammatory CD25⁺CD45RA^{neg} and decreased frequencies of regulatory TIGIT⁺ cells. Low frequencies of TIGIT⁺ cells (<25%) in CD38⁺ effector T cells identified a subgroup of IBD patients at diagnosis that had reduced duration of clinical remission during follow up. In patients responding to immunosuppressive treatment, frequencies of TIGIT and CD25 in CD38⁺ effector T cells of IBD patients normalized to frequencies similar to that seen in CD38⁺ effector T cells of age-matched healthy individuals.

IBD is a complex disease with a high degree of clinical heterogeneity and as a result an unpredictable response to therapy. Therefore, it is crucial to develop biological parameters to predict disease activity, disease course and response to treatment, enabling the design of tailored treatment strategies. As inflammatory CD4⁺ T cells drive IBD pathology, monitoring the inflammatory status of T cells in peripheral blood is highly desired to differentiate patients with a severe versus a mild disease course. It has, however, not been possible to date to monitor tissue-localized T-cell responses in peripheral blood of IBD patients. We are the first to show that phenotypic changes in peripheral blood CD38⁺ effector T cells instead of total CD4⁺ T cells reflect tissue-localized disease in IBD patients. In particular, IBD patients with active disease have increased frequencies of activated CD25⁺CD45RA^{neg} cells and decreased frequencies of TIGIT⁺ but not Foxp3⁺ cells compared to age-matched healthy controls. This increased frequency of activated CD38⁺ effector T cells was observed in both CD and UC patients (Figure S2). Crucially, heterogeneity in the percentage of TIGIT⁺ cells in CD38⁺ effector T-cell population prior to treatment differentiated patients with reduced duration of clinical remission during follow-up. Such differentiation was purely based on compositional changes of the CD38⁺ effector population as the total number of CD38⁺ effector T cells, the white blood cell count as well as the frequencies of CCR9⁺ and $\alpha4\beta7^+$ expressing cells within the CD38⁺ population, did not differ between IBD patients and controls (Figure S1B and S3).[8] The importance of analysis of the CD38⁺ effector T-cell population instead of total CD4⁺ T cells is further emphasized by a previous study that could not detect alterations in circulating TIGIT⁺ cells when monitoring total CD4⁺ T cells in IBD patients.[8, 13]

TIGIT has not been previously implicated in intestinal adaptive immune homeostasis. In healthy individuals 40% of peripheral blood CD38⁺ effector T cells expressed TIGIT and were enriched in inhibitory receptors and IL-10 expression, reflecting a preferential regulatory phenotype of these mucosally-imprinted T cells. TIGIT expression was stable during *in vitro* activation and proliferation of TIGIT⁺ cells (Figure S9). Although previous findings established a role for TIGIT in Treg-mediated immune suppression[14], we found the majority of CD38⁺ effector T cells to express TIGIT but not Foxp3. Our findings are in line with data showing TIGIT, but not Foxp3, is positively correlated with IL-10 expression by CD4⁺ T cells in models investigating tolerance induction in auto-immunity.[15] Likewise, we report changes in TIGIT but not Foxp3 in CD38⁺ effector T cells in IBD patients. In line with previous data showing that TIGIT-Fc modifies DC cytokine production[16], we show that TIGIT expressing CD38⁺ effector T cells have the capacity to modulate inflammatory cytokine expression by monocyte-derived DCs, suggesting that TIGIT expressing cells may have a local immunomodulatory effect on surrounding cells when reaching the intestinal tissue.

It is striking that in healthy individuals, with a balanced mucosal tolerance to microbiota, CD38⁺ effector T cells are enriched in other immune inhibitory receptors such as CTLA-4 and TIM-3 when compared to CD38^{neg} effector T cells. We chose to further analyze TIGIT because of the high percentage of CD38⁺ effector T cells that expressed TIGIT whereas we were unable to identify circulating CD38⁺ effector T cells that expressed surface TIM-3 when analyzed *ex vivo*, which is consistent with previous observations by others.[17] Moreover, in contrast to CTLA-4, TIGIT expression was stable upon differentiation and did not show transient upregulation in antigen experienced cells stimulated with anti-CD3. However, we do not exclude that analysis of multiple additional activational and regulatory markers on CD38⁺ effector T cells may further enable IBD patient stratification prior to treatment.

To conclude, we provide the first evidence that tracing the composition of the circulating CD38⁺ effector T-cell population of IBD patients allows to detect changes in the CD4⁺ T-cell response that correlate with disease course and therapy responsiveness. The identification of CD38⁺ effector T cells as targets for tracing chronic inflammation has immediate relevance for clinical trials in IBD but may be further exploited in other diseases such as graft versus host disease and checkpoint inhibitor-induced inflammation in the setting of cancer.

ACKNOWLEDGEMENTS

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REFERENCES

1. Sartor RB. Pathogenesis and immune mechanisms of chronic inflammatory bowel diseases. Am J Gastroenterol 1997; 92(12 Suppl): 5S-11S.
2. Maul J, Loddenkemper C, Mundt P, Berg E, Giese T, Stallmach A et al. Peripheral and intestinal regulatory CD4+ CD25(high) T cells in inflammatory bowel disease. Gastroenterology 2005; 128(7): 1868-1878.
3. Ban H, Andoh A, Shioya M, Nishida A, Tsujikawa T, Fujiyama Y. Increased number of Foxp3+CD4+ regulatory T cells in inflammatory bowel disease. Mol Med Rep 2008; 1(5): 647-650.
4. Li Z, Vermeire S, Bullens D, Ferrante M, Van Steen K, Noman M et al. Restoration of Foxp3+ Regulatory T-cell Subsets and Foxp3- Type 1 Regulatory-like T Cells in Inflammatory Bowel Diseases During Anti-tumor Necrosis Factor Therapy. Inflamm Bowel Dis 2015; 21(10): 2418-2428.
5. Veltkamp C, Anstaett M, Wahl K, Moller S, Gangl S, Bachmann O et al. Apoptosis of regulatory T lymphocytes is increased in chronic inflammatory bowel disease and reversed by anti-TNFalpha treatment. Gut 2011; 60(10): 1345-1353.
6. Guidi L, Felice C, Procoli A, Bonanno G, Martinelli E, Marzo M et al. Foxp3(+) T regulatory cell modifications in inflammatory bowel disease patients treated with anti-TNFalpha agents. Biomed Res Int 2013; 2013: 286368.
7. La Scaleia R, Morrone S, Stoppacciaro A, Scarpino S, Antonelli M, Bianchi E et al. Peripheral and intestinal CD4+ T cells with a regulatory phenotype in pediatric patients with inflammatory bowel disease. J Pediatr Gastroenterol Nutr 2010; 51(5): 563-572.
8. du Pre MF, van Berkel LA, Raki M, van Leeuwen MA, de Ruiter LF, Broere F et al. 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. American Journal of Gastroenterology 2011; 106(6): 1147-1159.
9. Deaglio S, Mallone R, Baj G, Donati D, Giraudo G, Corno F et al. Human CD38 and its ligand CD31 define a unique lamina propria T lymphocyte signaling pathway. Faseb J 2001; 15(3): 580-582.
10. Schneider M, Schumacher V, Lischke T, Lucke K, Meyer-Schwesinger C, Velden J et al. CD38 is expressed on inflammatory cells of the intestine and promotes intestinal inflammation. PLoS One 2015; 10(5): e0126007.
11. Wang J, Ioan-Facsinay A, van der Voort EI, Huizinga TW, Toes RE. Transient expression of Foxp3 in human activated nonregulatory CD4+ T cells. Eur J Immunol 2007; 37(1): 129-138.
12. Miyara M, Yoshioka Y, Kitoh A, Shima T, Wing K, Niwa A et al. Functional delineation and differentiation dynamics of human CD4+ T cells expressing the Foxp3 transcription factor. Immunity 2009; 30(6): 899-911.
13. Lord JD, Shows DM, Chen J, Thirlby RC. Human Blood and Mucosal Regulatory T Cells Express Activation Markers and Inhibitory Receptors in Inflammatory Bowel Disease. PLoS One 2015; 10(8): e0136485.
14. Joller N, Lozano E, Burkett PR, Patel B, Xiao S, Zhu C et al. Treg cells expressing the coinhibitory molecule TIGIT selectively inhibit proinflammatory Th1 and Th17 cell responses. Immunity 2014; 40(4): 569-581.
15. Burton BR, Britton GJ, Fang H, Verhagen J, Smithers B, Sabatos-Peyton CA et al. Sequential transcriptional changes dictate safe and effective antigen-specific immunotherapy. Nat Commun 2014; 5: 4741.
16. Yu X, Harden K, Gonzalez LC, Francesco M, Chiang E, Irving B et al. The surface protein TIGIT suppresses T cell activation by promoting the generation of mature immunoregulatory dendritic cells. Nat Immunol 2009; 10(1): 48-57.
17. Hastings WD, Anderson DE, Kassam N, Koguchi K, Greenfield EA, Kent SC et al. TIM-3 is expressed on activated human CD4+ T cells and regulates Th1 and Th17 cytokines. Eur J Immunol 2009; 39(9): 2492-2501.

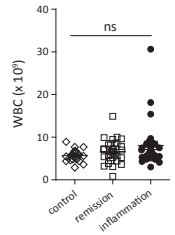
SUPPLEMENTARY DATA

A

Patient cohort I

	Remission	Inflammation	Control
Total patients, N	26	22	22
Gender			
Male, N (%)	8 (31%)	10 (45%)	11 (50%)
Female, N (%)	18 (69%)	12 (55%)	11 (50%)
Age, y (median, IQR)	16.4 (15.2-17.1)	14.3 (10.9-16.0)	9.9 (8.4-12.18)
IBD diagnosis			
CD, N (%)	16 (62%)	13 (59%)	NA
UC, N (%)	10 (38%)	9 (41%)	NA

B

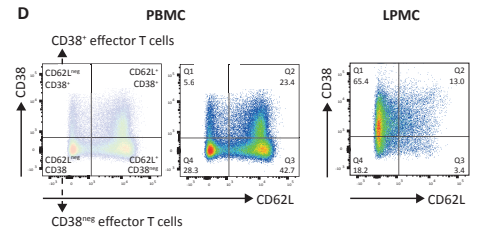


C

Patient cohort I (both treatment-naive and treated)

Patient cohort I	Fig 1A	Fig 1B	Fig 1D
Controls, N	**22**	**19**	**14**
No treatment	22	19	14
Inflammation, N	**22**	**11**	**18**
No treatment	22	11	18
Patients in remission, N	**26**	**7**	**23**
Monotherapy, N	***20***	***7***	***22***
Azathioprine	9	3	10
Mesalazine	6	3	7
Biological	2	0	1
Sulfasalazine	1	0	1
Methotrexate	1	0	2
Salazopyrine	1	1	1
Combination therapy, N	***6***	***0***	***1***
Azathioprine + biological	3	0	0
Azathioprine + mesalazine	1	0	1
Methorexate + biological	1	0	0
Prednison + mesalazine	1	0	0

D



E

Frequencies of CD45RO⁺ and CD45RA⁺ cells in PBMC:

	% CD45RO⁺ cells	% CD45RA⁺ cells
Q1 CD62L^neg CD38⁺ (%, IQR)	79.3 (73.7-82.1)	13.8 (9.0-20.1)
Q4 CD62L^neg CD38^neg (%, IQR)	93.7 (90.1-95.2)	2.6 (2.3-5.1)
Q2 CD62L⁺ CD38⁺ (%, IQR)	16.7 (9.9-27.4)	81.7 (75.6-83.0)
Q3 CD62L⁺ CD38^neg (%, IQR)	61.6 (48.1-76.0)	30.4 (24.1-40.1)

F

Patient cohort II (follow up in N=9 patients;
flow cytometric analyses before and during treatment)

	Treatment at moment of follow up analysis	Time between diagnosis and follow up (days)	Definition of remission
Patient 1	Prenisone and azathioprine	372	PGA: not in remission
Patient 2	Mesalazine	393	PUCAI<10
Patient 3	IFX (top-down) and azathioprine	223	PCDAI<10
Patient 4	Azathioprine	245	PCDAI<10
Patient 5	6-mercaptopurine	162	PGA
Patient 6	Prenisone and IFX	175	PGA
Patient 7	Mesalazine	198	PGA
Patient 8	Mesalazine and azathioprine	246	PUCAI<10
Patient 9	IFX (top-down) and azathioprine	108	PCDAI<10

Figure S1. Patient characteristics and CD38⁺ effector T-cell gating strategy. Information on **(A)** gender, age, IBD phenotype and **(B)** white blood cell counts in pediatric IBD patients and age-matched controls of cohort I. **(C)** Medication used by pediatric IBD patients in remission at moment of blood withdrawal (cohort I). None of the pediatric IBD patients with active disease ("inflammation") or the age-matched healthy controls were receiving any medication. **(D)** Gating strategy of CD3⁺CD4⁺ T cells based on the expression of CD62L and CD38. Left panel shows a representative stain on peripheral blood mononuclear cells (PBMC) and right panel show a representative stain on lamina propria mononuclear cells (LPMC) isolated from colonic tissue of the same individual. **(E)** Frequencies of CD45RO⁺ (n=19) and CD45RA⁺ cells in the CD62L^negCD38⁺, CD62L^negCD38^neg, CD62L⁺CD38⁺ and CD62L⁺CD38^neg T-cell populations (pre-gated on CD4⁺ T cells). **(F)** Information on drug exposure, duration of follow up and definitions of clinical remission in patients followed through time (follow up in n=9 patients from cohort II consisting of a total of n=18). CD, Crohn's disease; UC, ulcerative colitis; WBC, white blood cell; NA, not applicable; NS, not significant (p>0.05).

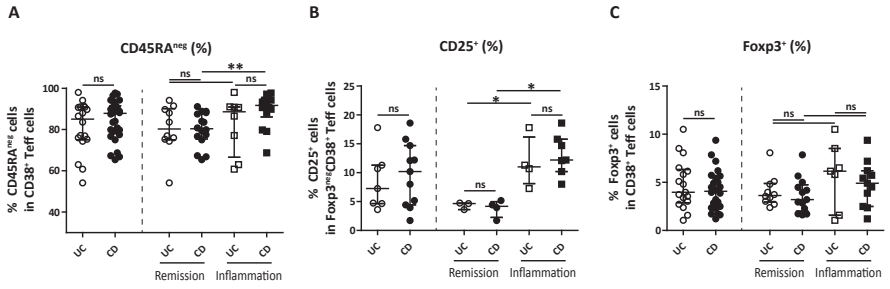


Figure S2. Composition of inflammatory versus regulatory cells in circulating CD38⁺ effector T cells of pediatric UC and CD patients. Flow cytometric analysis for CD3, CD4, CD62L, CD38, CD45RA, CD25 and Foxp3 was performed on peripheral blood from pediatric IBD patients (cohort I) with biopsy-proven intestinal inflammation at diagnostic endoscopy and prior to treatment (denoted as "inflammation") and pediatric IBD patients in clinical remission during treatment (denoted as "remission"). **(A)** CD45RAneg cell frequency in CD38⁺ effector T cells. **(B)** Frequency of CD25⁺ cells gated on Foxp3^{neg} cells in CD38⁺ effector T cells. **(C)** Foxp3^{neg} cell frequency in CD38⁺ effector T cells. UC, open symbols; CD, closed symbols. Data are expressed as median + IQR. p values were calculated using a Kruskall-Wallis H analysis followed by the Dunn's Multiple Comparison Test.

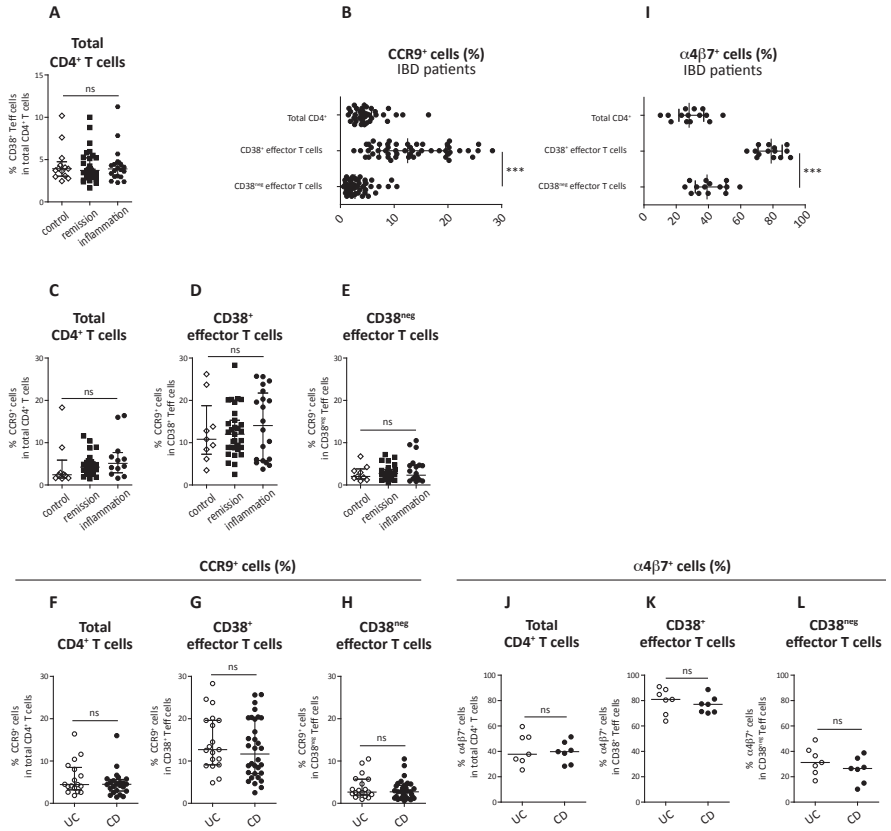


Figure S3. No differences in percentage of CD38⁺ effector T cells expressing gut-homing receptors CCR9 and α4β7 between pediatric UC and CD patients. (A-H) Flow cytometric analysis for CD3, CD4, CD62L, CD38 and CCR9 was performed on peripheral blood from pediatric IBD patients (cohort I) with biopsy-proven intestinal inflammation at diagnostic endoscopy and prior to treatment (denoted as "inflammation"), pediatric IBD patients in clinical remission during treatment (denoted as "remission") and age-matched healthy controls (denoted as "control"). Cells were gated on single cells, CD3⁺, CD4⁺, and CD62L^{neg}CD38⁺ cells (denoted as CD38⁺ effector T cells) or CD62L^{neg}CD38^{neg} cells (denoted as CD38^{neg} effector T cells). **(A)** Percentage of CD38⁺ effector T cells in CD4⁺ T cells of IBD patients and age-matched healthy controls. **(B)** Percentage of CCR9⁺ cells in total CD4⁺ T cells, CD38⁺ and CD38^{neg} effector T-cell populations in peripheral blood of IBD patients **(C-E)** Percentage of CCR9⁺ cells in total CD4⁺ T cells **(C)**, CD38⁺ **(D)** and CD38^{neg} **(E)** effector T-cell populations of IBD patients and age-matched healthy controls. **(F-H)** Percentage of CCR9⁺ cells in total CD4⁺ T cells **(C)**, CD38⁺ **(D)** and CD38^{neg} **(E)** effector T-cell populations of UC patients and CD patients. **(I-L)** Flow cytometric analysis for CD3, CD4, CD62L, CD38 and α4β7 was performed on peripheral blood from pediatric IBD patients with biopsy-proven intestinal inflammation (n=14 in total; consisting of patients prior to treatment, n=3; and patients receiving treatment, n=11). **(I)** Percentage of α4β7⁺ cells in total CD4⁺ T cells, CD38⁺ and CD38^{neg} effector T-cell populations in peripheral blood of IBD patients. **(J-L)** Median percentage of α4β7⁺ cells in total CD4⁺ T cells **(J)**, CD38⁺ **(K)** and CD38^{neg} **(L)** effector T-cell populations of UC patients and CD patients. Data are expressed as median +/- IQR. p values were calculated using Mann-Whitney analysis. Teff, effector T cell; UC, ulcerative colitis; CD, Crohn's disease; NS, not significant, ***p<0.001.

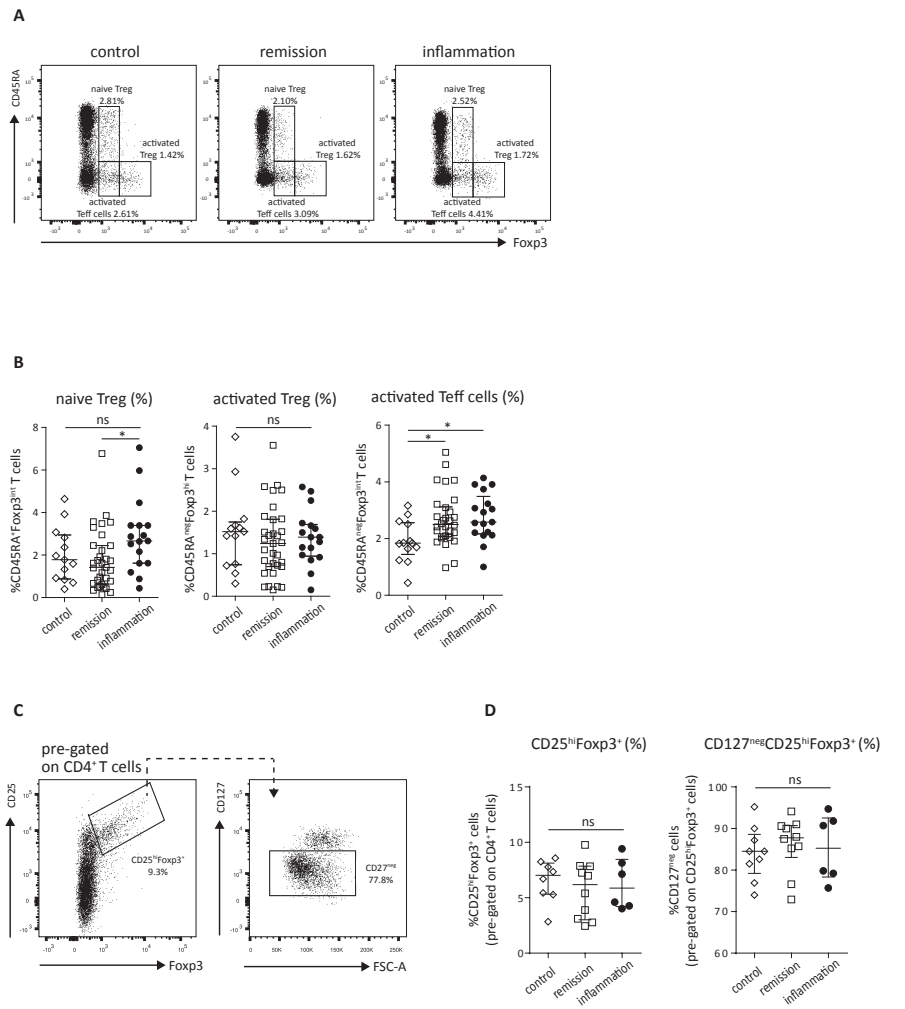


Figure S4. Combined analysis of Foxp3, CD45RA and CD127. Flow cytometric analysis for CD3, CD4, CD45RA, CD25, Foxp3 and CD127 was performed on peripheral blood of pediatric IBD patients with active disease (cohort I), pediatric IBD patients with disease in clinical remission (cohort I) and age-matched healthy controls. Cells were gated on single cells, $CD3^+$, $CD4^+$. **(A)** Representative dot-plots of the percentage of $CD45RA^+Foxp3^{int}$ (naive Treg), $CD45RA^{neg}Foxp3^{hi}$ (activated Treg) and $CD45RA^{neg}Foxp3^{int}$ (activated effector T cells) in $CD4^+$ T cells. **(B)** Frequencies of $CD45RA^+Foxp3^{int}$, $CD45RA^{neg}Foxp3^{hi}$ and $CD45RA^{neg}Foxp3^{int}$ in $CD4^+$ T cells. **(C)** Gating strategy for $CD25^{hi}Foxp3^+$ cells and $CD4^+CD25^{hi}CD127^{neg}Foxp3^+$ cells. **(D)** Frequencies of $CD25^{hi}Foxp3^+$ cells gated on $CD4^+$ T cells and $CD127^{neg}$ cells gated on $CD25^{hi}Foxp3^+CD4^+$ T cells in age-matched controls and IBD patients. Data are expressed as median + interquartile range (IQR). p values were calculated using a Kruskall-Wallis analysis. Treg, regulatory T cell; Teff, effector T cell; NS, not significant (p>0.05); *p<0.05.

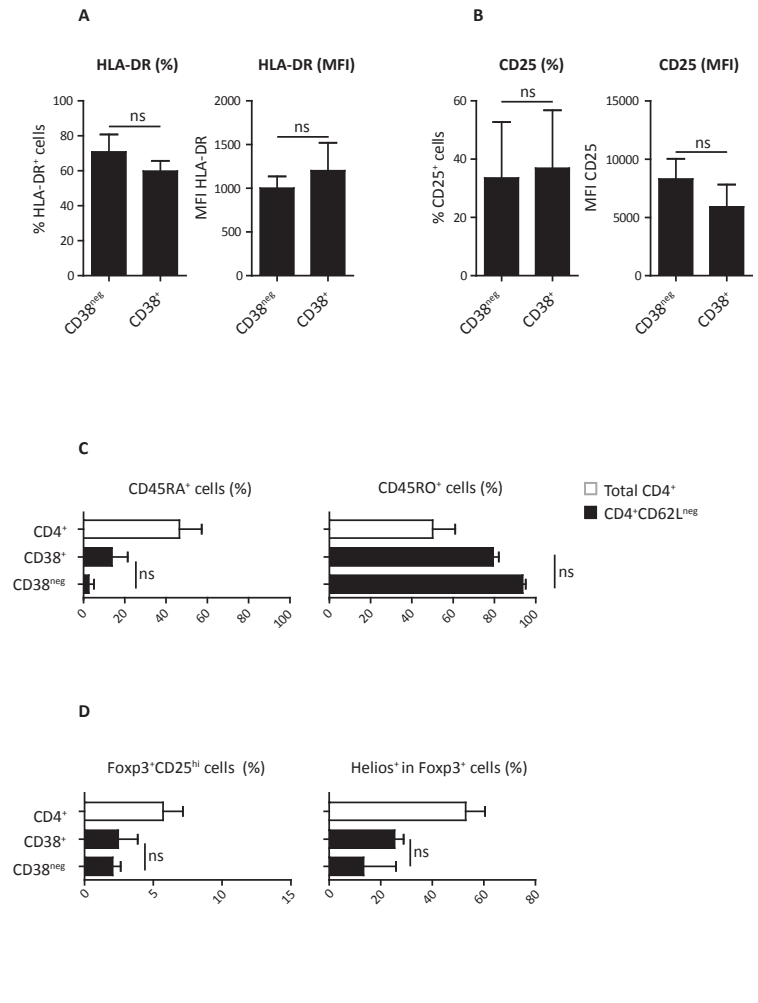


Figure S5. Similar frequencies of activated T cells between $CD38^+$ and $CD38^{neg}$ effector T cells after *in vitro* activation in an allogeneic MLR. (A) Purified $CD38^{neg}$ and $CD38^+$ effector T-cell populations (pre-gated on $CD4^+CD62L^{neg}$ cells) were cultured for 72 hours with allogeneic LPS-stimulated (100 ng/mL) monocyte-derived DCs (ratio 10:1). Percentage of $HLA\text{-}DR^+$ cells and HLA-DR MFI in $CD38^+$ and $CD38^{neg}$ T cells after 72 hours of culture. **(B)** Percentage of $CD25^+$ cells and CD25 MFI in $CD38^+$ and $CD38^{neg}$ T cells after 72 hours of culture. **(C)** Peripheral blood from adult healthy individuals was stained for flow cytometric analysis. Cells were gated on single cells, $CD3^+$, $CD4^+$, $CD38^{neg}CD62L^{neg}$ and $CD38^+CD62L^{neg}$ cells (denoted as $CD38^{neg}$ and $CD38^+$ effector T cells). Frequencies of $CD45RA^+$ (n=23) and $CD45RO^+$ (n=19) cells in the total $CD4^+$ T-cell population and $CD38^{neg}$ and $CD38^+$ T-cell populations (pre-gated on $CD4^+CD62L^{neg}$ cells). **(D)** Frequencies of $Foxp3^+CD25^{hi}$ and percentage $Helios^+$ cells in $Foxp3^+$ cells in the total $CD4^+$ T-cell population and $CD38^{neg}$ and $CD38^+$ T-cell populations (n=12). Data are expressed as median + IQR. p values were calculated using a Mann-Whitney analysis. MFI, mean fluorescence intensity; NS, not significant (p>0.05).

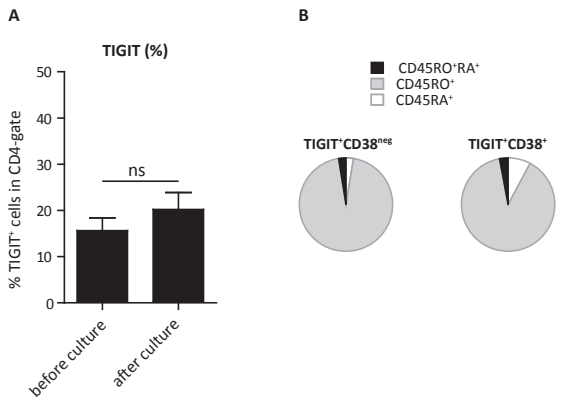


Figure S6. Frequencies of TIGIT-expressing CD4⁺ T cells are unaltered after *in vitro* activation with anti-CD3/28 for 96 hours. (A) PBMC of adult healthy individuals (n=4) were stimulated with anti-CD3/CD28 stimulation beads (0.5 bead per T cell) for 96 hours. Percentage of TIGIT⁺ cells in CD4⁺ T cells before and after 96 hours of culture are depicted (mean + SEM). **(B)** Pie charts depicting the average percentage of $CD38^{neg}TIGIT^{+}$ and $CD38^{+}TIGIT^{+}$ effector T cells positive for CD45RA and/or CD45RO. NS, not significant (p>0.05).

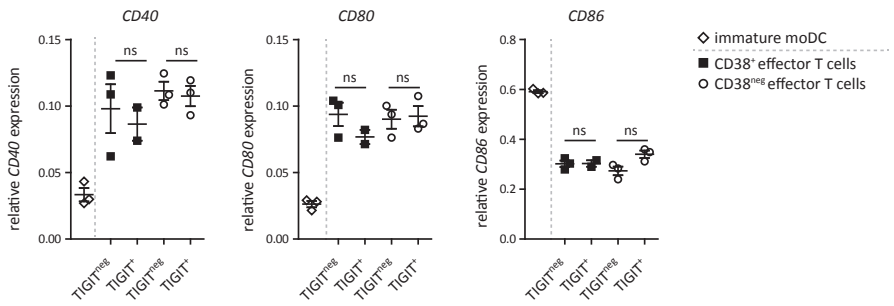


Figure S7. TIGIT expression on CD38⁺ effector T cells does not affect the maturation status of monocyte-derived DCs. Autologous immature monocyte-derived DCs were cultured together with purified $CD38^{+}TIGIT^{neg}$, $CD38^{+}TIGIT^{+}$, $CD38^{neg}TIGIT^{neg}$ or $CD38^{neg}TIGIT^{+}$ effector T cells for 24 hours in the presence of anti-CD3 (1 µg/ml) and LPS (10 ng/mL) in a ratio 2:1 (T cell : monocyte-derived DC). Analysis of *CD40, CD80* and *CD86* mRNA expression relative to *GAPDH* by quantitative PCR. One representative experiment shown out of two independent experiments. Data are represented as means + SEM. NS, not significant, *p<0.05**, p<0.01, ***p<0.001.

A

Patient cohort II (treatment-naive)

	Inflammation	Control
Total patients, N	18	9
Gender		
Male, N (%)	6 (33%)	2 (22%)
Female, N (%)	12 (67%)	7 (78%)
Age, y (median, IQR)	15.3 (11.1-16.9)	10.05 (6.0-12.0)
IBD diagnosis		
CD, N (%)	9 (50%)	NA
UC, N (%)	9 (50%)	NA

B

Follow up of patients from cohort II (duration to exacerbation)

		Time from diagnosis to first exacerbation (days)	Definition of exacerbation
Total group TIGIT<25% n=10	**TIGIT<25%**		
	Patient 1	245	PGA and FC (261 ug/g)
	Patient 2	87	PGA
	Patient 3	333	PGA, PUCAI>10 and FC (361 ug/g)
	Patient 4	204	PGA, PCDAI>10
	Patient 5	113	PGA
	Patient 6	79	PGA
	Patient 7	98	PGA and FC (1194.5 ug/g)
Total group TIGIT>25% n=8	**TIGIT>25%**		
	Patient 1	269	PGA and FC (725 ug/g)
	Patient 2	289	PGA, PCDAI>10, colitis on endoscopy
	Patient 3	365	Terminal ileitis and mild colitis on endoscopy

C

Patient	Diagnosis	Calprotectin (µg/g)	Exacerbation
1	CD	*1711 (H)*	No
2	UC	*619 (H)*	No
3	CD	*1300 (H)*	No
4	UC	*1000 (H)*	No
5	UC	*1800 (H)*	No
6	UC	*240*	No
7	UC	*458 (H)*	No
8	UC	*532 (H)*	No
9	CD	*1870 (H)*	Yes
10	CD	*540 (H)*	Yes
11	UC	*3000 (H)*	Yes
12	CD	*2200 (H)*	Yes
13	UC	*517 (H)*	Yes
14	UC	*2000 (H)*	Yes
15	CD	*554 (H)*	Yes
16	CD	*812 (H)*	Yes
17	CD	*1034 (H)*	Yes
18	CD	*812 (H)*	Yes

D

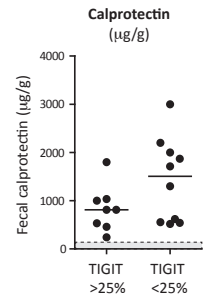


Figure S8. Individual time from diagnosis to exacerbation and definitions of exacerbation. (A) Information on gender, age and IBD phenotype in pediatric IBD patients (cohort II) and age-matched controls. **(B)** Individual patient information on time from diagnosis to exacerbation and definitions of exacerbation. **(C)** Fecal calprotectin levels (µg/g) of patients included in Figure 5. **(D)** Median baseline fecal calprotectin of patients with low (<25%) and high (>25%) TIGIT frequency in CD38⁺ effector T cells. The grey area indicates the normal reference interval for fecal calprotectin (<100 µg/g). CD, Crohn's disease; FC, fecal calprotectin; IFX, infliximab; PGA, physician global assessment; PUCAI, pediatric ulcerative colitis activity index; PCDAI, pediatric Crohn's disease activity index; UC, ulcerative colitis.

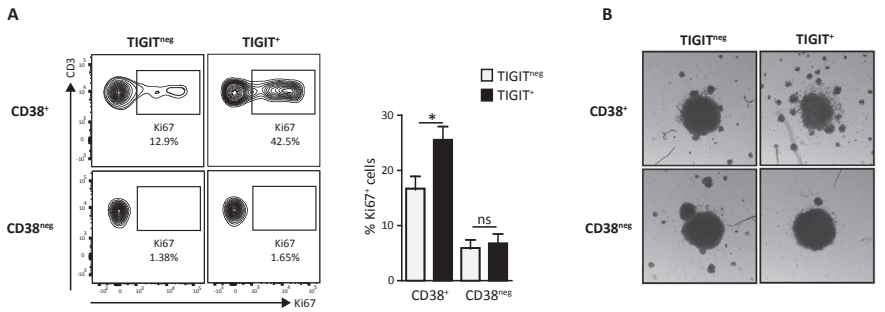


Figure S9. TIGIT expressing CD38⁺ effector T cells demonstrate high proliferative capacity *in vitro*. (A) Peripheral blood of adult healthy individuals was analyzed for CD3, CD4, CD62L, CD38, TIGIT and Ki67 by flow cytometry (n=13). Representative dot-plots (left) and frequencies of Ki67⁺ cells (right) are shown. **(B)** Purified $CD38^{+}TIGIT^{neg}$ and $CD38^{+}TIGIT^{+}$ T cells (pre-gated on $CD4^{+}CD62L^{neg}$ cells) were stimulated with anti-CD3/CD28 stimulation beads (0.5 bead per T cell). Microscopy of 14 hours after culture are shown. Data are expressed as median + IQR. p values were calculated using a Mann-Whitney analysis.

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4

MECHANISMS OF INDUCTION AND MICROBIAL REACTIVITY OF TIGIT$^+$CD4$^+$ T CELLS

Chapter 4

Manuscript in preparation.

4

MECHANISMS OF INDUCTION AND MICROBIAL REACTIVITY OF TIGIT$^+$CD4$^+$ T CELLS

ABSTRACT

T cell Ig and ITIM domain (TIGIT) is a coinhibitory receptor widely expressed by immune cells, amongst which CD4$^+$ T cells. Recently, we have demonstrated that frequencies of TIGIT$^+$ cells in circulating CD38$^+$ effector T cells, a CD4$^+$ T-cell population enriched for cells with specificity for mucosal antigens, are reduced inflammatory bowel disease (IBD), a chronic intestinal inflammation characterized by intestinal CD4$^+$ T-cell infiltration. In this study, using human and murine experimental approaches, we studied TIGIT expression in various CD4$^+$ T-cell subpopulations and determined the signals required for TIGIT expression. TIGIT expression was strongly associated with an antigen-experienced CD4$^+$ T-cell phenotype and was preferentially induced on TIGIT^{neg}CD4$^+$ T cells with a memory phenotype. Using the calcineurin inhibitor tacrolimus and calcium ionophore ionomycin, we show that calcium dependent signaling after T-cell receptor (TCR) ligation was required to induce TIGIT expression. Given the reduced TIGIT$^+$ cell frequencies in patients with IBD, we hypothesized that TIGIT expression on antigen-experienced CD4$^+$ T cells may be involved in regulating immune responses to intestinal bacteria. Indeed, a high percentage of intestinal CD4$^+$ T cells in both mice and human expressed TIGIT. Intestinal TIGIT expression depended on the presence of intestinal microbial antigens, as conventionalization of germ-free mice with specific pathogen free microbiota resulted in an increase in intestinal TIGIT expression. In agreement with the inhibitory function of TIGIT, human circulating TIGIT$^+$CD4$^+$ T cells displayed a functionally different response to bacterial antigen stimulation when compared to TIGIT^{neg}CD4$^+$ T cells, as evidenced by an increased frequency of IL-10-producing cells and undetectable expression of IL-17A. In conclusion, our data infer that TIGIT may have a role in regulating immune responses in the antigen-rich environment of the intestine. Analysis of immune responses in TIGIT deficient mice are needed to prove whether TIGIT maintains tolerance to intestinal bacteria.

INTRODUCTION

T cell Ig and ITIM domain (TIGIT) is a receptor of the Ig superfamily that is expressed by immune cells where it functions as a co-inhibitory receptor.[1] A wide variety of immune cells, including natural killer (NK) cells, CD4$^+$ and CD8$^+$ T cells, follicular T helper cells (Tfh) and regulatory T cells (Tregs), can express TIGIT on their surface.[2-6] TIGIT competes with the costimulatory receptor CD226 for two ligands, CD155 and CD112, which are expressed on the surface of antigen presenting cells (APCs).[7] TIGIT has an inhibitory effect on NK- and T-cell responses while CD226 delivers a costimulatory signal.[5, 8-10] Moreover, TIGIT modifies dendritic cell (DC) function via bi-directional signaling through CD155, promoting the generation of immunoregulatory DCs with decreased interleukin 12 (IL-12) and increased interleukin 10 (IL-10) production.[3, 11] In this manner, TIGIT exerts its immunosuppressive effects via direct and indirect mechanisms.

In line with its inhibitory functions, TIGIT deficiency or blockade exacerbates disease in models for autoimmune disease, including collagen-induced arthritis (CIA), graft versus host disease (GvHD) and experimental autoimmune encephalomyelitis (EAE).[5, 10] Moreover, genetic polymorphisms in the TIGIT-CD226 pathway have been associated with susceptibility to rheumatoid arthritis[12-16], juvenile idiopathic arthritis[17], autoimmune thyroid disease[16], multiple sclerosis[15, 16, 18], systemic lupus erythematodes[15], granulomatosis with polyangiitis[18], type 1 diabetes[15, 16], celiac disease[15], and inflammatory bowel disease (IBD).[19] IBD is a heterogeneous disease characterized by chronic intestinal inflammation. Although the precise etiology may differ per patient, the current theory is that IBD is caused by a dysregulated immune response to antigens of the intestinal bacteria in a genetically susceptible host.[20] The infiltration of CD4$^+$ T cells in the intestinal lamina propria is a critical step in the chronicity of the disease and causes a relapsing-remitting disease course that is characteristic for many CD4$^+$ T-cell mediated inflammatory diseases. Recently, our group has identified TIGIT as a key regulatory molecule in circulating CD38$^+$ effector T cells (CD62L^{neg}CD4$^+$), a CD4$^+$ T-cell population enriched for cells with specificity for mucosal antigens.[11, 21] In a subgroup of newly diagnosed patients with IBD, frequencies of TIGIT-expressing circulating CD38$^+$ effector T cells were two-fold reduced compared to healthy individuals (20% versus 40%, respectively) and identified patients with a reduced duration of clinical remission.[11] Similarly, it was recently demonstrated that frequencies of TIGIT$^+$CD4$^+$ T-cell frequencies were significantly reduced in peripheral blood of patients with psoriasis vulgaris, a CD4$^+$ T-cell mediated inflammatory skin disease, when compared to healthy individuals.[22] In patients with psoriasis, TIGIT expression on CD4$^+$ T cells negatively correlated with the Psoriasis Area and Severity Index, a frequently used disease score for the measurement of the severity of psoriasis.[22] Taken together, these data question whether reduced TIGIT expression in CD4$^+$ T cells may play a role in inflammatory disease.

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MECHANISMS OF INDUCTION AND MICROBIAL REACTIVITY OF TIGIT⁺CD4⁺ T CELLS

Among CD4⁺ T cells, TIGIT is highly expressed by Foxp3⁺ regulatory T cells (Tregs), in particularly on thymus-derived Foxp3⁺ Tregs, where it identifies a Treg subset that demonstrates selectivity for suppression of Th1 and Th17 but not Th2 cell responses.[3, 10, 23, 24] TIGIT is also expressed on a subpopulation of peripherally-derived Tregs with the capacity to produce IL-10.[25] A recent study demonstrated that regulatory macrophages can induce TIGIT expression on naive CD4⁺ T cells through a partly indoleamine 2,3-dioxygenase (IDO)-dependent mechanism.[25] In the same study, expression of TIGIT by naive CD4⁺ T cells was not induced by polyclonal anti-CD3/anti-CD28 stimulation, which is in contrast to several other studies that observed anti-CD3/anti-CD28 induced TIGIT expression on total CD4⁺ T cells, Tregs, naive and memory CD4⁺ T cells.[3, 5, 26, 27] Hence, it is unclear what stimulatory signals are required to induce TIGIT expression on CD4⁺ T cells and at what phase of T-cell differentiation this induction takes place. Moreover, it is unclear how these responses relate to intestinal microbial-host interaction. In this study, using human and murine experimental approaches, we studied TIGIT expression in various CD4⁺ T-cell subpopulations, including naive and memory CD4⁺ T cells, and determined the stimulatory signals required for TIGIT expression. Given the reduced TIGIT⁺ cell frequencies in patients with IBD[11], we hypothesized that TIGIT may be involved in regulating immune responses to intestinal bacteria. Hence, we tested whether intestinal TIGIT expression depended on the presence of intestinal microbial antigens and whether TIGIT expression could be identified on bacterial antigen-reactive T cells.

METHODS

Cell isolations (human).

Human venous blood was collected in EDTA tubes and peripheral blood mononuclear cells (PBMCs) were isolated using a Ficoll-Hypaque gradient according to standard protocol (Axis-Shield). CD4⁺ T cells were isolated from PBMCs by negative selection using the Dynabeads Untouched Human CD4⁺ T cell kit (11346D, ThermoFisher Scientific, Bleiswijk, the Netherlands). TIGIT^{neg}CD4⁺ cells, memory CD45RA^{neg}TIGIT^{neg}CD4⁺ T cells and naive CD45RO^{neg}TIGIT^{neg}CD4⁺ T cells were isolated using a DynabeadsTM Pan Mouse IgG kit (ThermoFisher). To obtain TIGIT^{neg}CD4⁺ cells, CD4⁺ T cells were incubated on ice for 30 min with 10 µg primary anti-TIGIT antibody (MBSA44, eBioscience, Bleiswijk, the Netherlands) per 1x10^7 cells. Memory and naive TIGIT^{neg}CD4⁺ cells were obtain by addition of either primary anti-CD45RA antibody (10 µg per 1x10^7 cells, clone HI100, Biolegend, San Diego, CA, USA) or primary anti-CD45RO antibody (clone UHCL1, Biolegend, 10 µg per 1x10^7 cells) to the first incubation step. Cells were cultured in Iscove's modified Dulbecco's medium (ThermoFisher) supplemented with heat inactivated fetal calf serum, Glutamax (ThermoFisher), 2-mercaptoethanol, penicillin and streptomycin.

Cell isolations (mice).

For *in vitro* stimulation experiments, cells were purified from spleens and lymph nodes of mice on a BABL/c background (Charles River, Maastricht, the Netherlands), which were kept under routine animal housing conditions in the Erasmus MC. Single-cell suspensions were enriched for CD4⁺ cells by depletion of B cells, macrophages, monocytes, and CD8⁺ cells with rat antibodies against B220 (clone 6B2), F4/80 (clone BM8), CD11b (clone MAC-1), MAC-2, MHCII (clone M5/114) and CD8 (clone 53.6.72) and anti-rat magnetic Dynabeads (ThermoFisher). TIGIT^{neg}CD4⁺ cells were isolated using 10 µg primary anti-TIGIT (clone GIGD7, eBioscience) per target 1x10^7 cells and sheep anti-rat IgG Dynabeads (ThermoFisher). For some experiments, TIGIT^{neg}CD4⁺ cells were separated into naive (CD62L⁺) and memory (CD62L^{neg}) cell populations using the CD4⁺CD62L⁺ T Cell Isolation Kit II (Miltenyi Biotec, Leiden, the Netherlands).

Cell cultures.

Human purified cell populations were cultured at 1x10^5 cells per well in a 96-well round bottom culture plate for 48 or 72 hours in IMDMc at 37°C with 5% CO_2. Cells were stimulated for the indicated time-points with: plate-bound anti-CD3 (clone OKT3, 0.5, 1 or 5 µg/ml, UltraLEAF, Biolegend), anti-CD28 (clone 15E8, 1 or 2 µg/ml, CD28 pure, functional grade, Miltenyi Biotec), anti-CD3/CD28 beads (bead-to-cell ratio 1:2, 1:4 or 1:10, Human T-Activator CD3/CD28, ThermoFisher), ionomycin (500 ng/ml, Sigma-Aldrich, Saint Louis, MO, USA), tacrolimus (5 mg/ml, Prograft, Astellas Pharma, Tokyo, Japan), retinoic acid (10nM, Sigma) or transforming growth factor-β (TGF-β, 20 ng/ml, R&D Systems).

Murine cell populations were cultured at 1x10^5 to 1.5x10^5 cells per well for 24, 72 or 96 hours in IMDMc at 37°C with 5% CO_2. Cells were stimulated for the indicated time-points with: plate-bound anti-CD3 (2 or 10 µg/ml, clone 145-2C11, BD), anti-CD28 (2 µg/ml, clone 37.51, BD), ionomycin (500 ng/ml, Sigma), tacrolimus (Prograft 5 mg/ml, Astellas Pharma, Tokyo, Japan), retinoic acid (10nM, Sigma) or TGF-β (20 ng/ml, R&D Systems). Cells stimulated with plate-bound anti-CD3 were cultured in 96-well flat bottom plates; other conditions were cultured in 96-well round plates.

CD154 based detection of flagellin-reactive CD4⁺ T cells.

A total of 3x10^6 PBMCs were plated at 6x10^6/ml and rested overnight in a 48-wells plate. The next day, cells were transferred to a 24-wells plate and stimulated for 7 hours with a mix of recombinant flagellins (A4-FlaX, A4-Fla2, A4-Fla3 and 14-2; total concentration of 10 µg/ml) in the presence of 1 µg/ml CD40 (HB14; Miltenyi Biotec) and 1 µg/ml CD28 functional grade pure Ab (CD28.6; Miltenyi Biotec). Anti-CD40 prevents CD154

downregulation and anti-CD28 optimizes the induction of CD 154 expression. Brefeldin A (3 µg/ml eBiosciences) was added during the last 2 hours of the stimulation. Stimulations were performed in Iscove's modified Dulbecco's medium (Lifetechnologies, Grand Island, NY, USA) supplemented with 5% human AB serum (SLBS8634; Sigma), Glutamax (Life Technologies), 2-mercaptoethanol, penicillin and streptomycin.

After stimulation, cells were stained for flow cytometry using monoclonal antibodies against CD3 (UCHT1, BD, or HIT3a, Biolegend), CD4 (SK3, BD), CD8 (RPA-T8, BD), CD14 (M5E2, Biolegend), CD20 (ZH7, Biolegend), and TIGIT (MBSA43, ThermoFisher), CD154 (5C8, Miltenyi), IL-10 (JES3-19F1, BD), IL-17A (N49-653, BD), IL-21 (eBio3A3-N2, eBiosciences), IFNγ (B27, BD), TNFα (Mab11, BD) or appropriate isotype controls (eBiosciences, BD or ThermoFisher). Flow cytometric analyses were performed on a FACS LSR II (BD Biosciences). For phenotypic analyses of $TIGIT^{+}$ and $TIGIT^{neg}$ flagellin-reactive $CD4^{+}$ T cells, a minimum of 5 cytokine-positive $CD4^{+}CD154^{+}$ T cells was used; individuals with lower events were excluded from the phenotypic analysis.

Lamina propria lymphocytes isolation.

Total intestinal tissues were incubated in 0.15% dithiothreithol (DTT)/HBSS for 15 minutes and in 1 mM EDTA/HBSS for 30 min at 60 rpm and 37C. Intestinal specimens without epithelial fractions were incubated in a digestive solution consisting of 10% FCS, 25 mM HEPES, 100 U/ml penicillin streptomycin, 30 µg/ml gentamycin, 0.5 µg/ml fungizone, 0.1 mg/ml collagenase III and 1 mg/ml DNase for 60 min at 37°C.

Flow cytometry.

Human cells were stained for flow cytometry *ex vivo* or after *in vitro* culture using monoclonal antibodies against CD3 (clone UCHT1, BD Biosciences, Franklin Lakes, NJ, USA; clone HIT3a, Biolegend), CD4 (clone SK3, BD), CD38 (clone HIT2, BD), CD62L (clone DREG-56, Biolegend), CD45RO/RPE (clone UCHL1, Agilent, Santa Clara, CA, USA), and TIGIT (clone MBSA43, eBioscience). Intracellular staining was performed with the Foxp3 fixation and permeabilization staining buffer kit, according to manufacturer's protocol (eBioscience), followed by staining with anti-TIGIT. Murine cells were stained for flow cytometry using monoclonal antibodies against CD3E (epsilon) (clone 500A2, BD), CD4 (clone RM4-5, BD), CD62L (clone MEL-14, Biolegend), CD38 (clone 90, Biolegend), CD44 (clone IM7, BD), CD45RB (clone 16A, BD), TIGIT (clone GIGD7, ThermoFisher) and live/dead markers. Cells were analyzed using the FACS Canto II and FlowJo software (BD).

Colonization of germ free mice.

Three independent biological experiments were performed using mice of different age as described before. In short, germ free mice were conventionalized by oral gavage with

0.5 ml of mixed fecal suspension obtained from 0.2 g of freshly obtained fecal material of conventionally raised mice (C57 BL/6 J) diluted 100-folds in brain heart infusion broth. Conventionalized mice were killed at days 1, 2, 4, 8, 16 and 30 post conventionalization. Jejunum, ileum and colon from each mouse were removed, RNA was extracted and transcriptome analysis was performed using the Affymetrix GeneChip Mouse Gene 1.1 ST array.

Statistics.

Significance between two groups was determined using Student's t-test and differences between multiple groups by using One-Way ANOVA, followed by correction for multiple testing (Bonferroni's Test). p Values of <0.05 were considered statistically significant. Prism software (GraphPad Software, Version 5.0, La Jolla, CA) was used for all statistical analysis.

RESULTS

TIGIT protein expression is strongly associated with a memory $CD4^{+}$ T-cell phenotype.

To gain insight in expression of TIGIT on human $CD4^{+}$ T-cell subpopulations, we analyzed TIGIT protein expression in human peripheral blood mononuclear cells (PBMCs) by flow cytometry. Surface TIGIT expression was detectable on 14.9% (median; interquartile range: 13-18%) of total circulating $CD4^{+}$ T cells (Figure 1A). Frequencies of $TIGIT^{+}$ cells were significantly higher in memory $CD45RO^{+}CD4^{+}$ T cells compared to naive $CD45RA^{+}CD4^{+}$ T cells (Figure 1B). We next assessed $TIGIT^{+}$ cell frequencies in naive T cells ($CD62L^{+}CD45RO^{neg}$, T_N), effector memory T cells ($CD62L^{neg}CD45RO^{+}$ T_{EM}), central memory T cells ($CD62L^{+}CD45RO^{+}$ T_{CM}) and effector T cells ($CD62L^{neg}CD45RO^{neg}$ T_{eff}). Frequencies of $TIGIT^{+}$ cells were significantly higher in T_{CM}, T_{EM} and T_{eff} cells compared to T_N cells (Figure 1C). Mean fluorescence intensity of TIGIT expression did not differ between T_{CM}, T_{EM} and T_{eff} cells (data not shown). When comparing $TIGIT^{+}$ and $TIGIT^{neg}$ T cells, $TIGIT^{+}$ cells mainly had a central memory phenotype, whereas the majority of $TIGIT^{neg}$ cells had a naive phenotype (Figure 1D and 1E). In order to investigate whether the TIGIT is similarly expressed on corresponding $CD4^{+}$ T-cell populations in both human and mice, we analyzed TIGIT expression on murine $CD4^{+}$ T cells isolated from the spleen (Figure 1 F and 1G). The majority of $TIGIT^{+}CD4^{+}$ splenocytes had an antigen-experienced phenotype, with particularly high percentages of effector memory T cells ($CD62L^{neg}CD44^{+}$ T_{EM}) and central memory T cells ($CD62L^{+}CD44^{+}$ T_{CM}). In line with the observations in human peripheral blood, the majority of $TIGIT^{neg}CD4^{+}$ splenocytes had a naive phenotype (Figure 1G). These data collectively show that TIGIT expression on human and murine $CD4^{+}$ T cells is strongly associated with an antigen-experienced phenotype.

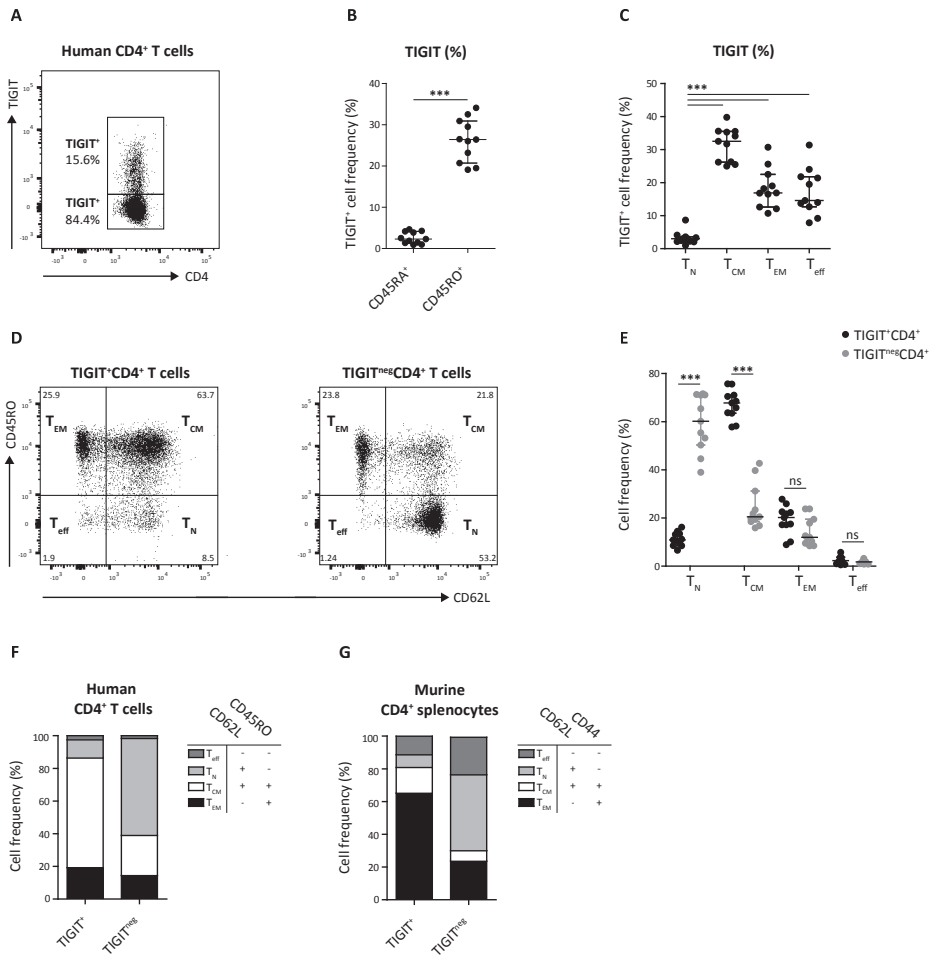

Figure 1. TIGIT expression is strongly associated with a memory CD4⁺ T-cell phenotype in both mice and humans. **(A-F)** Flow cytometric analysis for CD3, CD4, CD62L and CD45RO was performed on peripheral blood from adult healthy individuals (n=11). Cells were gated on single cells, CD3⁺ and CD4⁺. **(A)** Representative dot-plot of the percentage of TIGIT⁺ cells in the total CD4⁺ T-cell population in human peripheral blood. **(B)** Frequency of TIGIT⁺ cells in circulating memory CD45RO⁺CD4⁺ and naive CD45RA⁺CD4⁺ T cells. Data are expressed as median ± interquartile range. p values were calculated using a Kruskal-Wallis H analysis followed by the Dunn's Multiple Comparison Test. **(C)** Frequency of TIGIT⁺ cells in circulating CD62L⁺CD45RO^{neg} naive T cells (T_N), CD62L^{neg}CD45RO⁺ effector memory T cells (T_{EM}), CD62L⁺CD45RO⁺ central memory T cells (T_{CM}) and CD62L^{neg}CD45RO^{neg} effector T cells (T_{eff}). **(D)** Representative dot-plots of the percentage of T_N, T_{EM}, T_{CM} and T_{eff} in circulating TIGIT⁺CD4⁺ and TIGIT^{neg}CD4⁺ T cells. **(E)** Frequencies of T_N, T_{CM}, T_{EM} and T_{eff} in circulating TIGIT⁺CD4⁺ and TIGIT^{neg}CD4⁺ T cells in human peripheral blood. Data are expressed as median ± interquartile range. p values were calculated using a Mann Whitney analysis. **(F)** Bar graphs depicting median frequencies of T_N, T_{eff}, T_{CM} and T_{EM} cells in human peripheral blood TIGIT⁺CD4⁺ and TIGIT^{neg}CD4⁺ T cells (n=11). **(G)** Bar graphs depicting frequencies of T_N, T_{eff}, T_{CM} and T_{EM} cells in TIGIT⁺CD4⁺ and TIGIT^{neg}CD4⁺ murine splenocytes. Representative for two separate analyses. NS, not significant, *p<0.05, **p<0.01, ***p<0.001.

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TCR ligation induces TIGIT expression on murine and human TIGIT^{neg}CD4⁺ T cells.

Many of the proteins expressed by effector and memory CD4⁺ T cells are acquired soon after TCR ligation. The significantly higher frequency of TIGIT⁺ cells in T_{CM}, T_{EM} and T_{eff} compared to T_N (Figure 1C) could therefore imply that TIGIT expression on the cell surface is induced after antigen encounter.[3] To investigate whether TCR ligation induces TIGIT expression, we cultured TIGIT^{neg}CD4⁺ T cells isolated from murine splenocytes and lymph nodes in the presence of increasing concentrations of plate-coated anti-CD3 and determined the effect on TIGIT surface expression. TIGIT expression was induced on murine TIGIT^{neg}CD4⁺ T cells after stimulation with anti-CD3 in a dose dependent manner (Figure 2A and 2B). To test whether TCR-ligation induced TIGIT expression is a mechanism that is conserved among different species, we repeated the experiment with TIGIT^{neg}CD4⁺ T cells isolated from human PBMCs (Figure 2C and 2D). Importantly, very low frequencies of TIGIT⁺ cells were present at the start of the culture. In agreement with what we observed in murine cultures, anti-CD3 stimulation of human TIGIT^{neg}CD4⁺ T cells caused a dose-dependent increase in both the frequency (Figure 2C) and mean fluorescence intensity (Figure 2D) of TIGIT⁺ cells after 48 hours of culture. In all anti-CD3 stimulated murine and human cell cultures, the majority of TIGIT⁺CD4⁺ cells had a memory phenotype, as evidenced by high frequencies of CD44⁺ or CD45RO⁺ cells respectively (Figure S1). In contrast to naive CD4⁺ T cells that requires a costimulatory signal delivered by CD28 in addition to TCR ligation for full T-cell activation, memory CD4⁺ T cells are less dependent on accessory cell costimulation.[28] We therefore tested the effect of CD28 costimulation on TCR-ligation induced TIGIT expression. Addition of anti-CD28 antibodies to anti-CD3-stimulated cells significantly decreased the frequencies of TIGIT⁺ cells in murine cell cultures (Figure 2E), indicating that CD28 costimulation was not required for TIGIT induction.

To investigate the kinetics and stability of TIGIT expression, murine TIGIT^{neg}CD4⁺ T cells were analyzed after 24 and 96 hours of culture with anti-CD3 stimulation. Similar frequencies of TIGIT⁺ cells were observed at 24 hours and 96 hours of culture, indicating that the percentage of cells expressing TIGIT is stable for at least 96 hours on murine CD4⁺ T cells (Figure 2F). In contrast, the mean fluorescence intensity of TIGIT on stimulated murine cells was significantly higher at 96 hours compared to 24 hours (Figure 2F). Likewise, in cultures of human TIGIT^{neg}CD4⁺ T cells, the frequency of TIGIT expressing cells remained comparable between 48-72h of anti-CD3 stimulation while the mean fluorescence intensity of TIGIT further increased from 48 to 72 hours (Figure S2).

Taken together, TCR ligation induces TIGIT expression on the cell surface of TIGIT^{neg}CD4⁺ T cells in both mice and humans. TCR-induced expression of TIGIT is sustained for at least 72-96 hours and is primarily detected on CD4⁺ T cells with an antigen-experienced phenotype.

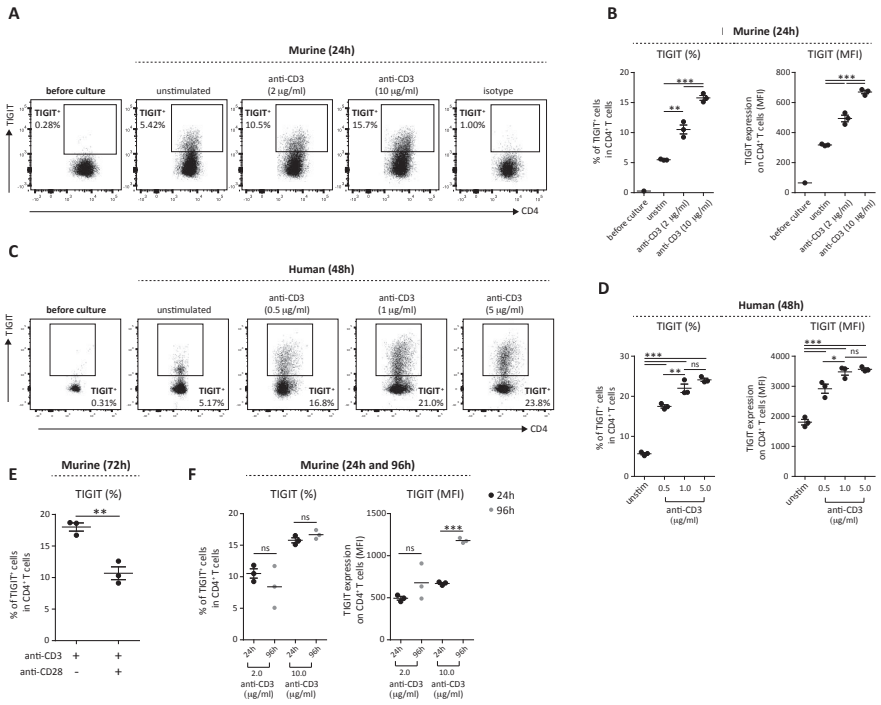

Figure 2. TCR ligation induces TIGIT on murine and human TIGIT^{neg}CD4$^+$ T cells. Murine TIGIT^{neg}CD4$^+$ cells were isolated from lymph nodes and splenocytes and cultured in triplicate for 24, 72 or 96h with different concentrations of anti-CD3 (2 and 10 µg/ml). Cells shown in this figure were pre-gated on single CD3$^+$CD4$^+$ lymphocytes. **(A)** Representative dot-plots of the percentage of TIGIT$^+$ cells after 24h of culture. **(B)** Frequency of TIGIT$^+$ cells and TIGIT expression per cell (MFI). The mean ± SD of a triplicate cell culture are shown. P values were calculated using One-way ANOVA followed by correction for multiple testing. Representative of 2 independent experiments. **(C-D)** Human TIGIT^{neg}CD4$^+$ T cells were isolated from peripheral blood of adult healthy individuals and cultured in triplicate for 48h with different concentrations of plate-coated anti-CD3 (0.5, 1 and 5 µg/ml). **(C)** Representative dot-plots of the percentage of TIGIT$^+$ cells after 48h of culture. **(D)** Frequency of TIGIT$^+$ cells and TIGIT MFI. The mean ± SD of a triplicate cell culture are shown. P values were calculated using One-way ANOVA followed by correction for multiple testing. Representative of 2 independent experiments. **(E)** Frequencies of TIGIT$^+$ cells in murine CD4$^+$ T cells after 72 h of culture in the presence of anti-CD3 (2 µg/ml) with or without anti-CD28 (2 µg/ml). **(F)** Frequencies of TIGIT$^+$ cells and TIGIT MFI on murine CD4$^+$ T cells after 24h and 96h. NS, not significant, *p<0.05, **p<0.01, ***p<0.001.

Calcium dependent signaling after TCR ligation induces TIGIT preferentially on memory TIGIT^{neg}CD4$^+$ T cells.

To investigate whether TIGIT expression is differentially induced on naive and memory CD4$^+$ T cells after TCR-mediated activation, we purified human naive (CD45RA$^+$) and memory (CD45RO$^+$) TIGIT^{neg}CD4$^+$ T cells and analyzed TIGIT expression after *in vitro* activation. TIGIT$^+$ cell frequencies did not increase within naive TIGIT^{neg}CD4$^+$ T cells after anti-CD3 ligation or combined anti-CD3/anti-CD28 stimulation (Figure 3A). The mean fluorescence

intensity of TIGIT-expressing cells in naive CD4$^+$ T-cell cultures did increase after anti-CD3/anti-CD28 stimulation. In contrast to naive CD4$^+$ T cells, anti-CD3 stimulation caused significant increase in both the frequency and mean fluorescence intensity of TIGIT$^+$ cells in memory CD4$^+$ T cells (Figure 3B). As observed in previous murine cell cultures (Figure 2E), addition of anti-CD28 antibodies to anti-CD3-stimulated cells significantly decreased the frequencies of TIGIT$^+$ cells in cultures of human memory CD4$^+$ T cells (Figure 3B). Similar results were obtained with murine naive (CD62L$^+$) and memory (CD62L^{neg}) TIGIT^{neg}CD4$^+$ T cells (Figure S3), corroborating that TCR ligation induces TIGIT preferentially on memory CD4$^+$ T cells.

TCR ligation initiates different intracellular signaling cascades, involving both Ca^{2+} and kinase signaling pathways (Figure S4). To investigate which intracellular pathway is

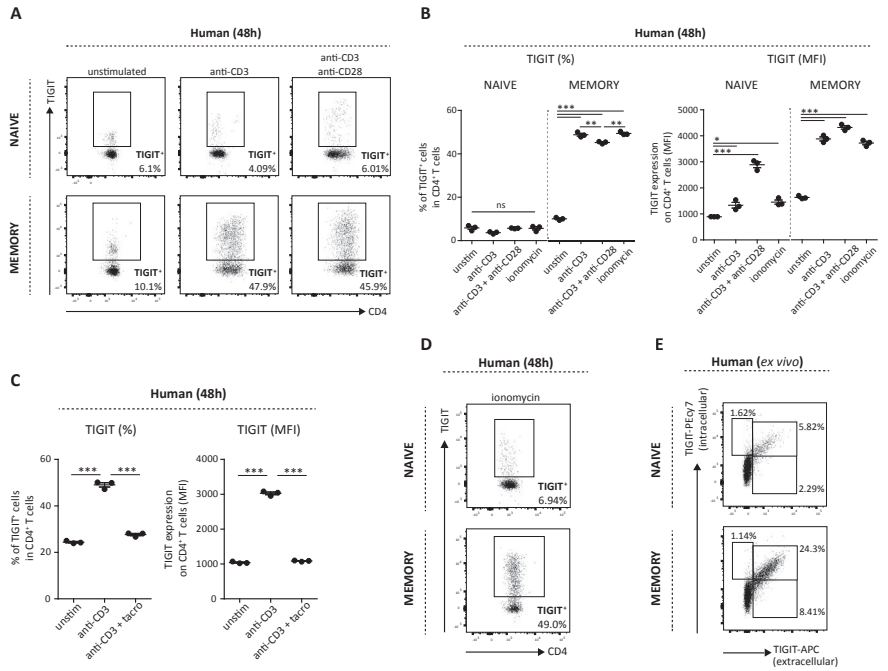

Figure 3. Ca^{2+} dependent signaling after TCR ligation induces TIGIT expression preferentially on memory CD4$^+$ T cells. (A-B) Human naive (CD45RA$^+$) T cells and memory (CD45RO$^+$) TIGIT^{neg}CD4$^+$ T cells were isolated from peripheral blood of adult healthy individuals and cultured in triplicate for 48h with different concentrations of plate-coated anti-CD3 (5 µg/ml) with or without anti-CD28 (2 µg/ml). **(A)** Representative dot-plots of the percentage of TIGIT$^+$ cells after 48h of culture. **(B)** Frequency of TIGIT$^+$ cells and TIGIT MFI. The mean ± SD of a triplicate cell culture are shown. **(C)** Memory TIGIT^{neg}CD4$^+$ cells were cultured with anti-CD3 (1 µg/ml) with or without tacrolimus (5 ng/ml) for 48h. Frequency of TIGIT$^+$ cells and TIGIT MFI (mean ± SD) are shown. **(D)** Representative dot-plots of the percentage of TIGIT$^+$ cells after 48h of ionomycin treatment (500 ng/ml). **(E)** Freshly isolated human PBMCs were stained for extracellular TIGIT protein (TIGIT-APC), fixed and permeabilized, and stained for intracellular TIGIT protein (TIGIT-PEcy7). P values were calculated using One-way ANOVA followed by correction for multiple testing. Representative of 2 independent experiments. NS, not significant, *p<0.05, **p<0.01, ***p<0.001.

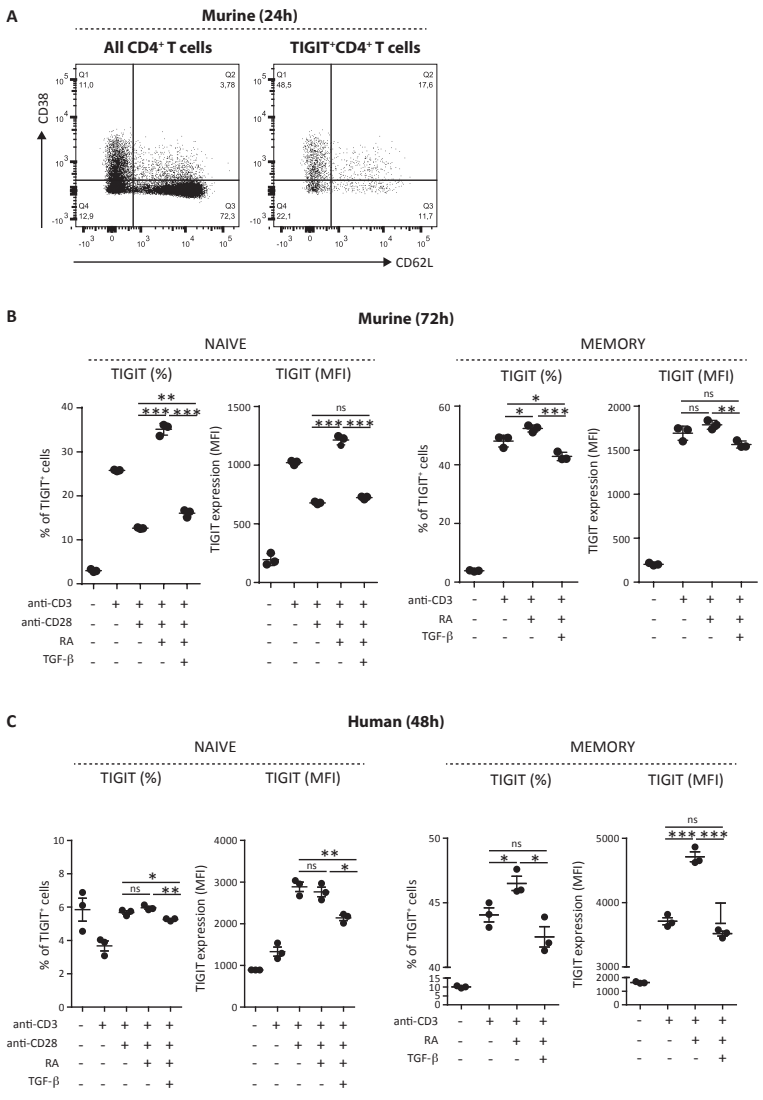

Figure 4. Environmental control of TIGIT induction on naive CD4⁺ T cells. (A) Murine TIGIT^{neg}CD4⁺ cells were isolated from splenocytes and cultured in triplicate for 24h with anti-CD3 (10 µg/ml), anti-CD28 (2 µg/ml). **(B)** Murine naive (CD62L⁺) and memory (CD62L^{neg}) TIGIT^{neg}CD4⁺ cells were isolated from lymph nodes and splenocytes and cultured in triplicate for 72h with anti-CD3 (10 µg/ml). In some conditions, anti-CD28 (2 µg/ml), RA (10 nM) and/or TGF-β (20 ng/ml) were added to the culture. **(C)** Human naive (CD45RA⁺) T cells and memory (CD45RO⁺) TIGIT^{neg}CD4⁺ T cells were isolated from peripheral blood of adult healthy individuals and cultured in triplicate for 48h with different concentrations of plate-coated anti-CD3 (5 µg/ml for naive cells; 1 µg/ml for memory cells). In some conditions, anti-CD28 (2 µg/ml), RA (10nM) and/or TGF-β were added to the culture.

mainly involved in TIGIT induction on memory CD4⁺ T cells, we blocked intracellular Ca^{2+} signaling downstream of the TCR using the calcineurin inhibitor tacrolimus. In both mice and humans, tacrolimus inhibited the induction of TIGIT in anti-CD3 activated memory TIGIT^{neg}CD4⁺ T cells (Figure 3C and S3), suggesting that TIGIT induction relies on the Ca^{2+} dependent signaling pathway downstream of the TCR. In line with this, selectively increasing intracellular Ca^{2+} levels by using ionomycin, a selective Ca^{2+} ionophore, induced high frequency of TIGIT⁺ cells on memory but not on naive TIGIT^{neg}CD4⁺ T cells (Figure 3B and 3D; Figure S3).

To investigate whether TIGIT on the cell surface of memory CD4⁺ T cells is recruited from intracellular stores or synthesized *de novo*, we determined the presence of intracellular TIGIT protein in freshly isolated circulating CD4⁺ T cells with and without cell surface TIGIT expression. Virtually all memory CD4⁺ T cells and the few naive T cells that expressed TIGIT on their cell surface had intracellular stores of TIGIT protein (Figure 3E). In contrast, no intracellular stores of TIGIT were detected in naive or memory CD4⁺ T cells without extracellular TIGIT expression. This in combination with the increased frequencies of TIGIT⁺ cells after *in vitro* anti-CD3 stimulation suggests that TIGIT is most likely synthesized *de novo* after TCR ligation.

Overall, these data show that Ca^{2+} signaling after TCR ligation induces *de novo* TIGIT expression preferentially in memory TIGIT^{neg}CD4⁺ T cells compared to naive TIGIT^{neg}CD4⁺ T cells.

Environmental control of TCR-induced TIGIT expression on TIGIT^{neg}CD4⁺ T cells.

Recently, we have shown that in healthy human peripheral blood the frequency of TIGIT⁺CD4⁺ T cells is approximately two-fold higher in CD38⁺CD62L^{neg} T cells enriched in gut homing potential when compared to CD38⁺CD62L^{neg} T cells that home to the skin.[11, 21] In keeping with this observation, *in vitro*-generated TIGIT⁺CD4⁺ cells contained a two-fold higher frequency of CD38⁺CD62L^{neg} compared to CD38^{neg}CD62L^{neg} cells (Figure 4A). As the CD38⁺CD62L^{neg} phenotype of CD4⁺ T cells is driven by the local mucosal factors retinoic acid (RA) and transforming growth factor beta (TGF-β), we next assessed the effect to RA and TGF-β on *de novo* TIGIT induction on naive and memory TIGIT^{neg}CD4⁺ T cells. In mice, the addition of RA further increased frequencies and mean fluorescence intensity of TIGIT⁺ cells after TCR ligation in both naive and memory CD4⁺ T cells (Figure 4B). In human CD4⁺ T cells, frequencies of TIGIT⁺ cells in activated memory but not naive CD4⁺ T cells were increased in the presence of RA. RA also potentiated the mean fluorescence intensity of TCR-induced TIGIT expression on memory but not on naive human CD4⁺ T cells. In both murine and human cell cultures, frequencies of TIGIT⁺ cells were significantly higher in the presence of RA alone compared to RA plus TGF-β (Figure 4B and 4C). This may be explained by decreased levels of intracellular Ca^{2+} when TGF-β is present, as others

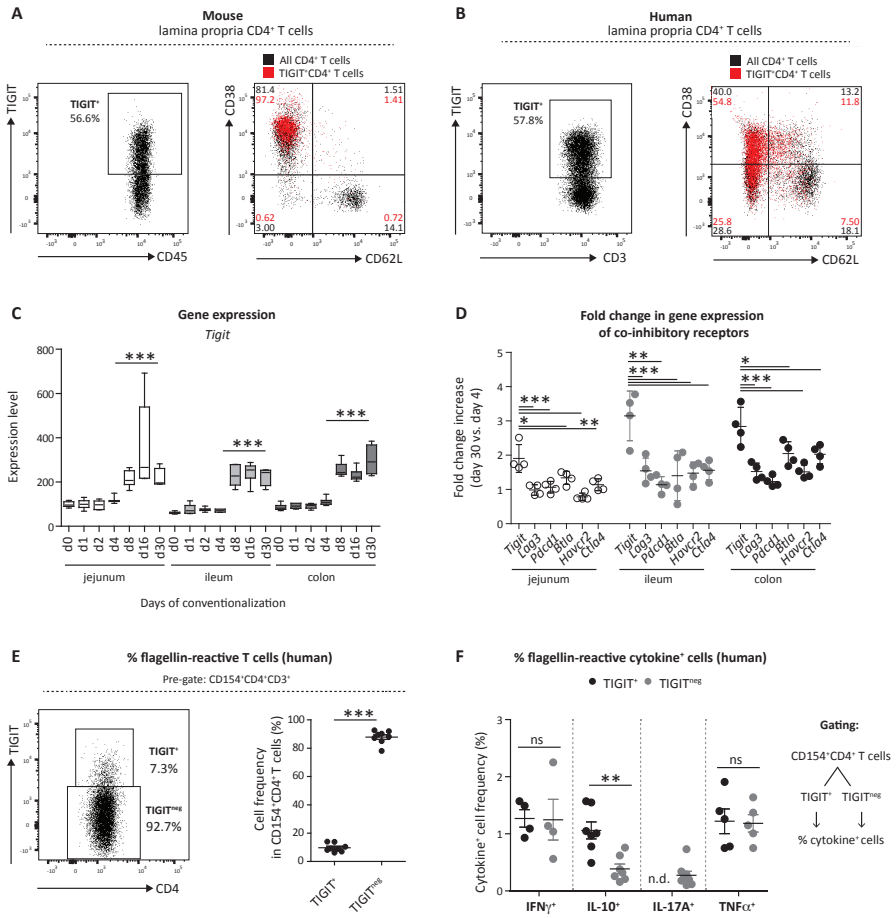

Figure 5. TIGIT expression increases upon bacterial colonization of germ-free mice. (A-B) Murine and human lamina propria lymphocytes were isolated from resected colonic tissue and analyzed with flow cytometry. Frequencies of TIGIT$^+$ cells and CD62L and CD38 expression in CD4$^+$ lamina propria lymphocytes are shown. **(C)** Jejunum, ileum, and colon gene expression levels of *Tigit* were analyzed in germ free and conventionalized mice at indicated days post conventionalization. Values are depicted as box and whisker diagram (top-to-bottom; maximum value, upper quartile, median, lower quartile, and minimal value, respectively). **(D)** Relative fold change in coinhibitory gene expression levels between day 30 and day 4 post conventionalization. Fold changes for *Tigit, Lag3, Pdcd1, Havcr2* and *Ctla4* are shown. One-way ANOVA followed by correction for multiple testing. **(E-F)** PBMCs from healthy individuals were stimulated with the a mix of recombinant flagellins and analyzed for CD154 expression. **(E)** Mean frequencies (± SEM) of TIGIT$^+$ and TIGIT^{neg} cells among flagellin-reactive CD154$^+$CD4$^+$ T cells are depicted (n=8). **(F)** Frequency of cytokine$^+$ cells in TIGIT$^+$ and TIGIT^{neg} flagellin-reactive CD154$^+$CD4$^+$ T cells after flagellin stimulation of PBMC derived from adult healthy individuals. A minimum of 5 cytokine$^+$ CD154$^+$CD4$^+$ T cells was used to calculate cytokine$^+$ cell frequencies; samples with lower number of events were excluded. The mean (± SEM) of cytokine$^+$ cells are shown. P values were calculated using Student's t Test. NS, not significant, *p<0.05, **p<0.01, ***p<0.001. Remark: "Chapter 4; Figure 5F" also appears in "Chapter 5; Figure 6B" of this thesis.

have observed that TGF-β prevents intracellular Ca^{2+} mobilization in stimulated CD4$^+$ T cells through inhibiting Tec kinase Itk activity.[29] Overall, these data demonstrate that CD38-inducing factors from the gut microenvironment such as RA but not TGF-b may potentiate TCR-induced TIGIT expression on memory CD4$^+$ T cells.

Intestinal *Tigit* expression increases after conventionalization of germ free mice and TIGIT expression on CD4$^+$ T cells is associated with a regulatory response to bacterial antigen.

In the healthy mouse intestine, a high percentage of lamina propria CD4$^+$ T cells has a CD38$^+$CD62L^{neg} phenotype.[21] To see whether this population of intestinal CD4$^+$ T cells contained TIGIT$^+$ cells, we analyzed TIGIT expression on lamina propria lymphocytes isolated from intestinal tissue of mice and humans. TIGIT protein was expressed on small intestinal and colonic CD4$^+$ T cells of both mice (Figure 5A) and humans (Figure 5B). The majority of murine TIGIT-expressing lamina propria CD4$^+$ T cells did not co-express Foxp3 and all had a CD62L^{neg}CD38$^+$ phenotype (Figure 5A and 5B). Given the high frequency of TIGIT$^+$ cells in the intestinal lamina propria, we questioned whether intestinal TIGIT expression depends on the presence of intestinal microbial antigens. To test this, we analyzed experimental data from previous experiments in which we conventionalized germ free mice with specific pathogen free microbiota[30] and determined intestinal *Tigit* expression before and after conventionalization. Conventionalization of germ free mice resulted in a two- to three-fold increase in intestinal *Tigit* expression starting at day 8 post conventionalization (Figure 5C). As shown in Figure S5A (data in part previously published[30]), the increase in *Tigit* expression coincided with the elevation of expression of tolerance-associated proteins, including *Il10*, *Foxp3* and cytotoxic T lymphocyte antigen-4 (*Ctla4*). In contrast to the expression of *Il10*, *Foxp3* and *Ctla4*, *Tigit* expression was very stable in both small intestine and colon during later time points (Figure 5C and S5A). The fold-change increase in *Tigit* expression observed between day 30 and day 4 was significantly higher when compared to other coinhibitory receptors, including Programmed Cell Death 1 (*Pdcd1*), Lymphocyte-activation gene 3 (*Lag3*), B- and T-lymphocyte attenuator (*Btla*) and Hepatitis A virus cellular receptor 2 (*Havcr2*, encoding T-cell immunoglobulin and Mucin 3, Tim-3; see Figure 5D and S5B).

As a large proportion of intestinal T cells is expected to have specificity to microbial antigen, we hypothesize that TIGIT may be expressed by microbiota reactive CD4$^+$ T cells. To test this hypothesis we first determined frequencies of TIGIT$^+$ cells in bacterial-antigen reactive CD4$^+$ T cells, which can be detected by upregulation of CD154 following antigen encounter.[31, 32] Thereto, PBMCs of human healthy individuals, which are known to possess circulating CD4$^+$ T cells reactive to intestinal bacterial antigens[33], were stimulated with recombinant flagellin and evaluated for CD154 expression. The majority

of bacterial flagellin-reactive CD4$^+$ T cells did not express TIGIT, however, a clear population of approximately 7%-10% TIGIT$^+$ cells could be identified among flagellin-reactive CD4$^+$ T cells (Figure 5E). To evaluate functional differences between flagellin-reactive TIGIT$^+$ and TIGIT^{neg} cells, CD154 detection was combined with intracellular cytokine analysis. In agreement with the inhibitory function of TIGIT, flagellin-reactive TIGIT$^+$CD4$^+$ T cells contained increased frequencies of IL-10-expressing cells compared to flagellin-reactive TIGIT^{neg}CD4$^+$ T cells (Figure 5F). Low frequencies of IL-17A$^+$ cells were present in flagellin-reactive TIGIT^{neg}CD4$^+$ T cells, whereas no IL-17A$^+$ cells could be detected in TIGIT$^+$CD4$^+$ cells (Figure 5F). IFNγ^+- and TNFα^+ cell frequencies were not significantly different between TIGIT$^+$CD4$^+$ and TIGIT^{neg}CD4$^+$ flagellin-reactive T cells (Figure 5D).

Taken together, these data demonstrate that TIGIT is expressed by high frequencies of intestinal CD4$^+$ T cells and this expression may be acquired during bacterial colonization of the gastrointestinal tract. Bacterial flagellin-reactive TIGIT$^+$CD4$^+$ cells are detectable and may be functionally different when compared to TIGIT^{neg}CD4$^+$ T cells, as suggested by an increased IL-10-producing cells and undetectable expression of IL-17A.

DISCUSSION

In this study, we show that TIGIT protein expression is strongly associated with an antigen-experienced CD4$^+$ T-cell phenotype. Using both human and murine experimental approaches, we demonstrate that calcium dependent signaling after TCR ligation induces TIGIT expression preferentially on antigen-experienced TIGIT^{neg}CD4$^+$ T cells with a memory phenotype. The intestinal tissue harbors a large population of antigen-experienced CD4$^+$ T cells. As we observed that TIGIT was expressed by a high percentage of intestinal CD4$^+$ T cells in both mice and human, we tested whether intestinal TIGIT expression depends on the presence of intestinal microbial antigens. Conventionalization of germ-free mice with specific pathogen free microbiota resulted in a two- to three-fold increase in intestinal TIGIT expression. A large proportion of intestinal T cells is expected to have specificity to microbial antigen. Therefore, we investigated whether TIGIT was expressed by microbiota-reactive CD4$^+$ T cells. Although the majority bacterial antigen-reactive CD4$^+$ T cells did not express TIGIT, a clear population of approximately 7%-10% TIGIT$^+$ cells could be identified among bacterial antigen-reactive CD4$^+$ T cells. Bacterial antigen-reactive TIGIT$^+$CD4$^+$ T cells contained higher frequencies of IL-10$^+$ cells compared to bacterial antigen-reactive TIGIT^{neg}CD4$^+$ cells, suggesting that TIGIT-expressing CD4$^+$ T-cell populations may be involved in regulating immune responses to intestinal bacteria.

It is well-established that naive and antigen-experienced CD4$^+$ T cells respond differently to TCR ligation.[28, 34] Some genes are more preferentially induced in antigen-

experienced CD4$^+$ T cells, rather than in naive CD4$^+$ T cells.[35] Here, we show that TIGIT expression is preferentially induced on antigen-experienced CD4$^+$ T cells with a memory phenotype. The rapid induction of *de novo* TIGIT expression by memory CD4$^+$ T cells may be linked to modulation of chromatin structure or demethylation of promoter regions and regulatory elements following initial naive CD4$^+$ T-cell activation, as the *TIGIT* gene locus is known to be hypermethylated in human naive CD4$^+$ T cells.[36] This is in line with our observation that TIGIT$^+$ cell frequencies did not increase on human naive TIGIT^{neg}CD4$^+$ T cells after anti-CD3 ligation with and without anti-CD28 costimulation. In contrast, Yu *et al.* have demonstrated that TIGIT$^+$ cell frequencies did increase after anti-CD3/anti-CD28 activation of purified human naive CD45RA$^+$CD4$^+$ T cells.[3] This discrepancy is likely explained by the fact we used purified TIGIT^{neg} cells in our cultures, excluding the possibility that the increased frequency of TIGIT$^+$ cells resulted from highly proliferative TIGIT$^+$ cells present at the start of culture.[11, 23] Interestingly, in both mice and humans, anti-CD28 costimulation decreased the frequency of TIGIT$^+$ cells in TCR-stimulated memory CD4$^+$ T cells. This is reminiscent of observations in naive CD4$^+$ T cells and CD8$^+$ T cells, where the presence of CD28-dependent signaling molecules decreases the induction of anergy- and exhaustion associated genes respectively.[37, 38] This suggests that the activation state of APCs may dictate the size of TIGIT$^+$ cell pool. Specifically, TIGIT upregulation may preferentially occur in non-inflammatory environments, where memory CD4$^+$ T cells receive incomplete priming signals through encounter of antigens presented by non-activated or nonprofessional APCs. Our *ex vivo* analyses of murine lamina propria CD4$^+$ T cells show that the majority of TIGIT$^+$CD4$^+$ T cells in the intestinal tissue do not co-express the regulatory transcription factor Foxp3. Thus, in addition to TIGIT expression on various Foxp3$^+$ Treg populations described in previous literature[23, 25], our data indicates that in certain environments, such as the non-inflamed intestinal environment, TIGIT is primarily expressed by Foxp3^{neg}CD4$^+$ T-cell populations.

We demonstrate that Ca^{2+} signaling after TCR ligation is required for TIGIT expression on memory CD4$^+$ T cells. Free intracellular Ca^{2+} induces calcineurin-mediated dephosphorylation and import of the transcription factor nuclear factor of activated T cells (NFAT) into the nucleus. Induction of most TCR-induced genes relies on cooperation of NFAT and other transcription factors such as activator protein-1 (AP-1).[39] However, NFAT activation on its own is sufficient to induce a two-fold change in TIGIT gene expression in human Jurkat T cells[39], suggesting that NFAT is a critical component of the signaling pathway that regulates TIGIT expression. Importantly, not all memory CD4$^+$ T cells express TIGIT after ionomycin treatment, indicating that additional factors are required for *TIGIT* gene transcription in particular subsets of memory CD4$^+$ T cells. Consistent with our previous studies[11], we show that TIGIT expression is strongly enriched on a subset of memory CD4$^+$ T cells with a CD38$^+$CD62L^{neg} phenotype. Future studies are needed to establish the role of

CD38, an ectoenzyme that increases intracellular Ca^{2+} concentrations, in the induction and maintenance of TIGIT expression.

Memory T cells have less stringent requirements for T-cell activation and exhibit enhanced functional properties compared to naive T cells.[40, 41] The significant difference in TIGIT expression between naive and memory T-cell subsets may therefore reflect the need to prevent unrestrained activation of memory T-cell responses. As TIGIT is expressed by a high percentage of intestinal $CD4^+$ T cells in both mice and human, TIGIT may have an important role in regulating immune responses in the antigen-rich environment of the intestine. In line with this idea, conventionalization of germ-free mice resulted in a two- to three-fold increase in intestinal TIGIT expression. We also demonstrate that circulating human $TIGIT^+CD4^+$ T cells display a preferential regulatory response to bacterial flagellin when compared to $TIGIT^{neg}CD4^+$ T cells, as evidenced by an increased IL-10-producing cells and undetectable expression of IL-17A. This is in agreement with our recently published human peripheral blood data, which show that TIGIT is positively correlated with IL-10 expression in circulating $CD38^+$ effector T cells ($CD4^+CD38^+CD62L^{neg}$), a population that is enriched for cells with mucosal antigen specificity.[21, 42] The association between TIGIT and IL-10 is specific for gut-homing $CD38^+$ effector T cells, as circulating $TIGIT^+CD38^{neg}$ effector T cells, that preferentially home to other organs such as the skin, do not have higher frequencies of $IL\text{-}10^+$ cells compared to $TIGIT^{neg}CD38^{neg}$ effector T cells.[11] The association between TIGIT and IL-10 has been observed before, as others have shown that knockdown of TIGIT expression in human $CD4^+$ T cells results in a significant reduction of IL-10 gene expression.[43] Moreover, TIGIT, but not Foxp3, was shown to be positively correlated with IL-10 expression by $CD4^+$ T cells in models investigating tolerance induction in auto-immunity.[44] Analysis of immune responses in TIGIT deficient mice at steady state and after induction of experimental colitis are needed to prove whether TIGIT maintains tolerance to harmless intestinal microbiota. In addition, detailed analysis of TIGIT function in different human disease settings, including IBD patients with altered $TIGIT^+$ cell frequencies, is needed to establish whether TIGIT is a target for immunomodulation.

In conclusion, our data demonstrates implicates a role for TIGIT in intestinal adaptive immune homeostasis. As TIGIT is highly and stably expressed on memory $CD4^+$ T cells, it may have the potential to modify or re-balance the immune system in chronic T-cell driven immune mediated inflammatory diseases, such as IBD.

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REFERENCES

1. Anderson AC, Joller N, Kuchroo VK. Lag-3, Tim-3, and TIGIT: Co-inhibitory Receptors with Specialized Functions in Immune Regulation. Immunity 2016;44:989-1004.
2. Boles KS, Vermi W, Facchetti F, et al. A novel molecular interaction for the adhesion of follicular CD4 T cells to follicular DC. Eur J Immunol 2009;39:695-703.
3. Yu X, Harden K, Gonzalez LC, et al. The surface protein TIGIT suppresses T cell activation by promoting the generation of mature immunoregulatory dendritic cells. Nat Immunol 2009;10:48-57.
4. Wang F, Hou H, Wu S, et al. TIGIT expression levels on human NK cells correlate with functional heterogeneity among healthy individuals. Eur J Immunol 2015;45:2886-97.
5. Joller N, Hafler JP, Brynedal B, et al. Cutting edge: TIGIT has T cell-intrinsic inhibitory functions. J Immunol 2011;186:1338-42.
6. Chauvin JM, Pagliano O, Fourcade J, et al. TIGIT and PD-1 impair tumor antigen-specific CD8(+) T cells in melanoma patients. J Clin Invest 2015;125:2046-58.
7. Bottino C, Castriconi R, Pende D, et al. Identification of PVR (CD155) and Nectin-2 (CD112) as cell surface ligands for the human DNAM-1 (CD226) activating molecule. J Exp Med 2003;198:557-67.
8. Stanietsky N, Simic H, Arapovic J, et al. The interaction of TIGIT with PVR and PVRL2 inhibits human NK cell cytotoxicity. Proc Natl Acad Sci U S A 2009;106:17858-63.
9. Liu S, Zhang H, Li M, et al. Recruitment of Grb2 and SHIP1 by the ITT-like motif of TIGIT suppresses granule polarization and cytotoxicity of NK cells. Cell Death Differ 2013;20:456-64.
10. Levin SD, Taft DW, Brandt CS, et al. Vstm3 is a member of the CD28 family and an important modulator of T-cell function. Eur J Immunol 2011;41:902-15.
11. Joosse ME, Menckeberg CL, de Ruiter LF, et al. Frequencies of circulating regulatory TIGIT(+)CD38(+) effector T cells correlate with the course of inflammatory bowel disease. Mucosal Immunol 2019;12:154-163.
12. Hashemi M, Zakeri Z, Eskandari-Nasab E, et al. CD226 rs763361 (Gly307Ser) polymorphism is associated with susceptibility to rheumatoid arthritis in Zahedan, southeast Iran. Iran Biomed J 2013;17:194-9.
13. Xi JS, Nie CL, Wang J, et al. Association of CD226 polymorphisms with the susceptibility to type 1 diabetes in Chinese children. Genet Mol Res 2015;14:15249-55.
14. Du Y, Shen LX, Yu LK, et al. The CD226 gene in susceptibility of rheumatoid arthritis in the Chinese Han population. Rheumatol Int 2012;32:1299-304.
15. Maiti AK, Kim-Howard X, Viswanathan P, et al. Non-synonymous variant (Gly307Ser) in CD226 is associated with susceptibility to multiple autoimmune diseases. Rheumatology (Oxford) 2010;49:1239-44.
16. Hafler JP, Maier LM, Cooper JD, et al. CD226 Gly307Ser association with multiple autoimmune diseases. Genes Immun 2009;10:5-10.
17. Reinards TH, Albers HM, Brinkman DM, et al. CD226 (DNAM-1) is associated with susceptibility to juvenile idiopathic arthritis. Ann Rheum Dis 2015;74:2193-8.
18. Wieczorek S, Hoffjan S, Chan A, et al. Novel association of the CD226 (DNAM-1) Gly307Ser polymorphism in Wegener's granulomatosis and confirmation for multiple sclerosis in German patients. Genes Immun 2009;10:591-5.
19. Jostins L, Ripke S, Weersma RK, et al. Host-microbe interactions have shaped the genetic architecture of inflammatory bowel disease. Nature 2012;491:119-24.
20. Sartor RB. Current concepts of the etiology and pathogenesis of ulcerative colitis and Crohn's disease. Gastroenterol Clin North Am 1995;24:475-507.
21. du Pre MF, van Berkel LA, Raki M, et al. 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;106:1147-59.
22. Wang FF, Wang Y, Wang L, et al. TIGIT expression levels on CD4+ T cells are correlated with disease severity in patients with psoriasis. Clin Exp Dermatol 2018;43:675-682.
23. Joller N, Lozano E, Burkett PR, et al. Treg cells expressing the coinhibitory molecule TIGIT selectively inhibit proinflammatory Th1 and Th17 cell responses. Immunity 2014;40:569-81.
24. Fuhrman CA, Yeh WI, Seay HR, et al. Divergent Phenotypes of Human Regulatory T Cells Expressing the Receptors TIGIT and CD226. J Immunol 2015;195:145-55.
25. Riquelme P, Haarer J, Kammler A, et al. TIGIT(+) iTregs elicited by human regulatory macrophages control T cell immunity. Nat Commun 2018;9:2858.

26. Chihara N, Madi A, Kondo T, et al. Induction and transcriptional regulation of the co-inhibitory gene module in T cells. Nature 2018;558:454-459.
27. Yan J, Zhao Q, Gabrusiewicz K, et al. FGL2 promotes tumor progression in the CNS by suppressing CD103(+) dendritic cell differentiation. Nat Commun 2019;10:448.
28. Croft M, Bradley LM, Swain SL. Naive versus memory CD4 T cell response to antigen. Memory cells are less dependent on accessory cell costimulation and can respond to many antigen-presenting cell types including resting B cells. J Immunol 1994;152:2675-85.
29. Chen CH, Seguin-Devaux C, Burke NA, et al. Transforming growth factor beta blocks Tec kinase phosphorylation, Ca2+ influx, and NFATc translocation causing inhibition of T cell differentiation. J Exp Med 2003;197:1689-99.
30. El Aidy S, van Baarlen P, Derrien M, et al. Temporal and spatial interplay of microbiota and intestinal mucosa drive establishment of immune homeostasis in conventionalized mice. Mucosal Immunol 2012;5:567-79.
31. Frentsch M, Arbach O, Kirchhoff D, et al. Direct access to CD4+ T cells specific for defined antigens according to CD154 expression. Nat Med 2005;11:1118-24.
32. Chattopadhyay PK, Yu J, Roederer M. A live-cell assay to detect antigen-specific CD4+ T cells with diverse cytokine profiles. Nat Med 2005;11:1113-7.
33. Hegazy AN, West NR, Stubbington MJT, et al. Circulating and Tissue-Resident CD4(+) T Cells With Reactivity to Intestinal Microbiota Are Abundant in Healthy Individuals and Function Is Altered During Inflammation. Gastroenterology 2017;153:1320-1337 e16.
34. Rogers PR, Dubey C, Swain SL. Qualitative changes accompany memory T cell generation: faster, more effective responses at lower doses of antigen. J Immunol 2000;164:2338-46.
35. Bevington SL, Cauchy P, Piper J, et al. Inducible chromatin priming is associated with the establishment of immunological memory in T cells. EMBO J 2016;35:515-35.
36. Zhang Y, Maksimovic J, Naselli G, et al. Genome-wide DNA methylation analysis identifies hypomethylated genes regulated by Foxp3 in human regulatory T cells. Blood 2013;122:2823-36.
37. Martinez GJ, Pereira RM, Aijo T, et al. The transcription factor NFAT promotes exhaustion of activated CD8(+) T cells. Immunity 2015;42:265-278.
38. Kamphorst AO, Wieland A, Nasti T, et al. Rescue of exhausted CD8 T cells by PD-1-targeted therapies is CD28-dependent. Science 2017;355:1423-1427.
39. Brignall R, Cauchy P, Bevington SL, et al. Integration of Kinase and Calcium Signaling at the Level of Chromatin Underlies Inducible Gene Activation in T Cells. J Immunol 2017;199:2652-2667.
40. Boesteanu AC, Katsikis PD. Memory T cells need CD28 costimulation to remember. Semin Immunol 2009;21:69-77.
41. Berard M, Tough DF. Qualitative differences between naive and memory T cells. Immunology 2002;106:127-38.
42. van Leeuwen MA, du Pre MF, van Wanrooij RL, et al. Changes in natural Foxp3(+)Treg but not mucosally-imprinted CD62L(neg)CD38(+)Foxp3(+)Treg in the circulation of celiac disease patients. PLoS One 2013;8:e68432.
43. Lozano E, Dominguez-Villar M, Kuchroo V, et al. The TIGIT/CD226 axis regulates human T cell function. J Immunol 2012;188:3869-75.
44. Burton BR, Britton GJ, Fang H, et al. Sequential transcriptional changes dictate safe and effective antigen-specific immunotherapy. Nat Commun 2014;5:4741.

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SUPPLEMENTARY DATA

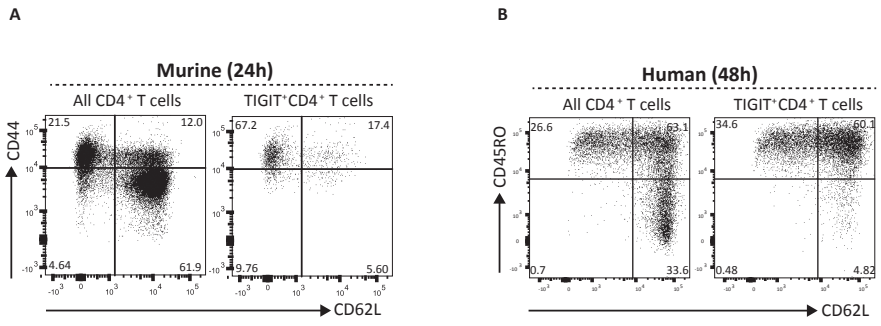


Figure S1. The majority of circulating TIGIT⁺CD4⁺ cells have a memory phenotype in both mice and humans. (A) CD44 and CD62L expression on total murine CD4⁺ T cells and TIGIT⁺CD4⁺ T cells after 24h of culture. **(B)** Human TIGIT^{neg}CD4⁺ T cells were isolated from peripheral blood of adult healthy individuals and cultured in triplicate for 48h with different concentrations of plate-coated anti-CD3 (0.5, 1 and 5 μg/ml). CD45RO and CD62L expression on total human CD4⁺ T cells and TIGIT⁺CD4⁺ T cells after 48h of culture with plate-coated anti-CD3 (1 μg/ml). Representative of 2 independent experiments.

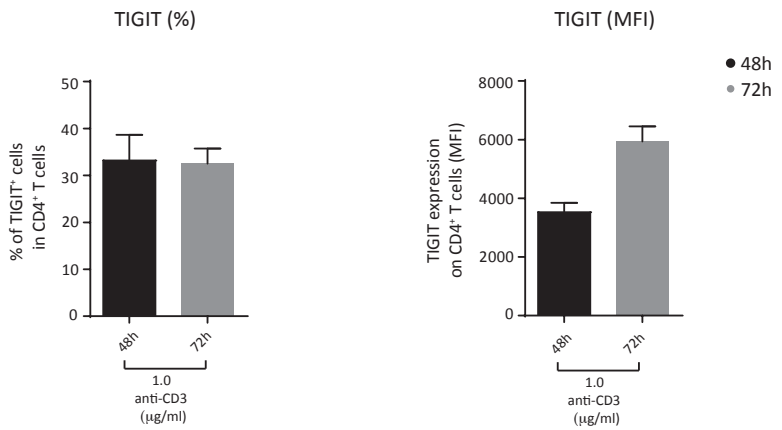
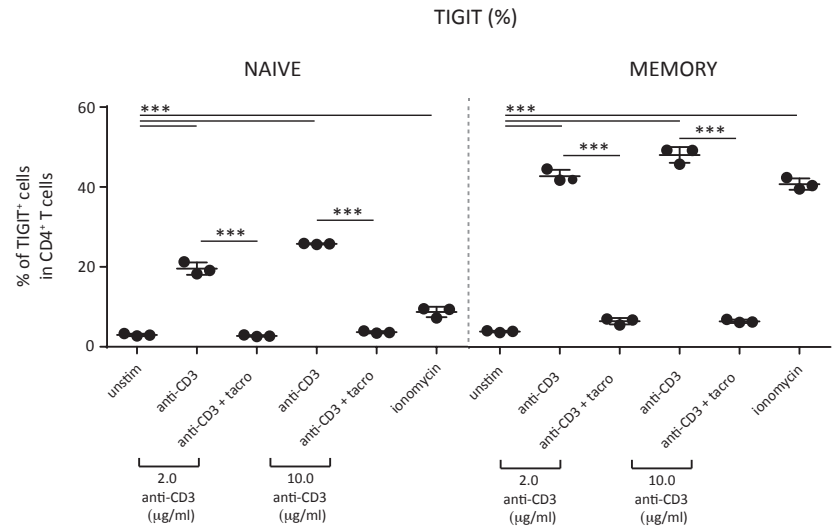


Figure S2. The mean fluorescence intensity, but not frequencies, of TIGIT⁺ cells increases from 48 to 72 hours. Human TIGIT^{neg}CD4⁺ T cells were isolated from peripheral blood of adult healthy individuals and cultured in triplicate for 48h with plate-coated anti-CD3 (1 μg/ml). Representative dot-plots of the percentage of TIGIT⁺ cells after 48h of culture (mean + SD, two independent donors).



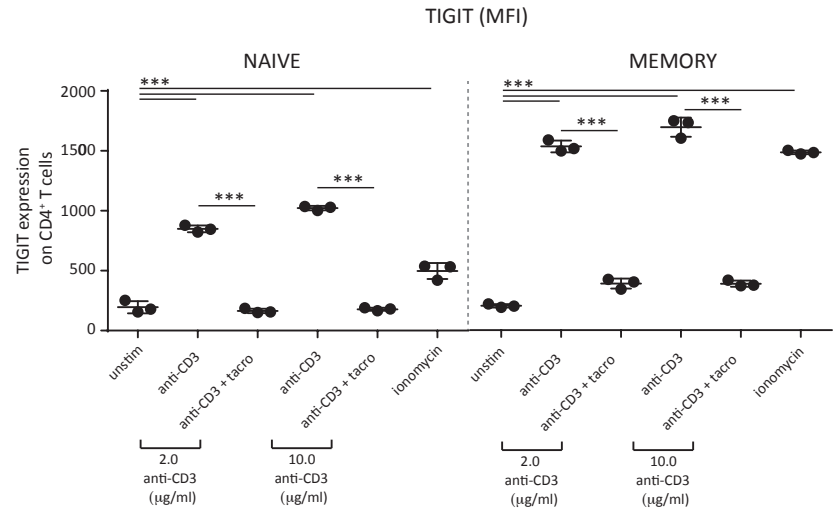


Figure S3. TCR ligation induces TIGIT expression preferentially on murine memory CD4⁺ T cells. Murine naive (CD62L⁺) T cells and memory (CD62L^{neg}) TIGIT^{neg}CD4⁺ T cells were isolated from spleens and lymph nodes of mice on a BABL/c background and cultured in triplicate for 72h with different concentrations of plate-coated anti-CD3 (2 and 10 μg/ml) with or without tacrolimus (5 ng/ml) and ionomycin (500 ng/ml). Frequency and mean fluorescent intensity (MFI) of TIGIT⁺ cells are shown. P values were calculated using One-way ANOVA followed by correction for multiple testing. NS, not significant, *p<0.05, **p<0.01, ***p<0.001.

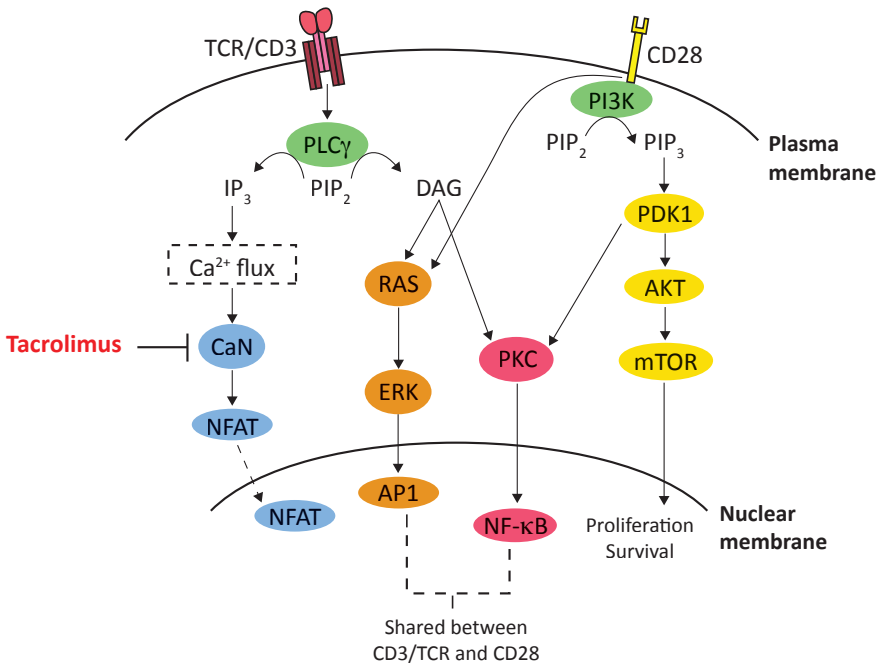


Figure S4. T-cell receptor and CD28 signaling pathways. TCR ligation transmits a signal through CD3 which activates phospholipase C gamma (PLCγ). PLCγ converts phosphatidylinositol 4,5-biphosphate (PIP2) to inositol 1,4,5-triphsphate (IP3) and diacylglycerol (DAG). IP3 then initiates the Ca^{2+} dependent signaling pathway that ends with translocation of transcription factor nuclear factor of activated T cells (NFAT) to the nucleus. Ca^{2+} dependent signaling can be mimicked through a calcium ionophore such as ionomycin, or inhibited with a calcineurin (CaN) inhibitor such as tacrolimus. DAG initiates the RAS-ERK-AP1 and PKC-NF-κB kinase signaling pathways. CD28 stimulation activates PI3K, which converts PIP2 into PIP3, initiating the PDK1-AKT-mTOR signaling pathway. CD28 stimulation also activates RAS-ERK-AP1 and DAG-NF-κB kinase signaling in a DAG-independent fashion. Based on references from: Kapturczak 2004 Transplant Proc., Smith-Garvin 2009 Annu Rev Immunol., Gaud 2018 Nat Rev Immunol, Diehn 2002 Proc Natl Acad Sci U S A., Esensten 2016 Immunity.

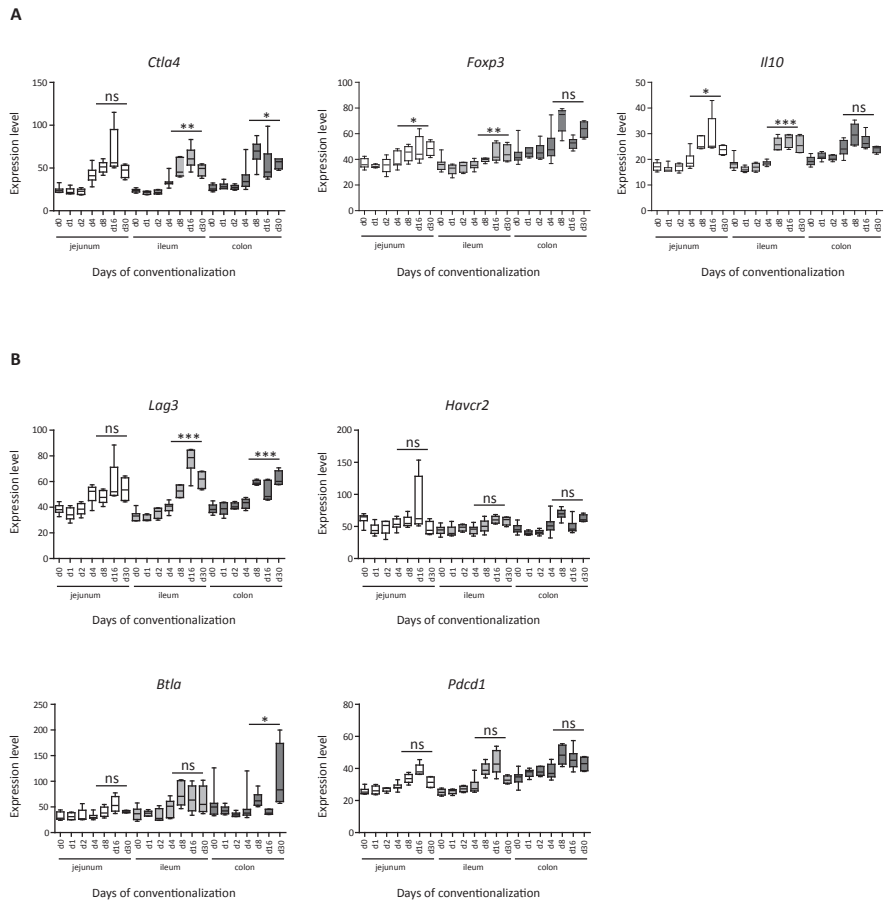


Figure S5. Jejunum, ileum, and colon gene expression levels were analyzed in germ free and conventionalized mice at indicated days post conventionalization. Values are depicted as box and whisker diagram (top-to-bottom; maximum value, upper quartile, median, lower quartile, and minimal value, respectively). **(A)** Gene expression for tolerance-associated genes *Ctla4, Foxp3* and *Il10* are shown. **(B)** Gene expression for coinhibitory receptors *Lag3, Btla, Pdcd1* and *Havcr2* are shown. Differences in expression between day 30 and day 4 post conventionalization were calculated using a Student's t Test. NS, not significant, *p<0.05, **p<0.01, ***p<0.001.

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5

CHARACTERIZATION OF CD4+ T-CELL RESPONSES TO BACTERIAL FLAGELLIN IN TREATMENT-NAIVE CROHN'S DISEASE PATIENTS WITH HIGH ANTI-MICROBIAL IgG TITERS

Chapter 5

Manuscript in preparation.

5

CHARACTERIZATION OF CD4⁺ T-CELL RESPONSES TO BACTERIAL FLAGELLIN IN TREATMENT-NAIVE CROHN'S DISEASE PATIENTS WITH HIGH ANTI-MICROBIAL IgG TITERS

ABSTRACT

In the intestine, a balance between regulatory and inflammatory CD4⁺ T-cell responses shapes the host's mutualism with its commensal microbiota. In Crohn's disease (CD) this microbial-host mutualism is lost leading to a dominant inflammatory T-cell response and tissue damage. However, CD4⁺ T-cell responses to intestinal bacteria in CD patients are poorly characterized. Humoral responses to bacterial flagellin are present in many individuals but particularly enhanced in a subgroup of CD patients. As antibody responses rely on the activation of CD4⁺ T cells, we hypothesized that CD patients with elevated anti-flagellin antibody titers have increased frequencies of flagellin-reactive CD4⁺ T cells. Moreover, we anticipated that these cells have a more dominant inflammatory phenotype compared to flagellin-reactive cells from healthy individuals and CD patients with low anti-flagellin antibody titers. Elevated IgG plasma antibodies to 8/8 flagellins were detected in 10 out of 47 pediatric CD patients, but not in ulcerative colitis (UC) patients and healthy controls. Short-term culture of PBMC from treatment naive patients having elevated anti-flagellin IgG revealed that these patients have increased frequencies of flagellin-reactive CD4⁺ T cells when compared to CD patients without elevated anti-flagellin IgG, UC patients and healthy controls. Preliminary phenotypic analysis suggests that frequencies of $\alpha 4\beta 7$ or CD38 expressing cells do not differ in flagellin-reactive versus non-reactive CD4⁺ T cells. However, patients with elevated anti-flagellin IgG had reduced frequencies of IL-10 secreting cells and cells expressing T cell immunoreceptor with Ig and ITIM domains (TIGIT) within the flagellin-reactive CD4⁺ T-cell population when compared to flagellin-reactive cells from healthy controls. In keeping with previous findings, TIGIT⁺ flagellin-reactive CD4⁺ T cells were enriched in IL-10 secreting cells when compared to TIGIT^{neg} flagellin-reactive CD4⁺ T cells. These data argue that flagellin-reactive CD4⁺ T cells from CD patients with elevated IgG plasma antibodies may have altered functional characteristics with reduced regulatory features.

INTRODUCTION

The gastrointestinal tract is the primary site of interactions between the host and microbiota.[1] Balanced inflammatory and regulatory intestinal T-cell responses to commensal microbiota are required to develop a healthy microbial-host mutualism.[2] The continuous communication between the microbiota and immune cells in the intestine is reflected by circulating microbiota reactive memory CD4⁺ T cells in peripheral blood[3] and the presence of antibody responses to commensal intestinal bacteria in healthy individuals.[4] In chronic intestinal inflammation as occurs in inflammatory bowel disease (IBD), inflammatory CD4⁺ effector T cells with a specificity for microbial antigens are thought to drive disease.[2, 5, 6] However, the microbial antigens recognized by these pathogenic CD4⁺ T cells and their phenotypic characteristics remain unknown.

Patients with IBD have variable serologically responses to microbial antigens, including bacterial flagellin.[7-10] Flagellin is a structural protein of the flagellum, a surface filament required for bacterial motility that is present on most motile bacteria in the intestine. Antibodies to CBir1 flagellin are elevated in approximately half of all Crohn's disease (CD) patients compared to healthy individuals and ulcerative colitis (UC) patients, and identify a subgroup of CD patients with a more rapid and aggressive disease phenotype.[9, 11-14] The elevated anti-flagellin IgG response in CD may reflect increased activation of flagellin-specific CD4⁺ T cells, as increased T-cell proliferation to recombinant flagellin has been detected in CD patients when compared to UC patients and healthy controls.[8, 15, 16] Moreover, murine studies using $\alpha\beta$ T-cell deficient mice have shown that generation of flagellin-specific Ig is T-cell dependent.[16] Innate immune activation contributes to this T-cell response through the Toll-like receptor 5 (TLR5)-MyD88 pathway.[16-18] Conversely, flagellin from *Helicobacter pylori* and *Campylobacter jejuni* which cannot activate TLR5 signaling does not elicit a flagellin-specific Ig response.[16, 17] Together, these data argue that studying the frequency and phenotype of flagellin-reactive CD4⁺ T cells in CD patients with elevated anti-flagellin IgG may provide insight into the mechanisms that disrupt intestinal tolerance to microbiota.

We hypothesize that CD patients with elevated anti-flagellin IgG antibodies have increased frequencies of flagellin-reactive CD4⁺ T cells with a more inflammatory phenotype compared to healthy individuals. In this study, we determined whether the presence of elevated anti-flagellin IgG can be used as a proxy for anti-flagellin CD4⁺ T-cell reactivity in therapy-naive pediatric CD patients. By using CD154 upregulation after flagellin stimulation to determine frequencies of flagellin-reactive CD4⁺ T cells in peripheral blood, we show that treatment-naive CD patients with elevated plasma IgG to flagellin have increased frequencies of flagellin-reactive CD4⁺ T cells when compared to healthy controls. These increased frequencies were not observed in CD patients without elevated

anti-flagellin antibodies or therapy-naive UC patients. Preliminary phenotypic analyses suggest that flagellin-reactive CD4⁺ T cells from CD patients with elevated anti-flagellin IgG may have altered functional characteristics with reduced regulatory features compared to flagellin-reactive CD4⁺ T cells of healthy controls.

METHODS

Patients.

Two cohorts of patients with biopsy-proven IBD were investigated. Cohort I consisted of pediatric UC patients (n=43) and pediatric CD patients (n=47). Age-matched controls without any inflammatory or intestinal disease who underwent orthopedic surgery at the time of blood withdrawal (n=10) were included in the control group. Peripheral blood was also obtained from adult healthy controls (n=10). The majority of both UC and CD patients in cohort I was treatment-naive at time of plasma and PBMC analysis (Table 1). Cohort II consisted of patients with new-onset, untreated CD with moderate-to-severe disease activity randomized to top-down (n=3) or step-up treatment (n=3). Top-down treatment consisted of 5 infliximab (IFX) infusions combined with azathioprine (AZA), and AZA as maintenance treatment. Patients randomized to step-up received standard induction treatment, either oral prednisolone or exclusive enteral nutrition, combined with AZA as maintenance treatment. The Medical Ethical Committee of the Erasmus University Medical Centre-Sophia Children's Hospital Rotterdam approved this study (METC 2007-335; cohort I) and (METC 2012-345; cohort II). Written informed consent was obtained from every patient and parents before study inclusion.

Plasma and PBMC collection.

Venous blood was collected in EDTA tubes. After centrifugation at 1000 rpm for 10 min at room temperature, plasma was obtained and stored at -80 °C. PBMCs were isolated using a Ficoll-Hypaque gradient according to standard protocol (Axis-Shield).

Microbiota antigen microarray.

Recombinant antigens and whole cellular lysates were diluted in 10 mM Tris buffer (pH 8.0) with 0.1% SDS and 20% glycerol at a concentration of 0.1-0.2 µg/mL. The proteins were printed onto FAST 16 nitrocellulose pad slides (Whatman, GE Healthcare Life Sciences, Pittsburgh, PA, USA) using a Micro-Grid II robot (Genomic Solutions, Ann Arbor, MI, USA) in duplicate in 2 different parts of the pad. The printed slides were allowed to air-dry overnight. Slides were blocked (Protein Array Blocking Buffer, Whatman), probed with human plasma at 1:100 dilution (SuperBlock, ThermoFisher Scientific, Bleiswijk, the

Netherlands), washed, and incubated with Alexa Fluor 647- or Alexa Fluor 546-labeled goat anti-human IgG or IgA (Kirkegaard & Perry Labs, Gaithersburg, MD, USA). The antigens and lysates included in the microarray are listed in Table S1.

The slides were read in an Axon GenePix 4000B dual laser microarray reader (Molecular Devices, Sunnyvale, CA, USA). The accompanying GenePix Pro 6.0 software determines the net median pixel intensities for each protein spot from a set of 10 measurements per spot. The instrument and software automatically subtract the pixel intensities of the background area surrounding the spot. The median value from 4 replicate protein spots was used to calculate the median net digital fluorescence unit value for each spot.

Detection of flagellin-reactive CD4⁺ T cells by Carboxy-Fluorescein Succinimidyl Ester (CFSE) dilution.

Flagellin proteins were isolated from commensal *(Lachnospiraceae bacterium A4)* and pathogenic bacteria (*Escherichia coli, Salmonella thyphimurium*) with a flagellin sequence with a strong homology to CBir1. The selected sequences were cloned, expressed in a vector and purified using GST-tag and his-tag purification in non-denaturing conditions. The selected sequences all contained the TLR5 binding domain and effectively induced TLR5 signaling and no TLR1/2 or TLR2/6 signaling. The flagellin isolated from *Salmonella thyphimurium* was the only flagellin that induced TLR4 signalling.

CFSE-labeled PBMCs ($2.5x10^5$) were cultured with different recombinant flagellins (50 µg/ml). Stimulations were performed in Iscove's modified Dulbecco's medium (Lifetechnologies, Grand Island, NY, USA) with heat inactivated fetal calf serum, Glutamax (Life Technologies), 2-mercaptoethanol, penicillin and streptomycin, supplemented with exogenous IL-2 (50 IU/ml; R&D). After 7 days, cells were stimulated for 4.5 hours with phorbol 12-myristate 13-acetate (PMA, 0.02 µg/ml, Sigma-Aldrich) and ionomycin (0.5 µg/ml, Sigma-Aldrich) in the presence of Brefeldin A (3 µg/ml eBiosciences) for the last 3.5 hours. After incubation with antibodies directed to surface proteins, cells were fixed in 2% formaldehyde and permeabilized with saponin (Sigma-Aldrich), and labeled with antibodies to IFNγ (4S.B3, BD) and the appropriate isotype controls (eBiosciences). Flow cytometric analysis was performed on a FACSCanto™II (BD Biosciences).

CD154 based detection of flagellin-reactive CD4⁺T cells.

A total of $3x10^6$ PBMCs were plated at $6x10^6$/ml and rested overnight in a 48-wells plate. The next day, cells were transferred to a 24-wells plate and stimulated for 7 hours with a mix of recombinant flagellins (A4-FlaX, A4-Fla2, A4-Fla3 and 14-2; total concentration of 10 µg/ml) in the presence of 1 µg/ml CD40 (HB14; Miltenyi Biotec) and 1 µg/ml CD28 functional grade pure Ab (CD28.6; Miltenyi Biotec). Anti-CD40 prevents CD154

downregulation and anti-CD28 optimizes the induction of CD 154 expression. Brefeldin A (3 µg/ml eBiosciences) was added during the last 2 hours of the stimulation. Stimulations were performed in Iscove's modified Dulbecco's medium (Lifetechnologies, Grand Island, NY, USA) supplemented with 5% human AB serum (SLBS8634; Sigma), Glutamax (Life Technologies), 2-mercaptoethanol, penicillin and streptomycin.

After stimulation, cells were stained for flow cytometry using monoclonal antibodies against CD3 (UCHT1, BD, or HIT3a, Biolegend), CD4 (SK3, BD), CD8 (SK1, BD), CD14 (M5E2, BD), CD20 (2H7, Biolegend), TIGIT (MBSA43, eBioscience), CD154 (24-31, Biolegend), IL-10 (JES2-19F1, BD), IL-17A (eBio64DEC17, eBiosciences), IL-21 (3A3-N2, Biolegend), IFNγ (4S. B3, BD), TNFα (Mab 11, Biolegend) or appropriate isotype controls (eBiosciences). Flow cytometric analyses were performed on a FACS LSR II (BD Biosciences). For phenotypic analyses of flagellin-reactive T cells, a minimum of 100 cytokine-positive $CD4^+CD154^+$ T cells was used; individuals with lower events were excluded from the phenotypic analysis.

Statistics.

Statistical analysis of data was performed using GraphPad Prism software (GraphPad Software, La Jolla, CA, USA) and IBM SPSS version 21. Significance between two groups was determined using Student's t-test or Mann-Whitney U-test and differences between multiple groups by using the Kruskal-Wallis H test. For categorical outcomes the Chi square test was used. P values <0.05 were considered statistically significant.

RESULTS

Plasma IgG antibodies to flagellin antigens are common in pediatric CD.

To investigate the humoral immune response to the intestinal commensal microbiota, we used a protein microarray containing recombinant protein antigens cloned from murine microbiota, including 8 recombinant flagellin antigens from the *Firmicutes* phylum (Table S1), one of the two major phyla found in the human gastrointestinal tract.[19] Plasma from pediatric CD and UC patients, as well as age-matched healthy controls was tested for IgG and IgA reactivity against this panel. The majority of the CD and UC patients tested were treatment-naive at the time of plasma isolation (57% and 67%, respectively; medication use is summarized in Table 1).

Regarding IgG reactivity, approximately half of CD patients (49%) had elevated IgG reactivity to at least one flagellin (Table 1). Elevated flagellin-specific IgG was significantly more common in CD patients compared to UC patients (Table 1, p=0.008; Fig. 1A). The proportion of patients with elevated flagellin-specific IgG reactivity was similar between treatment-naive and treatment-exposed patient groups (p=0.337 for CD; p=0.624 for

Table 1. Flagellin-specific IgG antibodies are common in pediatric CD.

	CD (n=47)	UC (n=43)	p value
Patient characteristics			
Sex (% male)			
Median age in years (IQR)†	14.3 (12-17)	13.3 (11-16)	NS
Median age in years (IQR)†	20/47 (43)	14/43 (33)	NS
Treatment-exposed (%)	1/20	4/14	-
Steroids	6/20	5/14	-
Azathioprine	11/20	2/14	-
Biologicals	2/20		-
Other			
Plasma IgG reactivity to flagellin, no. patients (%)*			
Total cohort	23/47 (49)	9/43 (21)	**0.008**
Treatment-naive	12/27 (44)	6/29 (21)	NS
Treatment-exposed	11/20 (55)	3/14 (21)	NS
IgG response to type of flagellin antigen, no. patients (%)*			
3-1-57	12/47 (26)	5/43 (12)	NS
14-2	15/47 (32)	1/43 (2)	**<0.0001**
CBir1	16/47 (34)	1/43 (2)	**<0.0001**
CBir11	16/47 (34)	3/43 (7)	**0.002**
CBir66	12/47 (26)	1/43 (2)	**0.002**
Fla2	11/47 (23)	1/43 (2)	**0.015**
Fla3	13/47 (28)	2/43 (5)	**0.004**
FlaX	11/47 (23)	1/43 (2)	**0.004**
IgG response to number of flagellin antigens, no. patients (%)*			
8 out of 8	10/47 (21)	0/43 (0)	**0.001**
7 out of 8	1/47 (2)	0/43 (0)	NS
6 out of 8	0/47 (0)	0/43 (0)	NS
5 out of 8	0/47 (0)	1/43 (2)	NS
4 out of 8	0/47 (0)	0/43 (0)	NS
3 out of 8	3/47 (6)	0/43 (0)	NS
2 out of 8	1/47 (2)	2/43 (5)	NS
1 out of 8	8/47 (17)	6/43 (14)	NS
0 out of 8	24/47 (51)	34/43 (79)	**0.008**

† Median in age-matched healthy control group was 11.7 years (8-14).

* Positive reactivity was defined as a value higher than 'mean + 2x SD' of the age-matched healthy control group. Mann-Whitney test (continuous variables) or Fisher's exact test (categorical variables) were used to test differences between 2 groups. Abbreviations: CD, Crohn's disease; UC, ulcerative colitis; No, number of; NS, not significant; IQR, interquartile range.

UC), which is in line with previous findings by others showing that antibody responses to flagellin do not change after therapy or surgery.[7, 9, 20] Frequencies of CD patients with elevated IgG reactivity for individual flagellins ranged from 23% to 34% (Table 1). Anti-flagellin antibody-positive CD patients often responded to multiple flagellins and strong positive correlations between IgG responses to these multiple flagellin antigens were observed (Fig. 1B-1C). Interestingly, eight patients had an elevated response to only one

flagellin; the average fluorescence signal intensity in these patients was often much lower compared to patients responding to multiple flagellins. In contrast to the differential IgG anti-flagellin reactivity between CD and UC, plasma IgA reactivity to bacterial flagellins was not significantly increased in CD patients compared to UC patients and age-matched healthy controls (Fig. 2).

Altogether, IgG antibodies to different recombinant flagellin antigens are common in pediatric CD patients, occur in similar frequencies between treatment-naive versus treatment-exposed patients, and strongly correlate to each other.

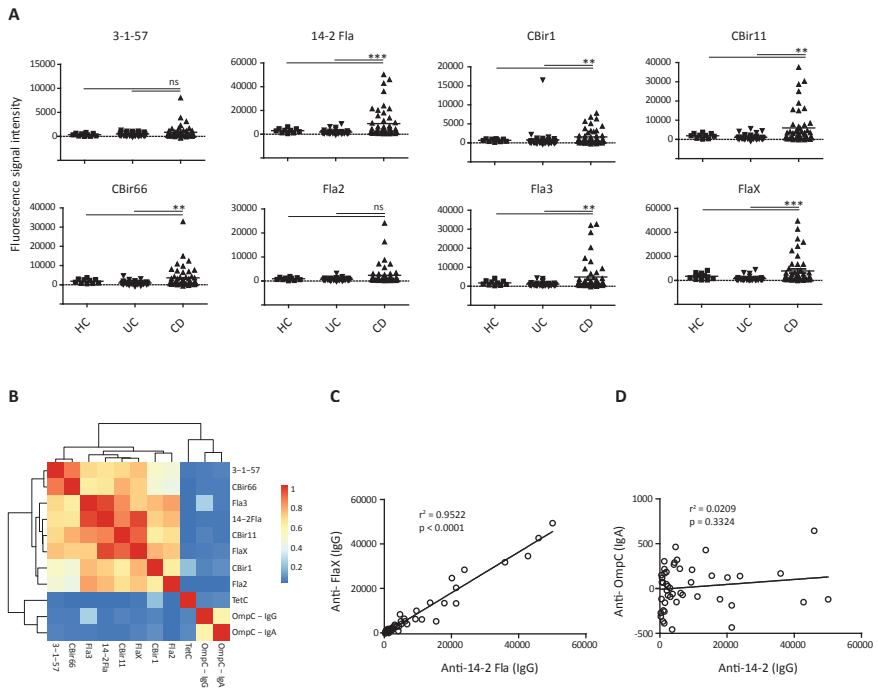

Figure 1. Plasma IgG antibodies to flagellin antigens are common in pediatric CD patients and are independent of other antimicrobial immune responses. Plasma was collected from children with IBD (UC, n=43, CD, n=47) and age-matched healthy controls (n=10). **(A)** The plasma IgG response to a panel of recombinant bacterial flagellin antigens was assessed using a protein microarray. P values were calculated using a Kruskall-Wallis test and corrected for multiple testing by Dunn's multiple comparisons test. **(B)** Heatmap representing the correlation coefficient for linear fits of flagellin IgG antibodies and IgG antibodies to OmpC and Tetanus C antigen in CD patients. Positive correlations are in red color; blue color depict no significant correlation. The numerical correlation coefficients can be found in Table S2. **(C)** The relationship between IgG anti-FlaX antibodies and IgG anti-14-2 Fla antibodies in CD patients. Correlation coefficient for linear fits are shown. **(D)** The relationship between IgA anti-OmpC antibodies and IgG anti-14-2 Fla antibodies in CD patients. Correlation coefficient for linear fits are shown. Abbreviations: NS, not significant; OmpC, *Escherichia coli* outer membrane protein C; TetC, Tetanus toxoid from *Clostridium tetani.*

CHARACTERIZATION OF CD4⁺ T-CELL RESPONSES TO BACTERIAL FLAGELLIN IN TREATMENT-NAIVE CROHN'S DISEASE PATIENTS WITH HIGH ANTI-MICROBIAL IgG TITERS

Reactivity to flagellin does not correlate to other humoral antimicrobial immune responses.

We next investigated the relationship between the presence of anti-flagellin antibodies and antibody responses to *Escherichia coli* outer membrane protein C (OmpC). IgA and IgG reactivity to OmpC was present in 6% (95% confidence interval (CI) 2-17) and 12% (95%CI 6-25) of CD patients, respectively. IgA and IgG responses to OmpC positively correlated (r^2=0.5620, p<0.0001; Figure 1B). There was no relationship between the IgA and IgG response to OmpC and any of the tested recombinant flagellin antigens (Fig. 1D). Moreover, there were no significant positive correlations between IgG responses to flagellins and any of the tested bacterial lysates (data not shown). These data indicate that plasma reactivity to flagellin does not correlate with serological responses to the previously defined bacterial antigen OmpC.

Increased frequencies of flagellin-reactive CD4⁺ T cells in therapy-naive CD patients with elevated anti-flagellin antibodies.

As IgG responses to peptide antigens require CD4⁺ T cells to stimulate isotype switching, the elevated IgG levels of to flagellin in CD may correlate with the presence of a flagellin-specific CD4⁺ T-cell response. Although flagellin-specific T-cell reactivity has been detected in peripheral blood of CD patients[8], flagellin-specific T cells are poorly characterized in patients with CD. The primary reason for this is the low frequency of these cells within the total CD4⁺ T-cell population in peripheral blood. Therefore, we first determined whether flagellin reactive CD4⁺ T cells could be observed using several complimentary culture techniques. Indeed, confirming previously published data, dividing flagellin-reactive

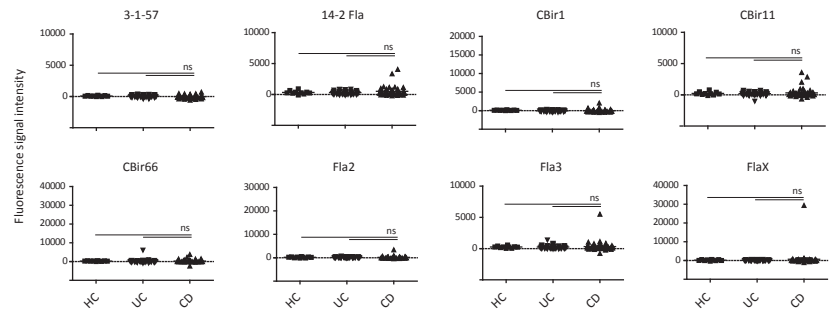

Figure 2. Plasma IgA reactivity to bacterial flagellins is not significantly increased in CD patients compared to UC patients. Plasma was collected from children with IBD (UC, n=43, CD, n=47) and age-matched healthy controls (n=10). The plasma IgA response to a panel of recombinant bacterial flagellin antigens was assessed using a protein microarray. P values were calculated using a Kruskall-Wallis test and corrected for multiple testing by Dunn's multiple comparisons test.

CD4⁺ T cells were detectable in PBMC from a seropositive CD patient after stimulation with recombinant flagellin for 7 days in the presence of exogenous IL-2 (Fig. 3A-C). As we have previously demonstrated that CD4⁺CD62L^{neg}CD38⁺ T cells (CD38⁺ effector T cells)

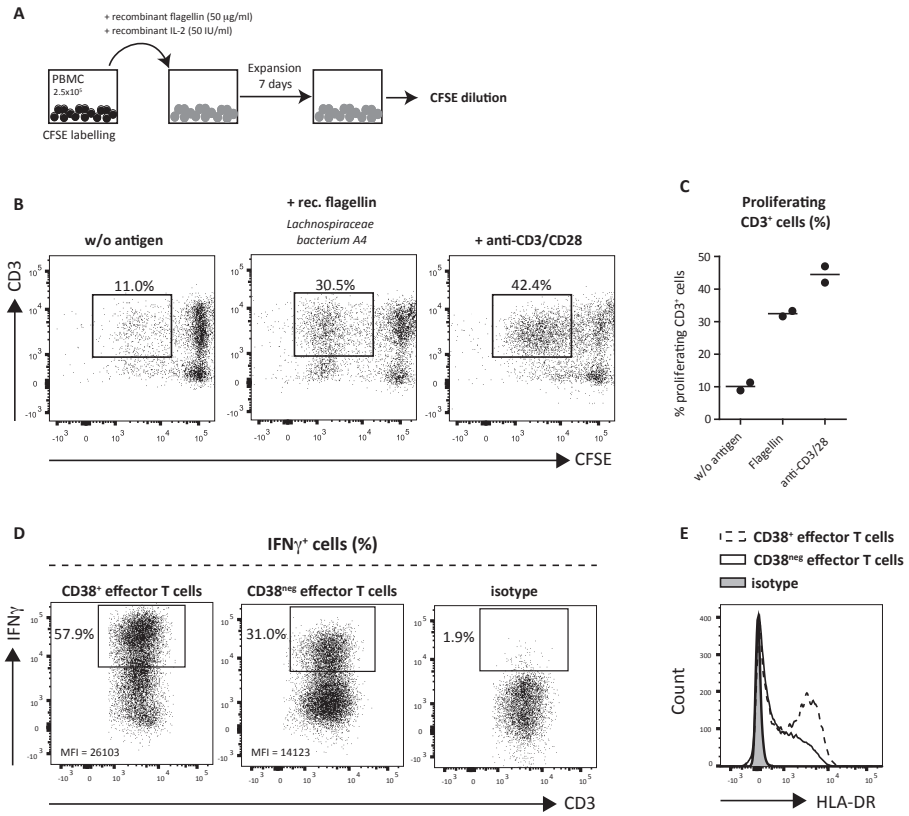

Figure 3. Flagellin-reactive CD4⁺ T-cell responses can be detected in peripheral blood and are present in both CD38⁺ and CD38^{neg} effector T-cell populations. (A-C) CFSE-labeled PBMCs ($2.5x10^5$) from a a treatment-naive seropositive CD patient were cultured in duplicate without antigen, with recombinant flagellin of *Lachnospiraceae bacterium A4* (50 µg/ml) or with anti-CD3/CD28 activator beads (T-to-bead ratio = 10:1). All cultures were supplemented with exogenous IL-2 (50 IU/ml). CD3⁺ T-cell responses were analyzed after 7 days. **(A)** Experimental setup. **(B)** Dot plots depicting the percentage of proliferating (CFSE^{low}) CD3⁺ T cells in a treatment-naive CD patient seropositive for flagellin-specific IgG. **(C)** Percentage proliferating CD3⁺ T cells after 7 days of culture. **(D-E)** Adult healthy control PBMC were separated into CD38⁺ and CD38^{neg} effector T cells (CD62L^{neg}CD4⁺) with flow cytometric sorting. Purified CD38⁺ and CD38^{neg} effector T cells were cultured with recombinant flagellin of *Lachnospiraceae bacterium A4* (50 µg/ml) in the presence of exogenous IL-2 (50 IU/mL). Intracellular IFNγ and HLD-DR expression were analyzed after 7 days. **(D)** Frequencies and MFI of IFNγ⁺ cells in CD38⁺ and CD38^{neg} effector T cells after 7 days of culture. **(E)** HLA-DR expression on CD38⁺ and CD38^{neg} effector T cells after 7 days of culture with recombinant flagellin.

in peripheral blood are enriched in α4β7 and reactivity to luminal antigen, we assessed whether flagellin reactivity is enriched in CD38⁺ effector T cells when compared to CD4⁺CD62L^{neg}CD38^{neg} T cells (CD38^{neg} effector T cells). Thereto, purified CD38⁺ and CD38^{neg} effector T cells from an adult healthy individual were cultured for 7 days with recombinant flagellin in the presence of exogenous IL-2 after which IFNγ secreting cells and HLA-DR expression were determined by flow cytometry (Fig. 3D-E). Again, flagellin-reactive CD4⁺ T-cell responses were detected in peripheral blood cells. Both IFNγ and HLA-DR expressing cells were most frequent in the CD38⁺ effector T-cell population but were also detectable in CD38^{neg} effector T cells. Together, using different methods we corroborate previous data that flagellin reactive T cells can be detected in peripheral blood. As flagellin-reactivity is not limited to CD38⁺ effector T cells only, we chose to further study these cells using short term stimulation of total PBMC without prior cell purification.

Thereto, the frequency and phenotype of flagellin-reactive CD4⁺ T cells in the total PBMC of CD and UC patients and healthy controls was determined using CD154 expression after short term stimulation with a mix of flagellin proteins (Fig. 4A; see Table S2 for information on patient selection). CD154 (also known as CD40L) is specifically expressed by all antigen-activated CD4⁺ T cells within 7h after T-cell receptor (TCR) stimulation.[21, 22] Stimulation with a mix of flagellin proteins, containing the recombinant flagellins 14-2, Fla-2, Fla3, and FlaX, induced detectable numbers of CD4⁺CD154⁺ T cells (Fig. 4B) but did not elicit CD154 upregulation in CD8⁺ T cells (Fig. S1). We next calculated the frequency of flagellin-reactive CD4⁺ T cells among the total CD4⁺ T-cell population (Fig. 4C; see Table S3 for method of calculation). Treatment-naive CD patients with elevated anti-flagellin IgG antibodies ("highly seroreactive" i.e. defined as having IgG reactivity to 8/8 flagellins) had significantly higher frequencies of flagellin-reactive CD154⁺ cells within the CD4⁺ T cell population when compared to healthy controls (Fig. 4C). No increased frequencies of CD154⁺ cells within the CD4⁺ T cell population were detected in treatment-naive CD and UC patients with low levels of IgG antibodies to flagellin. Frequencies of α4β7⁺ and CD38⁺ cells were not different in flagellin-reactive and flagellin-non reactive cells and varied equally in diseased and healthy individuals (Fig. 4D).

To assess possible changes in functional composition of the flagellin-reactive CD4⁺ T-cell population in CD patients, CD154 detection was combined with intracellular cytokine analysis (Fig. 5A). Frequencies of IL-10-expressing cells were decreased in flagellin-reactive CD4⁺CD154⁺ T cells of treatment-naive seropositive CD patients when compared to seronegative CD and UC patients and healthy controls (Fig. 5B). This decrease was specific for flagellin-reactive CD4⁺ T cells, as no differences in IL-10⁺ cell frequencies were observed in CD4⁺CD154^{neg} T cells (Fig. 5B). This decrease in CD154⁺IL-10⁺ cell frequencies in seropositive CD patients did not result from a generalized inability to produce IL-10, as CD154⁺IL-10⁺ cell frequencies were not decreased after polyclonal T-cell activation via anti-CD3/CD28

(data not shown). Moreover, TIGIT$^+$ cell frequencies in flagellin-reactive CD4$^+$CD154$^+$ T cells were decreased in seropositive CD patients compared to healthy controls, although some variation was also observed in CD4$^+$CD154^{neg} T cells (Fig. 5C). Interestingly, IL-17A$^+$ cell frequencies and TNFα^+ cell frequencies appeared higher in treatment-naive seronegative CD patients compared to healthy controls (Fig. 5D, 5F). These increases in IL-17A$^+$ cell and TNFα^+ cell frequency were not observed in CD4$^+$CD154$^+$ T cells from seropositive CD patients and UC patients compared to healthy controls. Frequencies of IFNγ^+ cells within CD4$^+$CD154$^+$ T cells had a large variation and were not significantly different between any of the groups (Fig. 5E).

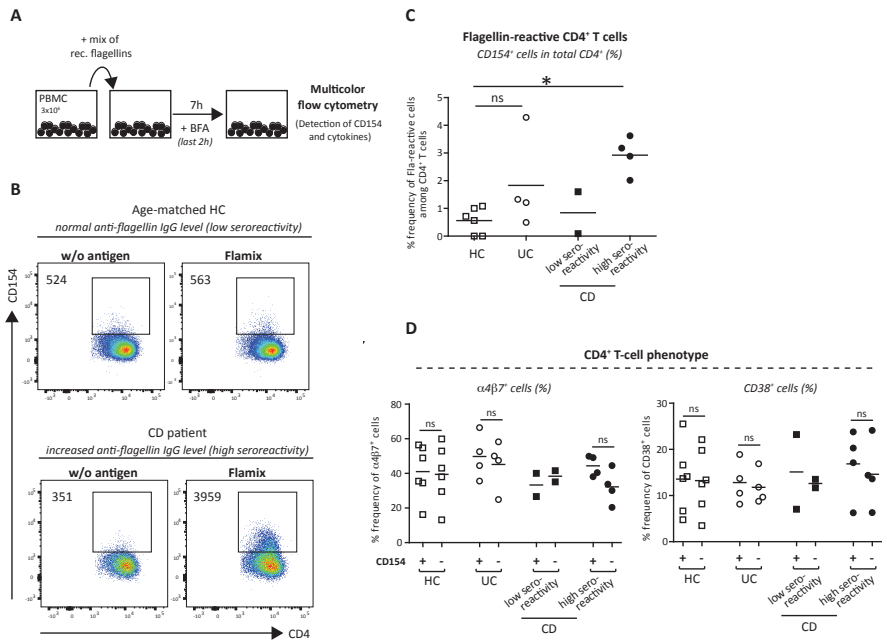

Figure 4. Increased frequencies of flagellin-reactive CD4$^+$CD154$^+$ T cells in treatment-naive CD patients with elevated anti-flagellin IgG. PBMCs were isolated from peripheral blood of healthy controls (n=6), treatment-naive pediatric UC patients (n=4), and treatment-naive pediatric CD patients, including CD patients with normal levels of anti-flagellin IgG antibodies (n=2) and CD patients with elevated anti-flagellin IgG antibody responses (n=4). **(A)** Experimental setup. PBMC were stimulated with a mix of recombinant flagellins (14-2, Fla2, Fla3 and FlaX; 10 µg/ml) for 7 hours and assessed for intracellular expression of CD154. **(B)** Representative dot plots showing the percentage CD154$^+$cells (pre-gated on live CD4$^+$ T cells) of an age-matched healthy control and CD patient with elevated flagellin-specific IgG levels. **(C)** Frequency of CD154$^+$ cells among CD4$^+$ T cells after flagellin stimulation in different groups of patients and healthy controls. See Table S3 for the Step-by-Step method of calculation. **(D)** Phenotype of CD154$^+$ cells (flagellin-reactive) and CD154^{neg} cells (not flagellin-reactive) in different groups of patients and healthy controls. Frequencies of α4β7$^+$ and CD38$^+$ cells are shown (pre-gated on live CD4$^+$ T cells; CD154$^+$ or CD154^{neg}).

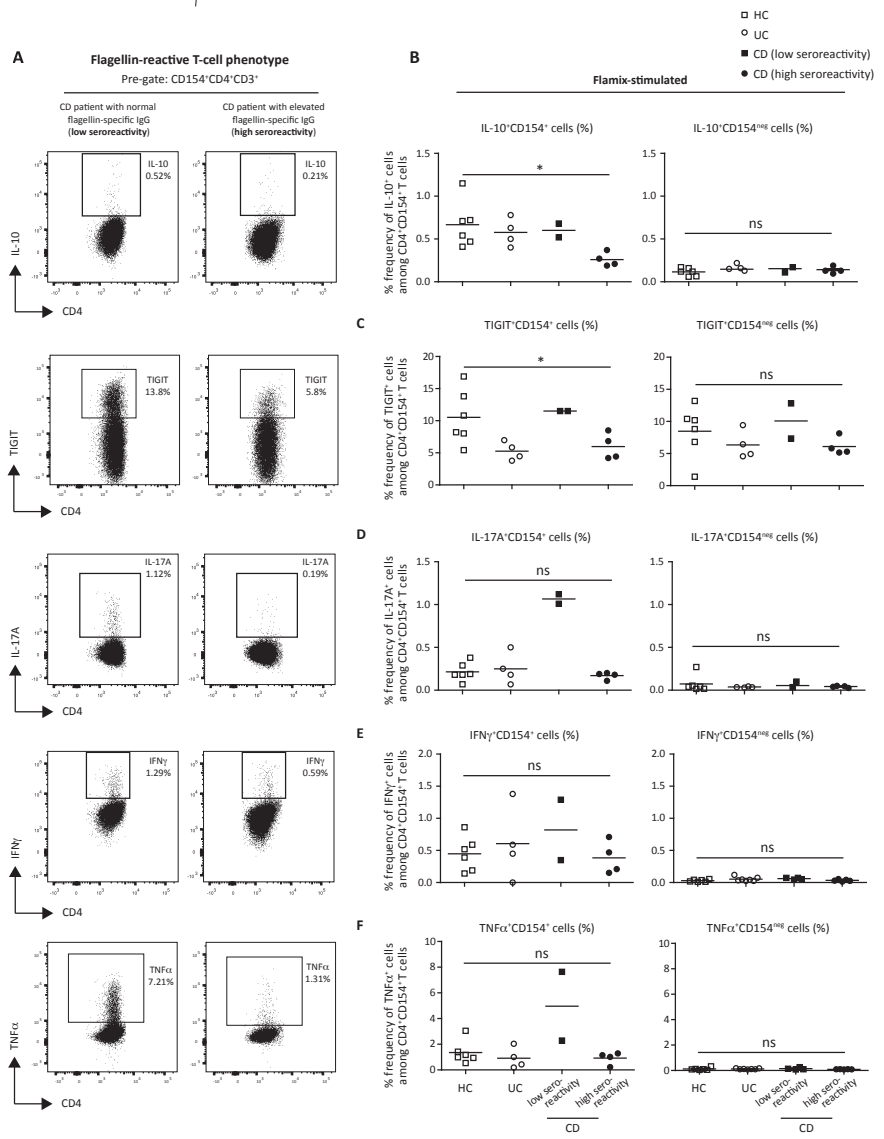

Figure 5. Frequencies of IL-10- and IL-17-expressing flagellin-reactive CD4$^+$CD154$^+$ T cells differ between CD patients with and without elevated flagellin-specific IgG levels. PBMCs were isolated from peripheral blood of healthy controls (n=6), treatment-naive pediatric UC patients (n=4), and treatment-naive pediatric CD patients, including CD patients with normal levels of anti-flagellin IgG antibodies (n=2) and CD patients with elevated anti-flagellin IgG antibody responses (n=4). **(A)** Representative dot plots showing the percentage cytokine$^+$ cells in flagellin-reactive CD4$^+$CD154$^+$ T cells in treatment-naive CD patients seronegative and seropositive for flagellin-specific IgG. **(B-F)** Frequencies of IL-10$^+$ **(B)**, TIGIT$^+$ **(C)**, IL-17A$^+$ **(D)**, IFNγ^+ **(E)** and TNFα **(F)** positive cells in CD4$^+$CD154$^+$ and CD4$^+$CD154^{neg} T cells after flagellin stimulation in different groups of patients and healthy controls.

Taken together, these data demonstrate that treatment-naive CD patients with elevated anti-flagellin antibodies have increased frequencies of flagellin-reactive CD4$^+$ T cells in peripheral blood. Preliminary phenotypic analyses of these cells suggest that frequencies of IL-10-, TIGIT and IL-17A-expressing flagellin-reactive CD4$^+$ T cells differ between seronegative and seropositive CD patients.

TIGIT-expressing CD4$^+$CD154$^+$ T cells with reactivity to flagellin have a preferential regulatory phenotype in both seropositive CD patients and healthy individuals.

In a previous study we have demonstrated that inhibitory receptor TIGIT expression identifies circulating CD38$^+$ effector T cells with immunoregulatory properties.[23] Notably, low frequencies of circulating TIGIT$^+$CD38$^+$ effector T cells at time of IBD diagnosis correlated with reduced duration of clinical remission during follow-up.[23] As both IL10$^+$ and TIGIT$^+$ cell frequencies in flagellin-reactive CD4$^+$CD154$^+$ T cells were decreased in seropositive CD patients compared to healthy controls, we determined whether TIGIT expression within the CD4$^+$CD154$^+$ T-cell population correlated with a specific cytokine profile at 7h after flagellin stimulation (Fig. 6A). Overall, taking into account the short stimulation time, the major difference between TIGIT$^+$CD4$^+$CD154$^+$ T cells and TIGIT^{neg}CD4$^+$CD154$^+$ T cells was the increased frequency of IL-10 secreting cells in the TIGIT$^+$ population which is detectable in all patient groups (Fig. 6B-E). Conversely, TIGIT^{neg}CD4$^+$CD154$^+$ T cells from seronegative CD patients, but not other patient groups, appeared to contain a higher frequency of TNFα secreting cells when compared to TIGIT$^+$CD4$^+$CD154$^+$ T cells (Fig. 6B-E). Overall, these data infer that within the flagellin-reactive CD4$^+$CD154$^+$ T-cell population TIGIT expression may associate with a preferential regulatory T-cell phenotype.

DISCUSSION

Here we show that treatment-naive pediatric CD patients with high anti-flagellin IgG titers have increased frequencies of flagellin-reactive CD4$^+$ T cells in peripheral blood when compared to UC patients and healthy controls. In both healthy and diseased individuals flagellin-reactive T cells have phenotypic regulatory and inflammatory features and can express $\alpha4\beta7$ and CD38. However, CD patients with elevated flagellin-specific IgG and reactivity to 8/8 flagellins have reduced frequencies of IL-10$^+$ and TIGIT$^+$ flagellin-reactive CD4$^+$ T cells when compared to those of healthy controls and patients with low levels of flagellin-reactive IgG. In line with previous findings, TIGIT and IL-10 expression appear related as TIGIT$^+$ flagellin-reactive CD4$^+$ T cells were enriched in IL-10 producing cells when compared to TIGIT^{neg}CD4$^+$ T cells, irrespective of health or disease.

Flagellin-specific IgG may be generated during gastro-intestinal assaults such as infections or epithelial barrier damage in which the compartmentalization of the mucosal T-cell response is lost and translocation of bacteria to systemic lymphoid tissue drives systemic T-cell responses.[16, 24] This is supported by several murine studies. In mice, repeated oral or rectal exposure of flagellin onto an intact mucosal surface does

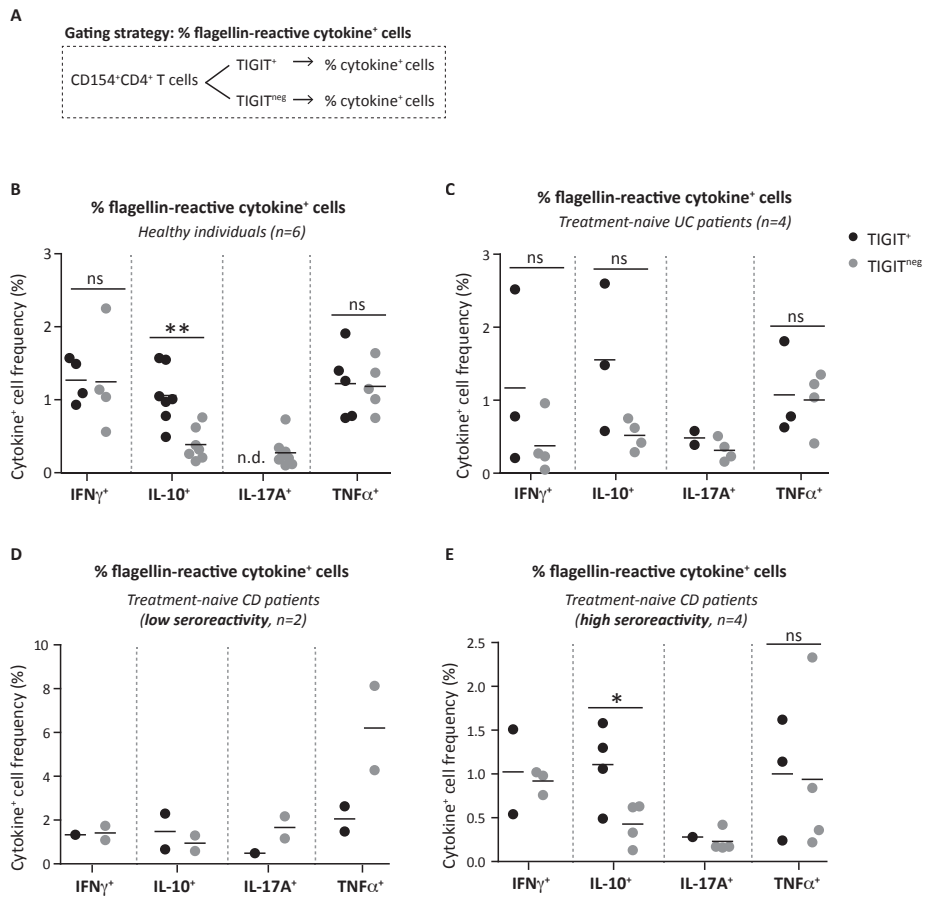

Figure 6. TIGIT-expressing CD4$^+$CD154$^+$ T cells with reactivity to flagellin have a preferential regulatory phenotype in both seropositive CD patients and healthy individuals. PBMCs were isolated from peripheral blood of healthy controls (n=6), treatment-naive pediatric UC patients (n=4), and treatment-naive pediatric CD patients, including CD patients with normal levels of anti-flagellin IgG antibodies (n=2) and CD patients with elevated anti-flagellin IgG antibody responses (n=4). **(A-D)** Frequency of cytokine$^+$ cells in TIGIT$^+$ and TIGIT^{neg} flagellin-reactive CD154$^+$CD4$^+$ T cells after flagellin stimulation of PBMCs. A minimum of 5 cytokine$^+$ CD154$^+$CD4$^+$ T cells was used to calculate cytokine$^+$ cell frequencies; samples with lower number of events were excluded. The mean (± SEM) of cytokine$^+$ cells are shown. P values were calculated using a Student's t test (only when ≥ 3 data points). NS, not significant, *p<0.05, **p<0.01, ***p<0.001. **Note:** Figure 6B also appears in chapter 4 of the thesis.

not result in a serum IgG response while intraperitoneal flagellin injection does.[16] In agreement, no detectable flagellin-specific T-cell activation occurs in the spleen during commensal microbial colonization at steady state.[24] However, intracolonic administration of flagellin during transient disruption of the epithelial barrier with ethanol does result in detectable levels of serum IgG antibodies to flagellin.[16] Increasing this barrier damage and inflammation with dextran sodium sulfate concomitant to flagellin exposure not only raises the magnitude of this systemic Ig response[16] but also drives a concomitant inflammatory T-cell proliferation and activation in the spleen.[24] Our human data extend these murine studies showing that in CD patients the frequency of flagellin-reactive T cells in peripheral blood correlates with the magnitude systemic anti-flagellin IgG antibody responses.

Our key finding that flagellin-reactive CD4$^+$ T cells harbor mixed pro-inflammatory and anti-inflammatory responses is in line with the general paradigm that harmless exogenous antigens elicit a balanced T-cell response in which regulatory cell populations counteract inflammatory cells. Upon loss of intestinal homeostasis, shifts in this balance due to a reduced anti-inflammatory phenotype appear directly associated to the magnitude of IgG antibody formation to flagellin. Regarding pro-inflammatory features, it is interesting that increased relative frequencies of IL-17A and TNFα secreting flagellin-reactive T cells were observed in CD patients with low anti-flagellin-IgG levels. This result agrees with a recent study showing increased IL-17A production in IBD patients after stimulation with heat-inactivated bacteria.[3] Instead of contributing to tissue pathology in these CD patients, these flagellin-reactive CD4$^+$ T cells could possibly promote intestinal barrier integrity through IL-17A and TNFα induced tissue repair in these patients.[3, 25, 26] In depth phenotypic analyses of flagellin-reactive CD4$^+$ T cells of a larger CD cohort is needed to investigate this. Regarding anti-inflammatory features of these cells, the reduced frequencies of IL-10$^+$ and TIGIT$^+$ flagellin-reactive CD4$^+$ T cells CD patients with high anti-flagellin-IgG are remarkable and raise the question whether a specific reduction in inhibitory CD4$^+$ T-cell responses to flagellin occurs in these CD patients. In these current analyses only a limited set of inhibitory T-cell features were assessed. Monitoring expression of other inhibitory receptors and Foxp3 expression is desired in future analyses. Despite this, it is of interest that TIGIT$^+$ flagellin-reactive T cells appear enriched in IL-10 expression especially as we have previously observed that reduced frequencies of TIGIT expressing cells identify treatment-naive IBD patients with reduced duration of clinical remission during follow up.[23]

Although the CD154 stimulation assay is a very potent tool to assess antigen-induced T-cell activation, a mix of multiple flagellins was required to reproducibly detect CD4$^+$ T-cell reactivity in peripheral blood. As such, it is unclear whether the increased frequency of flagellin-reactive CD4$^+$ T cells in CD patients with elevated anti-flagellin IgG reflects expansion of cells with defined limited TCR-specificity. A recent study has shown that

flagellin antigen stimulation of CD4$^+$ T cells from a flagellin-seropositive subject induces expansion of several TCRβ CDR3 sequences including sequences highly shared by or relatively private to CD (and UC) patients.[15] It would be of interest to ascertain whether cells with the same TCR can adopt both inflammatory and anti-inflammatory features in response to flagellin as murine studies have shown that inflammatory microenvironments can determine functional characteristics of flagellin-specific CD4$^+$ T cells with one transgenic flagellin-specific TCR.[27]

Although intestinal inflammation in both CD and UC patients causes excessive exposure of intestinal immune cells to common microbial antigens present in the intestine, antibody responses to microbial antigens, amongst which flagellins, are more common in CD than UC.[10] Increased frequencies of anti-flagellin T cell responses as observed in approximately half of the CD patients, may depend on a dysfunctional innate immune

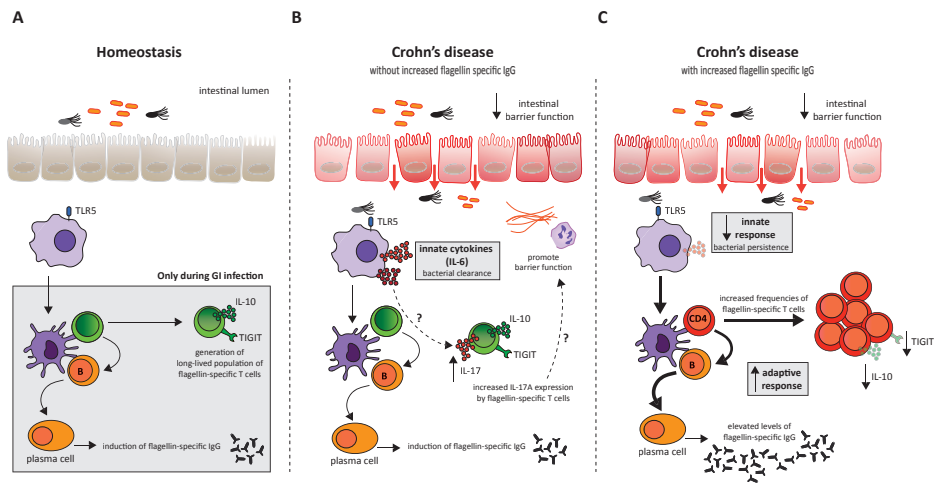

Figure 7. Working hypothesis on the generation of aberrant adaptive immune responses to flagellin. (A) Low frequencies of flagellin-reactive CD4$^+$ T cells and low levels of flagellin-specific IgG antibodies are detectable in peripheral blood of healthy humans. Based on data generated in mice, it is thought that these flagellin-specific CD4$^+$ T cells are activated during gastrointestinal infection and survive long-term as an effector memory CD4$^+$ T-cell population (Hand 2012 Science). As flagellin-specific antibodies depend on the presence of $\alpha\beta$ T cells (Sanders 2006 J Immunol), these T cells promote may plasma cell production of flagellin-specific antibodies. **(B)** In CD patients intestinal inflammation causes excessive exposure of intestinal innate immune cells to common microbial antigens such as flagellin. Flagellin activates innate immune cells through Toll-like receptor 5 (TLR5) leading to NFκB activation and pro-inflammatory cytokine secretion. The inflammatory environment could alter the phenotype of flagellin-specific CD4$^+$ T cells, resulting in elevated IL-17A expression. IL-17A is a critical driver of neutrophil recruitment and contributes to bacterial clearance (Ouyang 2008 Immunity). **(C)** Patients with increased flagellin-specific IgG levels have less IL-6 secretion and NFκB activation in response to flagellin (Takedatsu 2009 Gut, Shen 2008 IBD). This relative defect in the innate immune response could lead to an altered activation of flagellin-specific CD4$^+$ T cells resulting in increased frequencies of flagellin-specific CD4$^+$ T cells with decreased regulatory phenotype and elevated levels of flagellin-specific IgG.

response. In support of this hypothesis, flagellin-induced IL-6 production by monocytes is lower in CD patients with quantitatively higher anti-CBir1 antibody levels.[8] Elevated anti-CBir1 antibody levels are associated with the NFKB1 haplotype (H1) on chromosome 4, which results in lower expression of NFκB in EBV transformed B-cell lines generated from cells of CD patients.[28] Moreover, CD patients with NOD2 variants, resulting in a diminished innate response to bacterial muramyl dipeptide (MDP), have increased antibody responses to microbial antigens.[29] The hypothesis that defective innate responses result in unregulated adaptive microbiota-specific adaptive responses is further supported by the high levels of anti-microbial antibodies in patients with chronic granulomatous disease (CGD), a primary immunodeficiency disorder related to defective microbial killing.[30] Together, this suggests that decreased NFκB activation in response to bacteria could contribute to upregulated adaptive immune response to bacterial antigens in subpopulations of CD patients (see Fig. 6 for model). In addition to treatment aimed at immunosuppression to maintain disease remission, this particular subgroup of CD patients might benefit from agents used to boost innate immunity.[31, 32]

In summary, loss of intestinal tolerance as occurs in CD is associated with chronic inflammatory T-cell responses to components of commensal bacteria, amongst which the major antigen flagellin.[5, 6] Although disease in all patients is characterized by a deregulation of the mucosal immune response to bacterial proteins, the phenotype of the inflammatory T-cell response and the localization of the inflammation can be strikingly different between individuals.[33] This variability leads to large differences in disease severity and response to T-cell suppressive therapies. A better understanding of microbiota-specific T-cell responses may help uncover disease provoking immune mechanisms allowing for disease classification and treatment strategies to induce long lasting disease remission. We are the first to show that elevated levels of IgG antibodies to flagellin are associated with the presence of increased frequencies of flagellin-reactive $CD4^+$ T cells in treatment-naive CD patients. This finding provides a great technical advance allowing future in depth analyses of the precise nature of this T cell-response including T-cell receptor usage, clonality, molecular programming and function. Moreover, it can now be pursued how the frequency and phenotype of these flagellin-reactive $CD4^+$ T cells relates to response to immunosuppressive therapies in larger patient cohorts with detailed information on clinical disease and therapy response.

REFERENCES

1. Hooper LV, Littman DR, Macpherson AJ. Interactions between the microbiota and the immune system. Science 2012;336:1268-73.
2. Duchmann R, Kaiser I, Hermann E, et al. Tolerance exists towards resident intestinal flora but is broken in active inflammatory bowel disease (IBD). Clin Exp Immunol 1995;102:448-55.
3. Hegazy AN, West NR, Stubbington MJT, et al. Circulating and Tissue-Resident CD4(+) T Cells With Reactivity to Intestinal Microbiota Are Abundant in Healthy Individuals and Function Is Altered During Inflammation. Gastroenterology 2017;153:1320-1337 e16.
4. Haas A, Zimmermann K, Graw F, et al. Systemic antibody responses to gut commensal bacteria during chronic HIV-1 infection. Gut 2011;60:1506-19.
5. Kaser A, Zeissig S, Blumberg RS. Inflammatory bowel disease. Annu Rev Immunol 2010;28:573-621.
6. Sartor RB. Microbial influences in inflammatory bowel diseases. Gastroenterology 2008;134:577-94.
7. Landers CJ, Cohavy O, Misra R, et al. Selected loss of tolerance evidenced by Crohn's disease-associated immune responses to auto- and microbial antigens. Gastroenterology 2002;123:689-99.
8. Shen C, Landers CJ, Derkowski C, et al. Enhanced CBir1-specific innate and adaptive immune responses in Crohn's disease. Inflamm Bowel Dis 2008;14:1641-51.
9. Targan SR, Landers CJ, Yang H, et al. Antibodies to CBir1 flagellin define a unique response that is associated independently with complicated Crohn's disease. Gastroenterology 2005;128:2020-8.
10. Mitsuyama K, Niwa M, Takedatsu H, et al. Antibody markers in the diagnosis of inflammatory bowel disease. World J Gastroenterol 2016;22:1304-10.
11. Kugathasan S, Denson LA, Walters TD, et al. Prediction of complicated disease course for children newly diagnosed with Crohn's disease: a multicentre inception cohort study. Lancet 2017;389:1710-1718.
12. Dubinsky MC, Lin YC, Dutridge D, et al. Serum immune responses predict rapid disease progression among children with Crohn's disease: immune responses predict disease progression. Am J Gastroenterol 2006;101:360-7.
13. Papadakis KA, Yang H, Ippoliti A, et al. Anti-flagellin (CBir1) phenotypic and genetic Crohn's disease associations. Inflamm Bowel Dis 2007;13:524-30.
14. Lodes MJ, Cong Y, Elson CO, et al. Bacterial flagellin is a dominant antigen in Crohn disease. J Clin Invest 2004;113:1296-306.
15. Wu J, Pendegraft AH, Byrne-Steele M, et al. Expanded TCRbeta CDR3 clonotypes distinguish Crohn's disease and ulcerative colitis patients. Mucosal Immunol 2018.
16. Sanders CJ, Yu Y, Moore DA, 3rd, et al. Humoral immune response to flagellin requires T cells and activation of innate immunity. J Immunol 2006;177:2810-8.
17. de Zoete MR, Keestra AM, Wagenaar JA, et al. Reconstitution of a functional Toll-like receptor 5 binding site in Campylobacter jejuni flagellin. J Biol Chem 2010;285:12149-58.
18. Means TK, Hayashi F, Smith KD, et al. The Toll-like receptor 5 stimulus bacterial flagellin induces maturation and chemokine production in human dendritic cells. J Immunol 2003;170:5165-75.
19. Eckburg PB, Bik EM, Bernstein CN, et al. Diversity of the human intestinal microbial flora. Science 2005;308:1635-8.
20. Mow WS, Vasiliauskas EA, Lin YC, et al. Association of antibody responses to microbial antigens and complications of small bowel Crohn's disease. Gastroenterology 2004;126:414-24.
21. Chattopadhyay PK, Yu J, Roederer M. A live-cell assay to detect antigen-specific CD4+ T cells with diverse cytokine profiles. Nat Med 2005;11:1113-7.
22. Frentsch M, Arbach O, Kirchhoff D, et al. Direct access to CD4+ T cells specific for defined antigens according to CD154 expression. Nat Med 2005;11:1118-24.
23. Joosse ME, Menckeberg CL, de Ruiter LF, et al. Frequencies of circulating regulatory TIGIT(+)CD38(+) effector T cells correlate with the course of inflammatory bowel disease. Mucosal Immunol 2018.
24. Hand TW, Dos Santos LM, Bouladoux N, et al. Acute gastrointestinal infection induces long-lived microbiota-specific T cell responses. Science 2012;337:1553-6.
25. Kinugasa T, Sakaguchi T, Gu X, et al. Claudins regulate the intestinal barrier in response to immune mediators. Gastroenterology 2000;118:1001-11.
26. Song X, Dai D, He X, et al. Growth Factor FGF2 Cooperates with Interleukin-17 to Repair Intestinal Epithelial Damage. Immunity 2015;43:488-501.

27. Cong Y, Feng T, Fujihashi K, et al. A dominant, coordinated T regulatory cell-IgA response to the intestinal microbiota. Proc Natl Acad Sci U S A 2009;106:19256-61.
28. Takedatsu H, Taylor KD, Mei L, et al. Linkage of Crohn's disease-related serological phenotypes: NFKB1 haplotypes are associated with anti-CBir1 and ASCA, and show reduced NF-kappaB activation. Gut 2009;58:60-7.
29. Devlin SM, Yang H, Ippoliti A, et al. NOD2 variants and antibody response to microbial antigens in Crohn's disease patients and their unaffected relatives. Gastroenterology 2007;132:576-86.
30. Yu JE, De Ravin SS, Uzel G, et al. High levels of Crohn's disease-associated anti-microbial antibodies are present and independent of colitis in chronic granulomatous disease. Clin Immunol 2011;138:14-22.
31. Shiomi A, Usui T, Mimori T. GM-CSF as a therapeutic target in autoimmune diseases. Inflamm Regen 2016;36:8.
32. Roth L, MacDonald JK, McDonald JW, et al. Sargramostim (GM-CSF) for induction of remission in Crohn's disease: a cochrane inflammatory bowel disease and functional bowel disorders systematic review of randomized trials. Inflamm Bowel Dis 2012;18:1333-9.
33. Peloquin JM, Goel G, Villablanca EJ, et al. Mechanisms of Pediatric Inflammatory Bowel Disease. Annu Rev Immunol 2016;34:31-64.

CHARACTERIZATION OF CD4⁺ T-CELL RESPONSES TO BACTERIAL FLAGELLIN IN TREATMENT-NAIVE CROHN'S DISEASE PATIENTS WITH HIGH ANTI-MICROBIAL IgG TITERS

SUPPLEMENTARY DATA

Table S1. Flagellin represented on the microarray.

Phylum	Class	Flagellin	Protein ID	Primary species
Firmicutes	Clostridia	3-1-57	Flagellin	Lachnospiraceae
Firmicutes	Clostridia	14-2†	Flagellin from 14-2 isolate	Roseburia intestinalis
Firmicutes	Clostridia	CBir1	Flagellin	Butyrivibrio fibrisolvens
Firmicutes	Clostridia	CBir11	Flagellin	Roseburia inulinivorans
Firmicutes	Clostridia	CBir66	Flagellin	Roseburia intestinalis
Firmicutes	Clostridia	A4 Fla2†	Flagellin 2 from A4 isolate	Roseburia intestinalis
Firmicutes	Clostridia	A4 Fla3†	Flagellin 3 from A4 isolate	Roseburia inulinivorans
Firmicutes	Clostridia	FlaX*†	Flagellin	Roseburia inulinivorans

*FlaX is highly homologous to CBir1: 83.5% similarity to CBir1 at the NH2 conserved domain.
†Used for *in vitro* flagellin stimulations.

Table S2. Selected patients tested with the CD154 approach.

	Fla2	Fla3	FlaX	14-2
Positivity cut-off	1532,5	2079,8	4325	6997
UC patients without elevated anti-flagellin antibodies				
1	324	1012,5	1255,5	1394,25
2	1201,75	1131,5	1531,75	1335,5
3	215,25	124,5	376,25	310,25
4	457	758,5	610,25	1080,5
CD patients without elevated anti-flagellin antibodies				
1	929,25	482	673,5	1335,75
2	611,75	413,5	620,75	885,75
CD patients with elevated anti-flagellin antibodies				
1	7067	12918,5	24611,25	20100,25
2	7001,5	14670,5	28369,25	23844,5
3	16278,25	32593,5	49342,5	50068,75
4	2949	6502	13531,25	13641,75

Table S3. CD154⁺ T-cell frequency calculation (used for data in Figure 4C).

		CD	HC
A	Number of stimulated PBMCs	3000000	3000000
B	%CD4⁺CD3⁺ T cells in sample (of total)	14,6	4,17
C	Number of stimulated CD4⁺ T cells **(= A x B / 100)**	438000	125100
D	CD154⁺ events in Fla stimulated sample	563	3959
E	CD154⁺ events in non-stimulated sample	524	351
F	Number of specifically activated CD154⁺ cells **(= D - E)**	39	3608
G	% frequency of Fla-reactive cells among CD4⁺ T cells **(= F / C x 100)**	**0,0089041**	**2,8840927**

PBMC were stimulated with a mix of recombinant flagellins (14-2, Fla2, Fla3 and FlaX; 10 µg/ml) for 7 hours and assessed for intracellular expression of CD154. Calculation for the frequency of flagellin-reactive CD154⁺ T cells in the total circulating CD4⁺ T-cell pool.

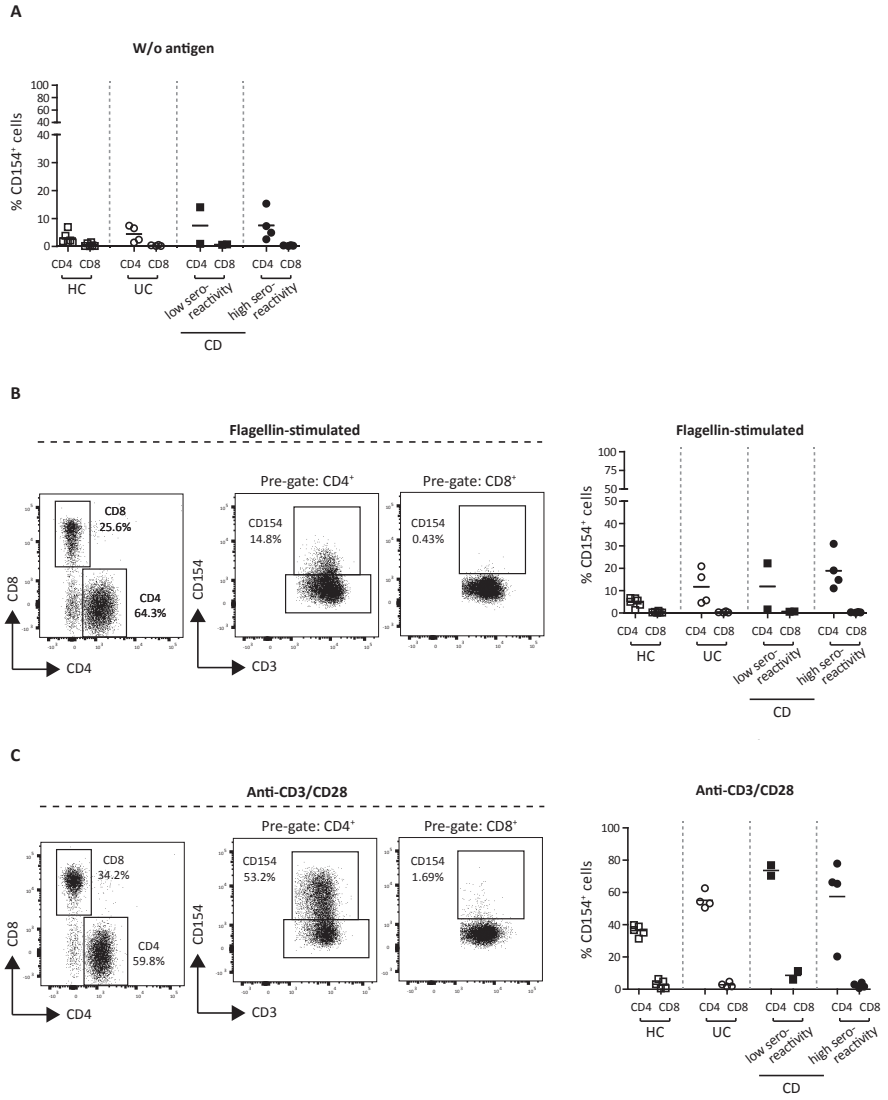

Figure S1. CD154 expression detects activated CD4⁺ but not CD8⁺ T cells. PBMCs were isolated from peripheral blood of healthy controls (n=6), treatment-naive pediatric UC patients (n=4), and treatment-naive pediatric CD patients, including CD patients with normal levels of anti-flagellin IgG antibodies (n=2) and CD patients with elevated anti-flagellin IgG antibody responses (n=4). **(A)** Frequencies of CD154⁺ cells in CD4⁺ and CD8⁺ T cells in cells cultured without antigen. **(B)** Representative dotplots and frequencies of CD154⁺ cells in CD4⁺ and CD8⁺ T cells in cells cultured with a mix of recombinant flagellin antigens (14-2, Fla2, Fla3 and FlaX; 10 µg/ml) for 7 hours. Contrary to Figure 4C, gate frequencies of CD154⁺ cells are depicted directly. **(C)** Representative dotplots and frequencies of CD154⁺ cells in CD4⁺ and CD8⁺ T cells in cells cultured with anti-CD3/CD38 activator beads (bead-to-cell ratio=1:2) for 7 hours.

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6

DUPLICATION OF THE *IL2RA* LOCUS CAUSES EXCESSIVE IL-2 SIGNALING AND PREDISPOSES TO VERY EARLY ONSET COLITIS

Chapter 6

Manuscript submitted for publication.

6

ABSTRACT

Single genetic mutations predispose to very early onset inflammatory bowel disease (VEO-IBD). Here, we identify a *de novo* duplication of the 10p15.1 chromosomal region, including the *IL2RA* locus, in a 2-year-old girl with treatment-resistant pancolitis that was brought into remission by colectomy. Strikingly, after colectomy while the patient was in clinical remission and without medication, the peripheral blood CD4:CD8 ratio was constitutively high and CD25 expression was increased on circulating effector memory, Foxp3^{+} and Foxp3^{neg} CD4^{+} T cells compared to healthy controls. This high CD25 expression increased IL-2 signaling, potentiating CD4^{+} T-cell derived IFNγ secretion after T-cell receptor (TCR) stimulation. Restoring CD25 expression using the JAK1/3-inhibitor tofacitinib controlled TCR-induced IFNγ secretion *in vitro.* As diseased colonic tissue, but not the unaffected duodenum, contained mainly CD4^{+} T cells with a prominent IFNγ-signature, we anticipate that local microbial stimulation may have initiated colonic disease. Overall, we identify a novel cause of VEO-IBD and demonstrate that increased IL-2 signaling predisposes to colonic intestinal inflammation.

DUPLICATION OF THE *IL2RA* LOCUS CAUSES EXCESSIVE IL-2 SIGNALING AND PREDISPOSES TO VERY EARLY ONSET COLITIS

INTRODUCTION

Inflammatory bowel disease (IBD) results from aberrant immune responses to intestinal microbiota and is maintained by inflammatory CD4^{+} effector T cells that have specificity for microbial antigens and reside in the intestinal lamina propria.[1, 2] There is a large variability in clinical disease patterns and, despite a growing availability of new therapeutic options, 40-50% of the patients suffer from frequent relapses or continuous inflammation. Identification of the patient's underlying immune disease and subsequent tailoring of treatment is therefore highly desired. Although genome-wide association studies have suggested that genetic control of inflammatory T-cell responses is linked to IBD susceptibility[3], the functional impact of most key susceptibility genes associated with IBD is currently not understood.[4, 5] Rare single genetic mutations can predispose to very early onset inflammatory bowel disease (VEO-IBD), an IBD-like disease presenting before the age of six. Monogenic defects, such as the *IL10, IL10R* and *Foxp3* loss-of-function mutations causing VEO-IBD, have uncovered pathways that are essential to prevent intestinal inflammation.[6, 7] As these monogenic defects fall within inflammatory immune networks that overlap with polygenic IBD loci, in depth immunological characterization of VEO-IBD patients provides key information to advance IBD patient classification.

One of the key genes shared between IBD susceptibility loci and monogenic VEO-IBD is the *IL2RA* gene encoding the interleukin-2 receptor alpha chain (CD25).[8] CD25 is constitutively expressed by CD4^{+}Foxp3^{+} regulatory T cells, on a subset of circulating CD4^{+} memory T cells and is rapidly induced on effector CD4^{+} T cells after T-cell receptor signaling.[9, 10] CD25 is the low-affinity IL-2 receptor which cannot function independently but forms a high-affinity IL-2 receptor when associated with the IL-2Rβ and common γ chain.[11] Signaling through the IL-2R induces T-cell proliferation and is critical for the development and peripheral expansion of CD4^{+}CD25^{+} regulatory T cells.[12, 13] As a result, IL-2 signaling is essential for intestinal homeostasis and both *Il2ra-/-* and *Il2-/-* mice develop spontaneous colitis, the latter with a predominant CD4^{+} T cell infiltration in the lamina propria.[14-16] In analogy, the clinical disease in *IL2RA* deficient patients resembles deficiency in the Foxp3 gene causing polyendocrinopathy, enteropathy, X-linked (IPEX) syndrome-like disease.[8] Paradoxically, excessive IL-2 signaling can also elicit intestinal disease demonstrated by high dose IL-2 in cancer therapy which is associated with gastrointestinal side effects in the majority of patients, including nausea, vomiting and diarrhea.[17] Moreover, excessive CD25 expression occurs in several autoimmune diseases and its inhibition effectively blocks clinical and inflammatory disease activity.[18, 19] Together, these data argue that a balanced IL-2 regulation is pivotal for intestinal homeostasis. However, it is unknown how intrinsically high IL-2 signaling would affect intestinal immune responses and to what degree it is detrimental for intestinal homeostasis.

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DUPLICATION OF THE *IL2RA* LOCUS CAUSES EXCESSIVE IL-2 SIGNALING AND PREDISPOSES TO VERY EARLY ONSET COLITIS

Here, we identify a de novo duplication of the 10p15.1 chromosomal region, including the *IL2RA* gene, in a 2-year-old female patient presenting with therapy-resistant VEO-IBD that was brought into remission by subtotal colectomy. Our data demonstrate that the patient's $CD4^+$ T cells exhibit constitutive activation of the IL-2R-pSTAT5 pathway leading to hyper-responsiveness of $CD4^+$ effector T cells and predisposing to T-cell driven pancolitis. As diseased colonic tissue, but not the unaffected duodenum, contained mainly $CD4^+$ T cells with a prominent IFNγ signature, we anticipate that local microbial stimulation may have initiated colonic disease. These findings shed new light on the role of IL-2 in intestinal homeostasis and direct further studies to examine the functional consequences of *IL2RA* genetic variation in IBD patients.

METHODS

Patients.
Peripheral blood was obtained from the VEO-IBD patient described below, her parents , a cohort of VEO-IBD patients (n=9), pediatric-onset IBD (PIBD) patients in clinical remission (n=4), pediatric IBD patients with active intestinal inflammation (n=4) and adult healthy controls (n=15). Small intestinal and colonic biopsies were obtained from the VEO-IBD patient at time of diagnosis. Specimens of resected colonic tissue were obtained from the VEO-IBD patient and PIBD patients (n=8) refractory to conventional and biological immunosuppressive therapy. The Medical Ethical Committee of the Erasmus University Medical Center-Sophia Children's Hospital Rotterdam approved this study (METC 2007-335). Written informed consent was obtained from every patient and parents before study inclusion.

Genetic analysis.
Targeted next-generation sequencing (TNGS) and whole exome sequencing (WES) were performed as described previously.[20, 21] Array-comparative genome hybridization (CGH) of DNA extracted from peripheral blood cells was performed on an Agilent 60K oligo-nucleotide microarray (Agilent Technologies, Santa Clara, California, USA).

Cell isolation and cultures.
Venous blood was collected in EDTA tubes and PBMCs were isolated using a Ficoll-Hypaque gradient according to standard protocol (Axis-Shield Diagnostics, Dundee,UK). PBMCs were labelled with CellTrace Violet (ThermoFisher Scientific, Bleiswijk, the Netherlands) and were stimulated with phytohemagglutinin (PHA, 5 µg/ml, ThermoFisher Scientific), ConA (10 µg/ml), anti-CD3 (0.5 µg/ml, Sanquin, Amsterdam, the Netherlands) or anti-CD3/CD28 stimulator beads (0.5 bead per PBMC) with or without recombinant human IL-2 (1, 10 or 100 IU/ml, R&D Systems, Minneapolis, MN, USA) or IL-15 (1, 10 or 100 µg/ml, R&D Systems) for the indicated time-points. In some experiments, tofacitinib (Pfizer, New York, NY, USA; 200 µM or 1000 µM) was added to the anti-CD3/CD28 stimulated conditions. Cells were cultured in Iscove's modified Dulbecco's medium (ThermoFisher Scientific) supplemented with heat inactivated fetal calf serum, Glutamax (ThermoFisher Scientific), 2-mercaptoethanol, penicillin and streptomycin.

Flow cytometry.
PBMCs were stained for flow cytometry ex vivo or after in vitro culture using monoclonal antibodies against CD3 (UCHT1, BD Biosciences, Franklin Lakes, NJ, USA; HIT3a, Biolegend, San Diego, CA, USA), CD4 (SK3, BD), CD8 (SK1, BD), CD38 (HIT2, BD), CD62L (DREG-56, Biolegend), CD25 (2A3, BD), CD45RA (HI100, BD), CD45RO/RPE (UCHL1, Agilent, Santa Clara, CA, USA), TIGIT (MBSA43, eBioscience, Bleiswijk, the Netherlands) and CCR7 (15053, R&D Systems). Intracellular staining was performed with the Foxp3 fixation and permeabilization staining buffer kit, according to manufacturer's protocol (eBioscience), followed by staining with anti-Foxp3 (236A/E7 or PCH101, eBioscience), anti-Helios (22F6, Biolegend) or anti-Ki67 (20Raj1, eBioscience) and the appropriate isotype controls (eBioscience).

For intracellular phosphorylated STAT5 staining, PBMCs were rested overnight at 37°C and stained for anti-CD4 (RPA-T4, BD), pSTAT5 (pY694, BD) or the appropriate isotype control according to the manufacturer's protocol (Alternative protocol 1, Fix-stain-perm, BD Phosflow). In several conditions, cells were stimulated with IL-2 (100 IU/ml) for 15 minutes prior to analysis. Cells were analyzed using the FACS Canto II and FlowJo software (BD).

Cytokine and soluble CD25 analysis.
Cytokine concentrations in cell supernatants or plasma were analyzed using an enzyme-linked immunosorbent assay set for IFNγ (eBioscience) according to the manufacturer's instructions. Soluble CD25 levels in plasma were determined using the human CD25/IL-2R alpha Quantikine ELISA kit according to the manufacturer's instructions (R&D Systems).

Epithelial cell and lamina propria lymphocytes isolation.
A specimen of total intestinal tissue was stored for immunohistochemistry (4% paraffin) and RNA isolation (RNA later, Sigma-Aldrich, Zwijndrecht, The Netherlands) prior to incubation. Next, total intestinal tissues were incubated in 0.15% dithiothreithol (DTT)/HBSS for 15 minutes and in 1 mM EDTA/HBSS for 30 minutes at 60 rpm and 37°C. Epithelial layers were stored in RA1 with DTT (1:100, Macherey-Nagel, Bethlehem, PA, USA) for RNA

isolation. Intestinal specimens without epithelial fractions were incubated in a digestive solution consisting of 10% FCS, 25 mM HEPES, 100 U/ml penicillin streptomycin, 30 µg/ml gentamycin, 0.5 µg/ml fungizone, 0.1 mg/ml collagenase III and 1 mg/ml DNase for 60 minutes at 37C. In several experiments, lamina propria lymphocytes (LPL) were stimulated for 5 hours with phorbol 12-myristate 13-acetate (PMA, 0.02 µg/ml, Sigma-Aldrich) and ionomycin (0.5 µg/ml, Sigma-Aldrich) in the presence of Brefeldin A (3 µg/ml eBioscience) for the last 2 hours and subsequently analyzed for intracellular cytokine expression.

RNA isolation and quantitative PCR.

Total RNA was extracted from total intestinal tissues or epithelial layers using the Nucleospin RNA II or XS kit (Macherey-Nagel) and reverse transcribed into cDNA using the SensiFAST cDNA synthesis kit (Bioline, London, UK). Real-time quantitative PCR was performed using SYBR Green on a AbiPrismR 7900 Sequence Detection system (Applied Biosystems, Foster City, CA, USA). The relative expression to *GAPDH* for each gene was measured as $2^{(-\Delta Ct)}$. Primer sets used were:

Gene	Forward primer	Reverse primer
GAPDH	5'-GTCGGAGTCAACGGATT-3'	5'-AAGCTTCCCGTTCTCAG-3'
CD3ε	5'-GGGCAAGAGTGTGTGAGA-3'	5'-CGGGAGGCAGTGTTCT-3'
CD4	5'-GGCATCTTCTTCTGTGTCA-3'	5'-CCTCGTGCCTCAAATG-3'
CD8α	5'-GAACCGAAGACGTGTTTG-3',	5'-CGCCCCCACTAAAATAAT-3'-3'
HLAE	5'-TCCGAGCAAAAGTCAAAT-3',	5'-GCCAGGTCAGTGTGATCT -3'
NKG2D	5'-AGCCAGGCTTCTTGTATGT-3'	5'-TTCCTGGCTTTTATTGAGAT -3'
MICA	5'-ATGGGAATGGAACCTACC-3'	5'-TCTGCCAATGACTCTGAAG-3'
GZMB	5'-TGGGGAAGCTCCATAAA -3'	5'-GGGCCTTGTTGCTAGG-3'
PRF1	5'-GAGCCTCGGTGAAGAGA-3'	5'-GCGCTTGCACTCTGAG-3'
IFNG	5'-CCAGGACCCATATGTAAAAG-3'	5'-TGGCTCTGCATTATTTTTC -3'
IL21	5'-AAGGCCCAACTAAAGTCAG-3'	5'-AGGGCATGTTAGTCTGTGTT-3'
CD25	5'-GCCGTCCTGAGAGTGAG-3'	5'-TTCCCGGCTTCTTACC-3'
IL15RA	5'-GCCGCCAGGTGTGTAT-3'	5'-TGGTCCCCCAAGTCAC-3'

Immunohistochemistry.

Paraffin embedded biopsies were sectioned, deparaffinized, and 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 EDTA buffer (1 mM, pH 8.0). The sections were blocked for 1h 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-Ki67 (monoclonal rabbit, D2H10, Cell Signaling Technology, Danvers, MA, USA), anti-CD3 (polyclonal rabbit, DakoCytomation), anti-pSTAT5 (Tyr694, C11C5, monoclonal rabbit, Cell Signaling Technology), anti-CD4 (4B12, monoclonal mouse, Thermo Fisher Scientific, Waltham, MA, USA), anti-CD8 (SP16, monoclonal rabbit, Thermo Fisher Scientific), anti-IL21 (polyclonal rabbit, LifeSpan Biosciences) or proper isotype antibodies. Immunoreactive sites were detected with biotinylated secondary antibodies using the Vectastain ABC Elite Kit (Vector Laboratories, Burlingame, CA, USA) and 3,3'-diaminobenzidine tetrahydrochloride (Sigma-Aldrich). Nuclei were counterstained with hematoxylin (Vector Laboratories). For immunofluorescence, sections were stained with anti-Ki67 Alexa-488 (rabbit, D3B5, Cell Signaling Technology), followed by anti-CD3 (polyclonal rabbit, DakoCytomation) with a secondary biotin-labelled goat-anti-rabbit Ab and Streptavidin-DyLight 594 (Vector Laboratories). The sections were mounted with medium for fluorescence containing 4,6-diamidino-2-phenylindole (DAPI, Vector). Images were acquired using a Leica DM5500B upright microscope and LAS image acquisition software (Leica Microsystems, Rijswijk, The Netherlands).

Statistics.

Significance was determined using Student's t-test or one-way performed on GraphPad Prism 5.0 software (GraphPad Software, La Jolla, CA, USA), as indicated in the figure legends. P values of <0.05 were regarded as significant. In case of multiple comparisons, an adjusted significance level was used according to the Bonferroni correction (significance level = 0.05/number of comparisons).

RESULTS

In a case of VEO colitis, CGH identifies a de novo 374 kb duplication of the 10p15.1 region containing the *IL2RA* locus.

The patient was a girl who developed acute severe colitis at 2 years of age. She was the first child of non-consanguineous Caucasian parents and the only significant family history was left-sided ulcerative colitis (UC) of benign course diagnosed in her mother at 32 years (figure 1A). The child had no history of recurrent or opportunistic infections and diagnostic tests for cow's milk protein allergy and celiac disease were negative. Endoscopic examination showed severe pancolitis with edematous and fragile mucosa and multiple erosions and pangastritis of mild intensity. In contrast, esophagus, duodenum, jejunum and terminal ileum were macroscopically normal and there were no peri-anal or extra-intestinal manifestations. Histology showed chronic active colitis with crypt

Table 1. Time points of sample collection.

Visit		Sample collection (peripheral blood and intestinal tissue)	Treatment	CRP (mg/L)	Calprotectin (ug/g)	PUCAI
February 2015	S0	Presentation with severe acute colitis. Diagnostic endoscopy with biopsies in small intestine and colon.	No treatment	19	2861	55
August 2015	S1	Persistent disease activity using infliximab 10 mg/kg every 4 weeks and azathioprine 25 mg/day. Received blood sample.	Infliximab 5 mg/kg/day per 4 weeks, Azathioprine 1dd30mg, Allopurinol 1dd50mg, Cholecalciferol 1dd800IE, Omeprazol 1dd10mg, Cotrimoxazol 1dd30mg	< 3	ND	45
December 2016	S2	Subtotal colectomy with ileostoma. Received blood sample and intestinal tissue of patient.	Infliximab 5 mg/kg/day per 4 weeks (last infusion on 21st of December 2015), Azathioprine 1dd30mg, Allopurinol 1dd50mg, Cholecalciferol 1dd800IE, Omeprazol 1dd10mg, Vancomycin 2dd250mg, Gentamicin 2dd50mg	209	ND	55
January 2017	S3	Closure of double-barreled ileostomy, one month protectomy and ileoanal pouch anastomosis. No symptoms of intestinal disease. Received blood samples of patient and parents.	No immunosuppressive treatment. No recent antibiotic use.	ND	ND	0
August 2017	S4	Regular visit at outpatient clinic. No symptoms of intestinal disease. Received blood samples of patient and parents.	No immunosuppressive treatment. No recent antibiotic use.	ND	ND	0

Abbreviations: CRP, C-reactive protein; PUCAI, pediatric ulcerative colitis activity index; ND, not determined.

architectural distortion, destructive cryptitis and crypt abscesses (figure 1B, left panel) and immunohistochemisitry revealed an important lymphocytic infiltrate containing $CD4^+$ but almost no $CD8^+$ cells (figure 1B, right panel). Ileal biopsies at diagnosis showed a mixed inflammatory infiltrate and cryptitis that, in contrast to the colonic inflammation, resolved during immunosuppressive treatment. At diagnosis and before start of immunosuppression, leukocyte count and absolute number of T, NK and B lymphocytes in peripheral blood were within normal range.[22] Serum concentrations of immunoglobulins were also normal.

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As her disease was refractory to standard immunosuppressive-, immunomodulatory-, and biological therapy, subtotal colectomy with temporary ileostomy was performed one year after initial diagnosis, followed 12 months later by proctectomy and ileoanal pouch anastomosis. Subtotal colectomy resulted in drastic clinical improvement without further need for immunosuppressive therapy (figure 1A). After surgery and without immunosuppression, the patient displayed a high CD4:CD8 ratio in peripheral blood when compared to age-matched VEO-IBD patients (figure 1C) and to an age-matched reference population, despite normal absolute number of lymphocytes.[22] The patient's clinical course and time points at sample collection are depicted in figure 1A and Table 1.

To identify a possible underlying genetic defect, the patient was screened for known mutations associated with VEO-IBD using targeted next-generation sequencing (TNGS) as described.[21] DNA from the patient and both parents were next analyzed by whole exome sequencing (WES, figure S1A). TNGS and WES failed to reveal any single gene mutation. Yet, increased DNA copy number was suggested by an excess in the number of reads derived from *IL2RA* encoding exons in the patient compared to other individuals simultaneously tested by TNGS or compared to the parents in WES analysis. Further analyses using array comparative genome hybridization (CGH, figure S1B), revealed a 374 kb duplication of the 10p15.1 chromosomal region, including the *IL2RA* and *IL15RA* genes (figure 1D, Table 2). CGH analyses were normal in both parents, confirming the de novo origin of the duplication.

Table 2. Genes involved in duplication on the 10p15.1 chromosomal region.

Gene symbol	Gene name	Function
IL2RA	IL-2 receptor alpha subunit	The IL-2 receptor alpha (IL2RA) and beta (IL2RB) chains, together with the common gamma chain (γ), constitute the high-affinity IL-2 receptor.
IL15RA	IL-15 receptor alpha subunit	The IL-15 receptor alpha subunit specifically binds interleukin 15 (IL-15) with high affinity. The receptors of IL15 and IL2 share two subunits, IL2R beta and common γ chain.
FBXO18	F-box protein, helicase 18	Constitutes one of the four subunits of an ubiquitin protein ligase complex called SCFs (SKP1-cullin-F-box), which function in phosphorylation-dependent ubiquitination.
ANKRD16	Ankyrin repeat domain-16	An ANK repeat is a protein containing at least one ANK repeat, a conserved domain of approximately 33 amino acids, that was originally identified in ankyrin. Associated with protein-protein interactions.
RBM17	RNA binding motif protein 17	Part of spliceosome complex and functions in the second catalytic step of mRNA splicing.

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To define whether increased *IL2RA* and/or *IL15RA* expression may play a role in the patient's intestinal disease, mRNA expression was compared in colonic tissue resected from the patient and from treatment-resistant PIBD patients. Strikingly, *IL2RA* (figure S1C) but not *IL15RA* (figure S1D) mRNA expression was elevated in the patient's colonic tissue compared to PIBD controls. We therefore focused our analyses on the possible role of IL-2R in modulating immune function and promoting colonic inflammation in the patient.

Duplication of the *IL2RA* locus is associated with increased CD25 expression and activation of the IL-2 pathway in peripheral CD4⁺ T cells.

As IL-2 has important roles in T-cell survival and proliferation[23-25], we hypothesized that the observed duplication may potentiate T-cell activation. Flow cytometric analysis of peripheral blood cells was first performed one year after colectomy, when the child was in clinical remission and without medication (figure 1A, visits S3-S4). While there was no change in the frequency of CD25⁺ T cells (figure S2A), the mean fluorescence intensity of CD25 was significantly increased in CD4⁺ and CD8⁺ T cells from the patient compared to T cells from her parents and healthy adult individuals (figure 1E, left panel, and S2B). In particular, CD25 expression was increased on circulating effector memory CD4⁺ T cells compared to central memory and naive CD4⁺ T cells from the patient (figure 1E, right panel). In contrast, CD25 expression did not differ between naive, effector memory and central memory CD4⁺ T cells from the patient's parents and adult healthy individuals (data not shown). In the patient, CD25 expression was also increased on circulating regulatory CD4⁺Foxp3⁺ T cells and non-regulatory CD4⁺Foxp3^{neg} T cells compared to controls (figure 1F), but the frequency of regulatory CD4⁺Foxp3⁺ T cells remained unchanged (figure S2C). Altogether, these data suggested that the patient's T cells may overexpress CD25 after antigen stimulation. In line with these results, high concentrations of soluble CD25 were detected in the patient's plasma at multiple visits, including after colectomy when the patient was free of clinical symptoms and without medication (figure S2D, visits S1-S3).

One major signaling cascade downstream IL-2 involves the activation of Janus kinase 3 (JAK3) and the subsequent phosphorylation of signal transducer and activator of transcription 5 (STAT5).[11] Therefore, to assess whether increased CD25 expression was associated with enhanced IL-2 signaling in the patient's T cells, STAT5 phosphorylation was compared in peripheral blood CD4⁺ T cells from the patient (visit S3) and from pediatric IBD patients with active intestinal inflammation ("PIBD inflammation") or in clinical remission ("PIBD remission"). A fraction of circulating CD4⁺ T cells from the patient was activated as evidenced by their increased STAT5 phosphorylation compared to circulating CD4⁺ T cells from pediatric IBD patients at baseline (figure 1G). Moreover, STAT5 phosphorylation after a 15-minute stimulation with exogenous IL-2 was significantly increased in the patient's CD4⁺ T cells compared to T cells from PIBD controls

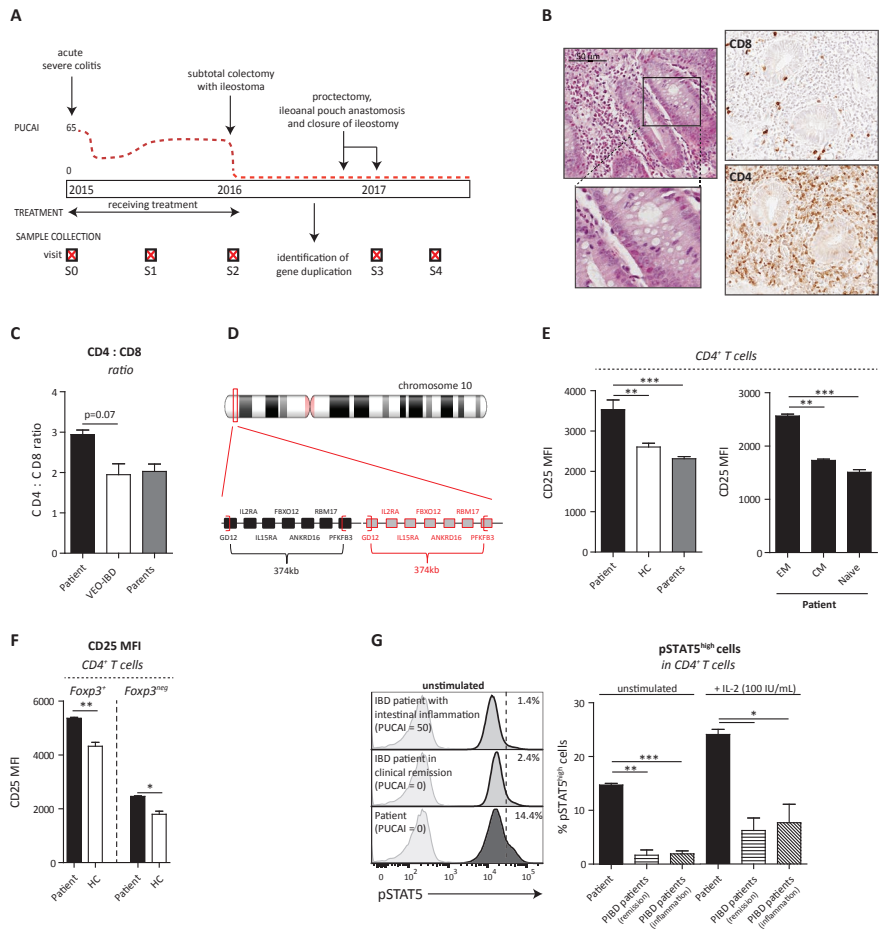

Figure 1. A 374 kb duplication on 10p15.1 including the *IL2RA* locus leads to intrinsically increased CD25 and enhanced IL-2 signaling in CD4⁺ T cells. (A) Timeline depicting the patient's clinical course and time points of sample collection (denoted as visits S0-S4). PUCAI, pediatric ulcerative colitis activity index. **(B)** H&E staining on paraffin-embedded colonic tissue at time of diagnosis (left, time point S0) and immunohistochemical detection of CD4 and CD8 in paraffin-embedded resected colonic tissue (right, time point S2). **(C)** Flow cytometric analysis of CD4:CD8 ratio in peripheral blood of the patient, her parents (time points S3 and S4) and VEO-IBD patients (n=9). Median (5th to 95th percentiles) for the 2-5 year age category is 1.6 (0.9-2.9).[1] **(D)** Localization of the patient's duplication on chromosome 10. **(E-G)** Flow cytometric analysis of CD3, CD4, CD38, CD62L, CD45RA, CCR7, CD25, Ki67 and/or Foxp3 expression was performed on peripheral blood from the patient, her parents (time points S3 and S4) and healthy adult controls (HC, n=4). **(E)** CD25 expression (MFI) on total CD4⁺ T cells (left) and on effector memory (CD45RA^{neg}CCR7^{neg}), central memory (CD45RA^{neg}CCR7⁺) and naive (CD45RA⁺CCR7⁺) CD4⁺ T cells in the patient (right). **(F)** CD25 expression on regulatory Foxp3⁺CD4⁺ T cells and Foxp3^{neg}CD4⁺ T cells. **(G)** PBMCs of the patient, PIBD patients with active intestinal inflammation (n=3) and PIBD patients in remission (n=3) were stimulated with IL-2 (100 IU/mL) for 15 min followed by quantification of STAT5 phosphorylation (pY694) in CD4⁺ cells by flow cytometry (visit S3). *p<0.05, **p<0.01, ***p<0.001 using one-way ANOVA followed by the Bonferron's Multiple Comparison Test.

(figure 1G). Overall, these data led us to conclude that *de novo* duplication of *IL2RA* gene promoted CD25 expression on circulating effector memory CD4⁺ T cells and enhanced their responsiveness to IL-2.

CD4⁺ T cells of the patient with duplication of IL2RA display increased responsiveness to TCR stimulation.

As indicated above, cellular infiltration in the inflamed colon of the patient consisted predominantly of CD4⁺ T cells (Figure 1B), suggesting that enhanced IL-2 responsiveness may preferentially promote expansion of CD4⁺ T cells of mucosal origin. Analyses of CFSE dilution demonstrated that patient CD4⁺ T cells contained a significantly higher frequency of spontaneously dividing cells than CD4⁺ T cells from controls (figure 2A). Moreover, the patient's CD4⁺ T-cell proliferation remained enhanced upon anti-CD3 and anti-CD3/CD28 activation (figure 2A). To investigate whether the increased CD4:CD8 ratio observed the in peripheral blood may indeed reflect the expansion of mucosal CD4⁺ T cells, we analyzed expression of the proliferation marker Ki67 in CD62L^{neg}CD38⁺CD4⁺ T cells which are enriched in gut-homing T cells specific for mucosal antigens[26]. In keeping with our hypothesis, circulating CD62L^{neg}CD38⁺CD4⁺ T cells, but neither total CD4⁺ T cells nor CD62L^{neg}CD38^{neg}CD4⁺ T cells, contained higher frequencies of proliferating Ki67⁺ cells in the patient than in controls (figure 2B). We therefore concluded that the increased Ki67 expression likely reflected enhanced in vivo proliferative rate of CD4⁺ T cells of mucosal origin.

We next examined whether the increased proliferative rate of circulating CD4⁺ T cells in the patient with *ILR2A* duplication may be explained by their enhanced responsiveness to IL-2. In the absence of TCR ligation, addition of exogenous IL-2 induced a comparable increase in CD25 expression in CD4⁺ and CD8⁺ T cells from the patient and from controls (data not shown). However, following ligation of CD3, IL-2 significantly potentiated more CD25 upregulation in CD4⁺ T cells from the patient than from controls (figure 2C). Moreover, a low concentration of exogenous IL-2 (10 IU/ml) was sufficient to cause a strong increase in CD25 expression in CD3-stimulated CD4⁺ T cells from the patient (figure 2C). Ligation of CD3 together with CD28 also caused a more pronounced up-regulation of CD25 in CD4⁺ T cells from the patient than from controls (figure 2D). Of note, percentages of live cells were similar between patient and control cell cultures (data not shown). Comparable results were observed in CD8⁺ T cells but the differences between patient and control cells were less prominent (figure S3), a result agreeing with the higher absolute numbers of CD4⁺ T cells in all culture conditions (data not shown) as well as *in vivo* in peripheral blood and colonic tissue of the patient.

Taken together, these data show how increased T-cell responsiveness to IL-2 in the patient with *IL2RA* duplication can amplify the proliferative response of CD4⁺ T cells to

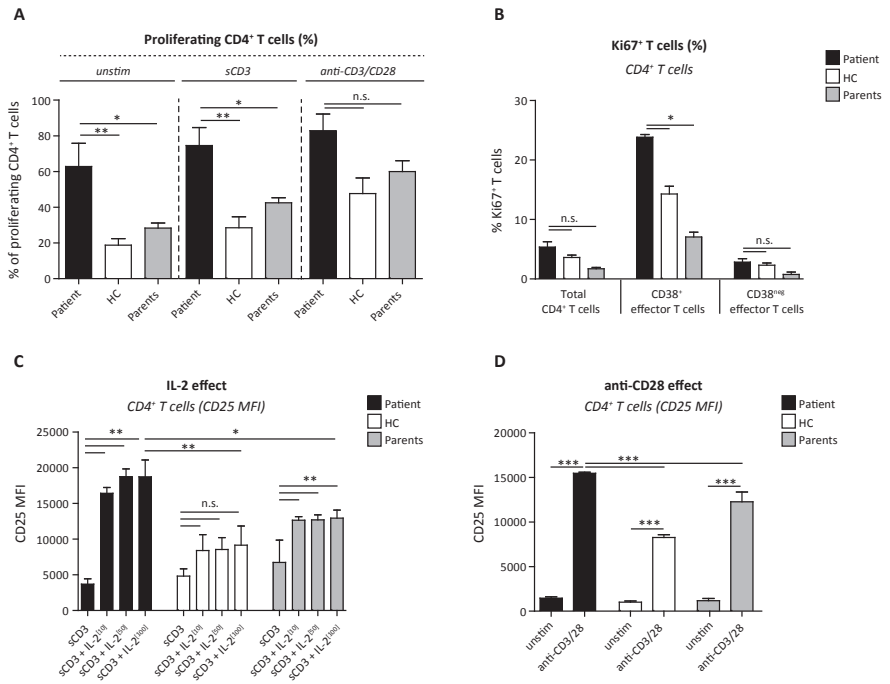

Figure 2. CD25 overexpression is associated with increased response to IL-2. (A) Patient PBMCs (visits S3 and S4) and healthy adult control (n=4) were stimulated with anti-CD3 (500 ng/ml) or anti-CD3/CD28 beads (bead-to-cell ratio 1:2) for 48 hours. Percentage of proliferating CD4⁺ T cells was analyzed by CellTrace Violet dilution. **(B)** Frequencies of Ki67⁺ cells gated on CD4⁺ T cells, CD62L^{neg}CD38⁺CD4⁺ T cells and CD62L^{neg}CD38^{neg}CD4⁺ T cells. Data are mean ± SD (patient) or mean ± SEM (healthy adult controls, n=10). **(C)** PBMCs were stimulated with anti-CD3 (500 ng/ml) in the absence or presence of IL-2 (1, 50 or 100 IU/ml). After 48h, CD3, CD4 and CD25 expression was determined by flow cytometry. **(D)** PBMCs were stimulated with anti-CD3/CD28 beads (bead-to-cell ratio 1:2). CD25 expression on CD4⁺ T cells was analyzed at 48 hours. Data are mean ± SD (patient) or mean ± SEM (healthy adult controls, n=4). Representative of two independent experiments (time points S3 and S4); N.s., not significant, *p<0.05, **p<0.01, ***p<0.001 using one-way ANOVA followed by the Bonferron's Multiple Comparison Test.

antigenic stimulation and license the expansion of peripheral T cells initially activated in the antigen-rich intestinal environment.

Colonic CD4⁺ IEL and LPL of the patient with *IL2RA* duplication show increased proliferation and activation compared to T cells of treatment-resistant pediatric-onset IBD patients.

To investigate whether and how increased T-cell responsiveness to IL-2 may predispose to colonic inflammation, T-cell infiltration and activation were compared in colonic tissue resected from the patient with the *IL2RA* duplication and from 8 pediatric-onset

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treatment-resistant IBD patients (4 ulcerative colitis (UC) patients, 4 Crohn's disease (CD) patients, denoted as "PIBD resection controls"). In the patient, colonic inflamed biopsies were also compared to non-inflamed duodenal tissue obtained during initial assessment at diagnosis.

As already indicated and as depicted in figures 1B, the patient's colon was strongly infiltrated by $CD3^+$ T cells in both *lamina propria* and epithelium (figure 3A). In order to precisely compare the density and nature of the colonic T-cell infiltrate in the patient and in the inflamed controls, mRNA encoding *CD3*, *CD4* and *CD8* were quantified by RT-PCR. Expression of *CD3* and *CD4* but not *CD8* mRNA was strikingly higher in whole colonic tissue derived from the patient than from PIBD resection controls (figure 3B). Comparable results were obtained when mRNA was extracted from the epithelial layer (figure 3C), which normally contains predominantly $CD8^+$ T cells[27], sustaining evidence that $CD4^+$ T cells were more particularly prone to expand in the patient. Accordingly, immunohistochemistry showed a marked increase in the number of proliferating $Ki67^+CD3^+$ T cells in the *lamina propria* and epithelium of the patient's colon compared to inflamed colon resected from PIBD controls (figure 3D, S4A-C). Flow cytometry further indicated that most $Ki67^+$ *lamina propria* lymphocytes (LPL) isolated from the patient's colon were $CD4^+$ (figure 3E). Consistent with the hypothesis that proliferation of colonic T cells resulted from their increased responsiveness to IL-2, many $CD4^+$ but no $CD8^+$ colonic LPL from the patient expressed CD25 (figure 3E). Moreover, multiple clusters of cells displaying nuclear pSTAT5 were observed in the patient's colon while they were rare in colonic tissues resected from PIBD controls (figure 3F, S4B-C). Of note, the number of proliferating $CD3^+Ki67^+$ cells and $pSTAT5^+$ cells was very low in the histologically normal duodenum of the patient (figure 3D, S4D), suggesting that expansion of colonic T cells may be driven by the microbiota that is considerably more abundant in the distal intestine than in duodenum.

In order to define how the $CD4^+$ T cells, which proliferate in the patient's colon, may contribute to tissue damage, we next examined mRNA expression of effector molecules and inflammatory cytokines. Confirming the lack of colonic $CD8^+$ T-cell activation, there was no change in the amounts of mRNA encoding Natural Killer Group 2D *(NKG2D)*, a NK receptor expressed by $CD8^+$ IEL, nor in mRNA encoding granzyme B *(GZMB1)* and perforin *(PRF1)*, two effector molecules mediating the cytolytic capacity of IEL, in the patient when compared to PIBD controls (data not shown). Also, very few $IL\text{-}17^+$ cells were detected in the patient's affected colonic tissue (data not shown). In contrast, *IFNG* and *IL21* mRNA were increased in the inflamed colonic tissue of the patient compared to inflamed PIBD resection controls (figure 3G, S5A-B) and numerous $T\text{-}bet^+$ and $IL\text{-}21^+$ cells were detected by immunohistochemistry in the lamina propria and epithelial layer of the colon (but not of duodenum; figure 3H, S5C). In agreement, $IFN\gamma^+$- and $IL\text{-}21^+$ were detectable by flow cytometry in lamina propria $CD4^+$ T cells isolated from the patient's colon (figure S5D).

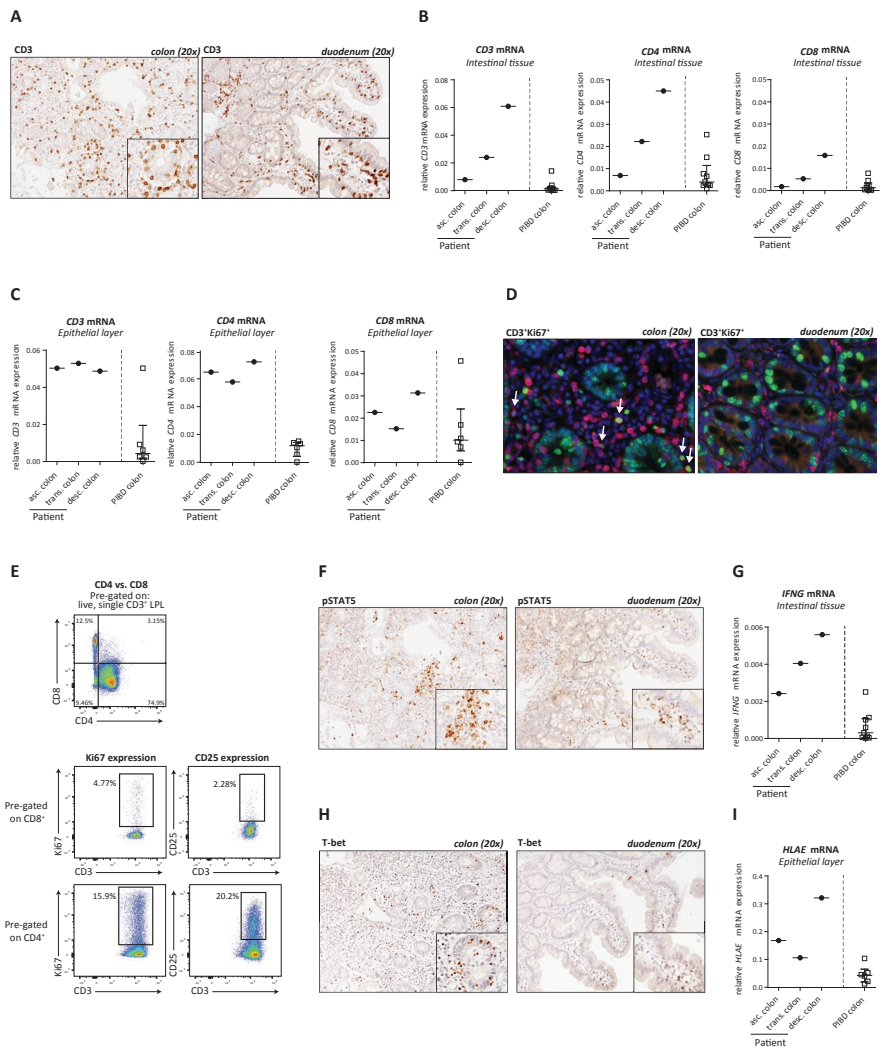

Figure 3. Increased proliferation and activation of colonic $CD4^+$ IEL and LPL in the patient with IL2RA duplication compared to treatment-resistant pediatric-onset IBD. (A, F, H) Representative immunohistochemical staining for CD3, pSTAT5 and Tbet in paraffin-embedded resected inflamed colonic tissue (visit S2) and paraffin-embedded tissue of the unaffected duodenum at time of diagnosis (visit S0). **(B)** *CD3*, *CD4* and *CD8* mRNA and **(G)** *IFNG* mRNA expression in total resected colonic tissue of the patient and PIBD controls. **(C)** *CD3*, *CD4* and *CD8* mRNA and **(I)** *HLAE* mRNA expression in the epithelial layer isolated from resected colonic tissue of the patient and PIBD controls. **(D)** Representative immunofluorescent double staining of paraffin-embedded resected colonic tissue of the patient. Green=Ki67, red=CD3, blue=4',6-diamidino-2-phenylindole (DAPI) nuclear staining. **(E)** LPL were isolated from inflamed colonic tissue of the patient. Frequencies of $CD4^+$ and $CD8^+$ cells in live $CD3^+$ LPL, and Ki67 and CD25 expression by $CD4^+$ and $CD8^+$ LPL were analyzed by flow cytometry.

Finally, mRNA encoding the non-classical MHC-I molecule *HLAE* (figure 3I), was higher in the patient's colonic epithelial layer compared to pediatric IBD controls, likely reflecting the increased local production of IFNγ.

Altogether, these data indicated that increased T-cell responsiveness to IL-2 in the patient with *IL2RA* duplication licensed expansion and activation of colonic CD4⁺ T cells expressing T-bet and secreting large amounts of IFNγ and IL-21.

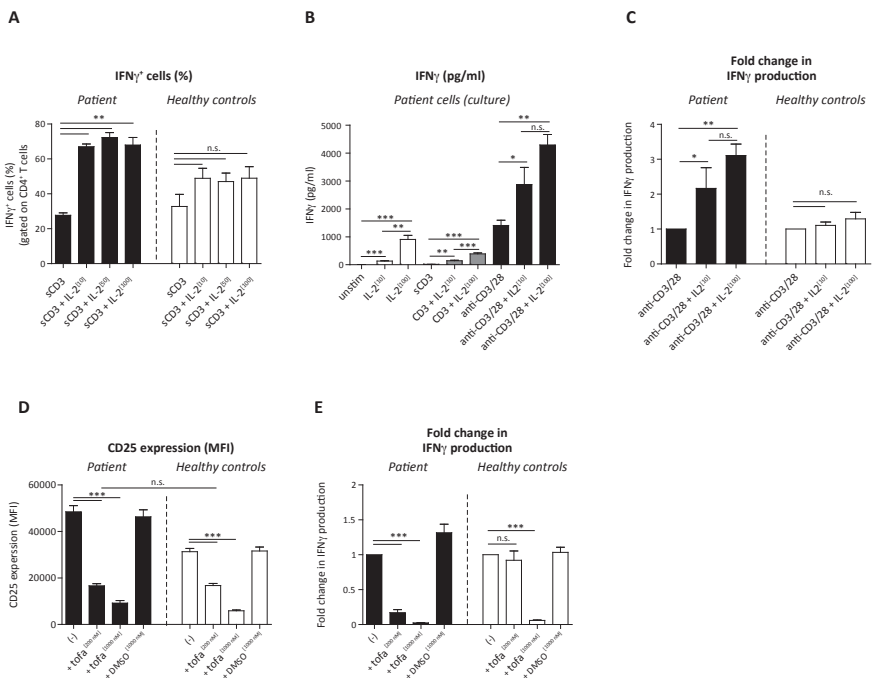

Figure 4. Increased IL-2 signaling enhances IFNγ production and is reversed by JAK1/3 inhibition. (A-C) PBMCs of the patient and healthy adult controls were stimulated with anti-CD3 (500 ng/ml) or anti-CD3/anti-CD28 beads (bead-to-cell ratio 1:2) in the absence or presence of IL-2 (1, 50 or 100 IU/ml) for 48 hours. **(A)** Percentage of IFNγ-expressing CD4⁺ T cells were analyzed by flow cytometry. **(B)** IFNγ secretion by patient cells was analyzed using ELISA. **(C)** IFNγ response of patient and healthy adult donors are shown. The relative increase in IFNγ secretion between anti-CD3/CD28 and cultures with IL-2 is shown (considering the percentage of cytokine secretion upon anti-CD3/CD28 stimulation as 100%). **(D-E)** PBMCs of the patient and healthy adult controls were stimulated with anti-CD3/anti-CD28 beads (bead-to-cell ratio 1:2) in the absence or presence of tofacitinib (200 nM or 1000 nM) for 48 hours. **(D)** CD25 expression on CD4⁺ T cells was analyzed by flow cytometry. **(E)** Supernatants were assayed for IFNγ using an ELISA. The relative difference in IFNγ secretion between anti-CD3/CD28 and cultures with tofacitinib is shown (considering the percentage of cytokine secretion upon anti-CD3/CD28 stimulation as 100%). Data are mean ± SD (patient) and mean ± SEM (adult healthy controls, n=4); N.s., not significant, *p<0.05, **p<0.01, ***p<0.001 using one-way ANOVA followed by the Bonferron's Multiple Comparison Test.

Increased IL-2 signaling enhances TCR-driven IFNγ production in CD4⁺ T cells of the patient and this effect is reversed by JAK1/3 inhibition.

To assess if this high IFNγ response was related to the increased CD25 expression and IL-2 signaling observed in the patient's T cells, we next compared the effect of IL-2 on IFNγ production in PBMC from the patient and from controls. A significant enhancing effect of IL-2 on the frequency of IFNγ-producing CD4⁺ T cells was observed in the patient's PBMC stimulated with anti-CD3, but not in control PBMC (figure 4A). Along the same line, exogenous IL-2 potentiated the secretion of IFNγ induced by CD3 or anti-CD3/CD28 stimulator beads in PBMC of the patient but not of controls (figures 4B, 4C). To further demonstrate the role of IL-2 signaling in potentiating IFNg secretion by patient's T cells, we used the JAK1/3 inhibitor tofacitinib to inhibit IL-2 signaling. As shown in figure 4D, tofacitinib restored normal CD25 expression (figure 4D) and significantly reduced IFNγ secretion by patient CD4⁺ T cells (figure 4E). This result was achieved at suboptimal concentration of tofacitinib (200 nM), which had no effect on IFNγ secretion by control PBMC (figure 4E). A higher concentration of tofacitinib (1000 nM) equally reduced IFNγ secretion in PBMC from healthy controls and from the patient (figure 4E). These data support the view that the enhanced IFNγ response observed in the patient's colon resulted from increased CD25 expression and subsequent enhanced IL-2 signaling.[28, 29]

DISCUSSION

To our knowledge, this is the first description of a patient with an *IL2RA* locus duplication presenting with treatment-resistant colitis at 2 years of age. The patient's colon, but not the unaffected small intestine was infiltrated with proliferating CD3⁺, predominantly CD4⁺ T cells, and contained numerous T-bet⁺ cells expressing high levels of *IFNG* mRNA. After subtotal colectomy and during complete clinical disease remission, CD25 expression was increased in circulating effector memory CD4⁺ T cells and on-going activation of peripheral CD4⁺ T cells was evidenced by increased STAT5 phosphorylation and proliferation. In keeping with their enhanced expression of CD25, peripheral CD4⁺ T from the patient showed increased responsiveness to IL-2, which potentiated their production of IFNg after TCR stimulation. Conversely, inhibiting IL-2 signaling with the JAK1/3 inhibitor tofacitinib restored normal CD25 expression and ablated TCR-induced IFNγ secretion in patient's T cells. Altogether, these results indicate that duplication of the *IL2RA* gene causes increased upregulation of CD25 in activated CD4⁺ T cells, which, as a consequence, undergo excessive stimulation in the antigen-rich environment of the colon and induce inflammation.

Our results showing that duplication of the *IL2RA* locus potentiates CD25 expression and IL-2 signaling are reminiscent of previous reports showing that CD25 surface

expression on memory T cells is variable and can be correlated with haplotypes of the *IL2RA* region, conferring susceptibility to type I diabetes and multiple sclerosis.[30] Along the same line, susceptibility to UC and CD associates with single nucleotide polymorphisms (SNPs) in the *IL2RA* locus[3] although quantitation of CD25 surface expression on $CD4^+$ T cells of individuals bearing this IBD-associated SNP has not yet been reported. Understanding the functional consequences of genetic variation in the *IL2RA* locus in IBD and determining associating disease patterns and therapy responsiveness may therefore have relevance for IBD.

As subtotal colectomy has now effectively induced clinical remission in the patient for more than 27 months without further need of medication but did not correct the increased levels of CD25 on T cells, we conclude that the *IL2RA* locus duplication alone is not sufficient to drive disease. As development of the microbial-host mutualism is a key immunological process in the intestine at young age, we anticipate that microbial stimulation and subsequent antigenic triggering of T cells may have initiated colonic disease. Accordingly, the diseased colon contained many proliferating $CD3^+Ki67^+$ cells with increased STAT5 phosphorylation, which were absent in the duodenum where the concentration in bacteria is considerably less. Despite elevated CD25 expression on both $CD4^+$ and $CD8^+$ T cells, infiltration of the colonic mucosa was dominated by $CD4^+$ T cells, further suggesting that microbiota-derived antigen may have triggered disease. Supporting this hypothesis, anti-microbial antibodies with a wide range of specificity were detectable in the patient's plasma (data not shown). Interestingly, the cellular infiltrate in the affected colonic tissue comprised many T-bet^+, $IFNg^+$ and $IL\text{-}21^+$ cells, but only very few $IL\text{-}17^+$ cells. These observations are in line with previous studies demonstrating that IL-2 signaling supports Th1 responses, induces *Tbx21* and IFNγ[28, 31] but may inhibit Th17 responses.[32, 33]

The patient's duplication consisted of a 374 kb genomic segment including the *IL2RA* gene, the *IL15RA* gene and three additional genes (see Table 2 for all genes and their functions contained in the duplication). To date, none of the additional three genes *(FBXO18, ANKRD16* and *RBM17)* have been reported to be involved in immune responses. In contrast, it cannot be formally excluded that, besides functional changes in the CD25 pathway, increased IL-15 signaling may have participated in the patient's disease. However, no enhanced *IL15RA* mRNA could be detected in the inflamed colon compared to PIBD controls and cell numbers of leukocyte populations preferentially maintained by IL-15, such as NK cells and $CD8^+$ memory T cells[34, 35], were increased neither in the patient's peripheral blood nor in colonic tissue. Moreover, *in vitro* stimulation assays with IL-15 did not reveal increased IL-15 receptor function. Of note, IL-15 signaling in T cells mainly depends on a signaling module consisting of the IL-2Rβ chain and of the common γ chain that are shared with IL-2. Yet, and in contrast with IL-2, IL-15 binds the $\beta\gamma_c$ complex with high avidity and does not require IL-15RA for downstream activation of the JAK/STAT pathway and IL-15Rα seems mainly involved *in vivo* in the transpresentation of IL-15/IL-15Rα complexes to lymphocytes.[36]

Phenotypic stratification of the clinically heterogeneous group of pediatric IBD patients on the basis of their underlying disease mechanisms is pivotal to enable targeted therapy. Although, the here discovered pattern of hyper-CD25 associated inflammation was not dominant in the eight control pediatric-onset IBD patients used in this study, future studies are aimed to identify IBD patients with a similar pattern of hyper-IEL, IFNγ and pSTAT5 positive lesions. This may uncover a specific subgroup of patients with predominant $CD4^+$ T-cell driven disease, who might benefit from IL-2/IL-2R signaling targeting therapies.

Taken together, our data show how increased CD25 expression and enhanced IL-2 signaling can predispose to colonic inflammation and identify a novel mechanism of monogenic form of IBD. These findings contrast with the severe autoimmune enteropathy that can develop in patients lacking $CD25^8$ and highlight the importance of tightly controlled of IL-2 signaling to preserve intestinal homeostasis.

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REFERENCES

1. Sartor RB. Pathogenesis and immune mechanisms of chronic inflammatory bowel diseases. Am J Gastroenterol. 1997 Dec;92(12 Suppl):5S-11S.
2. Aranda R, Sydora BC, McAllister PL, et al. Analysis of intestinal lymphocytes in mouse colitis mediated by transfer of CD4+, CD45RBhigh T cells to SCID recipients. J Immunol. 1997 Apr 1;158(7):3464-73.
3. Jostins L, Ripke S, Weersma RK, et al. Host-microbe interactions have shaped the genetic architecture of inflammatory bowel disease. Nature. 2012 Nov 1;491(7422):119-24.
4. Uhlig HH. Monogenic diseases associated with intestinal inflammation: implications for the understanding of inflammatory bowel disease. Gut. 2013 Dec;62(12):1795-805.
5. Uhlig HH, Schwerd T. From Genes to Mechanisms: The Expanding Spectrum of Monogenic Disorders Associated with Inflammatory Bowel Disease. Inflamm Bowel Dis. 2016 Jan;22(1):202-12.
6. Glocker EO, Kotlarz D, Boztug K, et al. Inflammatory bowel disease and mutations affecting the interleukin-10 receptor. N Engl J Med. 2009 Nov 19;361(21):2033-45.
7. Bennett CL, Christie J, Ramsdell F, et al. The immune dysregulation, polyendocrinopathy, enteropathy, X-linked syndrome (IPEX) is caused by mutations of Foxp3. Nat Genet. 2001 Jan;27(1):20-1.
8. Caudy AA, Reddy ST, Chatila T, et al. CD25 deficiency causes an immune dysregulation, polyendocrinopathy, enteropathy, X-linked-like syndrome, and defective IL-10 expression from CD4 lymphocytes. J Allergy Clin Immunol. 2007 Feb;119(2):482-7.
9. Baecher-Allan C, Wolf E, Hafler DA. Functional analysis of highly defined, FACS-isolated populations of human regulatory CD4+ CD25+ T cells. Clin Immunol. 2005 Apr;115(1):10-8.
10. Paliard X, de Waal Malefijt R, Yssel H, et al. Simultaneous production of IL-2, IL-4, and IFN-gamma by activated human CD4+ and CD8+ T cell clones. J Immunol. 1988 Aug 1;141(3):849-55.
11. Liao W, Lin JX, Leonard WJ. Interleukin-2 at the crossroads of effector responses, tolerance, and immunotherapy. Immunity. 2013 Jan 24;38(1):13-25.
12. Siegel JP, Sharon M, Smith PL, et al. The IL-2 receptor beta chain (p70): role in mediating signals for LAK, NK, and proliferative activities. Science. 1987 Oct 2;238(4823):75-8.
13. Sakaguchi S. Naturally arising Foxp3-expressing CD25+CD4+ regulatory T cells in immunological tolerance to self and non-self. Nat Immunol. 2005 Apr;6(4):345-52.
14. Sadlack B, Merz H, Schorle H, et al. Ulcerative colitis-like disease in mice with a disrupted interleukin-2 gene. Cell. 1993 Oct 22;75(2):253-61.
15. Willerford DM, Chen J, Ferry JA, et al. Interleukin-2 receptor alpha chain regulates the size and content of the peripheral lymphoid compartment. Immunity. 1995 Oct;3(4):521-30.
16. Ma A, Datta M, Margosian E, et al. T cells, but not B cells, are required for bowel inflammation in interleukin 2-deficient mice. J Exp Med. 1995 Nov 1;182(5):1567-72.
17. Schwartzentruber D, Lotze MT, Rosenberg SA. Colonic perforation. An unusual complication of therapy with high-dose interleukin-2. Cancer. 1988 Dec 1;62(11):2350-3.
18. Martin R. Anti-CD25 (daclizumab) monoclonal antibody therapy in relapsing-remitting multiple sclerosis. Clin Immunol. 2012 Jan;142(1):9-14.
19. Liang D, Zuo A, Shao H, et al. Role of CD25+ dendritic cells in the generation of Th17 autoreactive T cells in autoimmune experimental uveitis. J Immunol. 2012 Jun 1;188(11):5785-91.
20. Parlato M, Charbit-Henrion F, Pan J, et al. Human ALPI deficiency causes inflammatory bowel disease and highlights a key mechanism of gut homeostasis. EMBO Mol Med. 2018 Apr;10(4).
21. Fabienne Charbit-Henrion MP, Sylvain Hanein , Rémi Duclaux-Loras, Jan Nowak, Bernadette Begue, Sabine Rakotobe, Julie Bruneau, Cécile Fourrage, Olivier Alibeu, Frédéric Rieux-Laucat, Eva Lévy, Marie-Claude Stolzenberg, Fabienne Mazerolles, Sylvain Latour, Christelle Lenoir, Alain Fischer, Capucine Picard, Marina Aloi, Jorge Amil Dias, Mongi Ben Hariz, Anne Bourrier, Christian Breuer, Anne Breton, Jiri Bronski, Stephan Buderus, Mara Cananzi, Stéphanie Coopman, Clara Crémilleux, Alain Dabadie, Clémentine Dumant-Forest, Odul Egritas Gurkan, Alexandre Fabre, Aude Fischer, Marta German Diaz, Yago Gonzalez-Lama, Olivier Goulet, Graziella Guariso, Neslihan Gurcan, Matjaz Homan, Jean-Pierre Hugot, Eric Jeziorski, Evi Karanika, Alain Lachaux, Peter Lewindon, Rosa Lima, Fernando Magro, Janos Major, Georgia Malamut, Emmanuel Mas, Istvan Mattyus, Luisa M. Mearin, Jan Melek, Victor Manuel Navas-Lopez, Anders Paerregaard, Cecile Pelatan, Bénédicte Pigneur, Isabel Pinto Pais, Julie Rebeuh, Claudio Romano, Nadia Siala, Caterina Strisciuglio, Michela Tempia-Caliera, Patrick Tounian, Dan Turner, Vaidotas Urbonas, Stéphanie Willot, Frank M. Ruemmele, Nadine Cerf-Bensussan. Diagnostic yield of next-generation sequencing in very early-onset inflammatory bowel disease: a multicenter study. Journal of Crohn's and Colitis. 2018(In press).

22. Comans-Bitter WM, de Groot R, van den Beemd R, et al. Immunophenotyping of blood lymphocytes in childhood. Reference values for lymphocyte subpopulations. J Pediatr. 1997 Mar;130(3):388-93.
23. Kelly E, Won A, Refaeli Y, et al. IL-2 and related cytokines can promote T cell survival by activating AKT. J Immunol. 2002 Jan 15;168(2):597-603.
24. Smith KA. Interleukin-2: inception, impact, and implications. Science. 1988 May 27;240(4856):1169-76.
25. Zambricki E, Shigeoka A, Kishimoto H, et al. Signaling T-cell survival and death by IL-2 and IL-15. Am J Transplant. 2005 Nov;5(11):2623-31.
26. du Pre MF, van Berkel LA, Raki M, et al. 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.
27. Jarry A, Cerf-Bensussan N, Brousse N, et al. Subsets of CD3+ (T cell receptor alpha/beta or gamma/delta) and CD3- lymphocytes isolated from normal human gut epithelium display phenotypical features different from their counterparts in peripheral blood. Eur J Immunol. 1990 May;20(5):1097-103.
28. Shi M, Lin TH, Appell KC, et al. Janus-kinase-3-dependent signals induce chromatin remodeling at the Ifng locus during T helper 1 cell differentiation. Immunity. 2008 Jun;28(6):763-73.
29. Shatrova AN, Mityushova EV, Vassilieva IO, et al. Time-Dependent Regulation of IL-2R alpha-Chain (CD25) Expression by TCR Signal Strength and IL-2-Induced STAT5 Signaling in Activated Human Blood T Lymphocytes. PLoS One. 2016;11(12):e0167215.
30. Dendrou CA, Plagnol V, Fung E, et al. Cell-specific protein phenotypes for the autoimmune locus IL2RA using a genotype-selectable human bioresource. Nat Genet. 2009 Sep;41(9):1011-5.
31. Liao W, Lin JX, Wang L, et al. Modulation of cytokine receptors by IL-2 broadly regulates differentiation into helper T cell lineages. Nat Immunol. 2011 Jun;12(6):551-9.
32. Laurence A, Tato CM, Davidson TS, et al. Interleukin-2 signaling via STAT5 constrains T helper 17 cell generation. Immunity. 2007 Mar;26(3):371-81.
33. Yang XP, Ghoreschi K, Steward-Tharp SM, et al. Opposing regulation of the locus encoding IL-17 through direct, reciprocal actions of STAT3 and STAT5. Nat Immunol. 2011 Mar;12(3):247-54.
34. Ranson T, Vosshenrich CA, Corcuff E, et al. IL-15 is an essential mediator of peripheral NK-cell homeostasis. Blood. 2003 Jun 15;101(12):4887-93.
35. Kennedy MK, Glaccum M, Brown SN, et al. Reversible defects in natural killer and memory CD8 T cell lineages in interleukin 15-deficient mice. J Exp Med. 2000 Mar 6;191(5):771-80.
36. Meresse B, Korneychuk N, Malamut G, et al. Interleukin-15, a master piece in the immunological jigsaw of celiac disease. Dig Dis. 2015;33(2):122-30.

6

SUPPLEMENTARY DATA

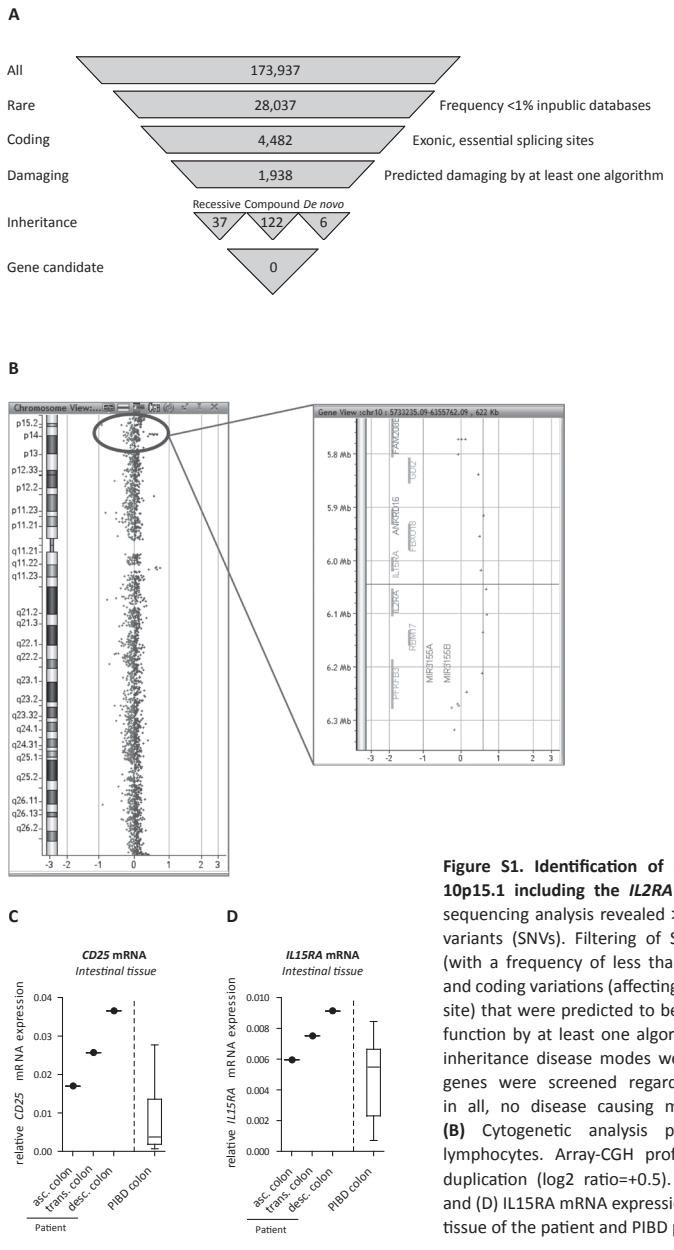


Figure S1. Identification of a 374 kb duplication on 10p15.1 including the *IL2RA* locus. (A) Whole exome sequencing analysis revealed >170,000 single nucleotide variants (SNVs). Filtering of SNVs is shown. First, rare (with a frequency of less than 1% in public databases) and coding variations (affecting exons or essential splicing site) that were predicted to be damaging for the protein function by at least one algorithm were selected. Then, inheritance disease modes were applied and candidate genes were screened regarding protein function. All in all, no disease causing mutations were identified. **(B)** Cytogenetic analysis performed on peripheral lymphocytes. Array-CGH profile showing the 10p15.1 duplication (log2 ratio=+0.5). **(C)** Relative CD25 mRNA and (D) IL15RA mRNA expression in total resected colonic tissue of the patient and PIBD patients (n=8).

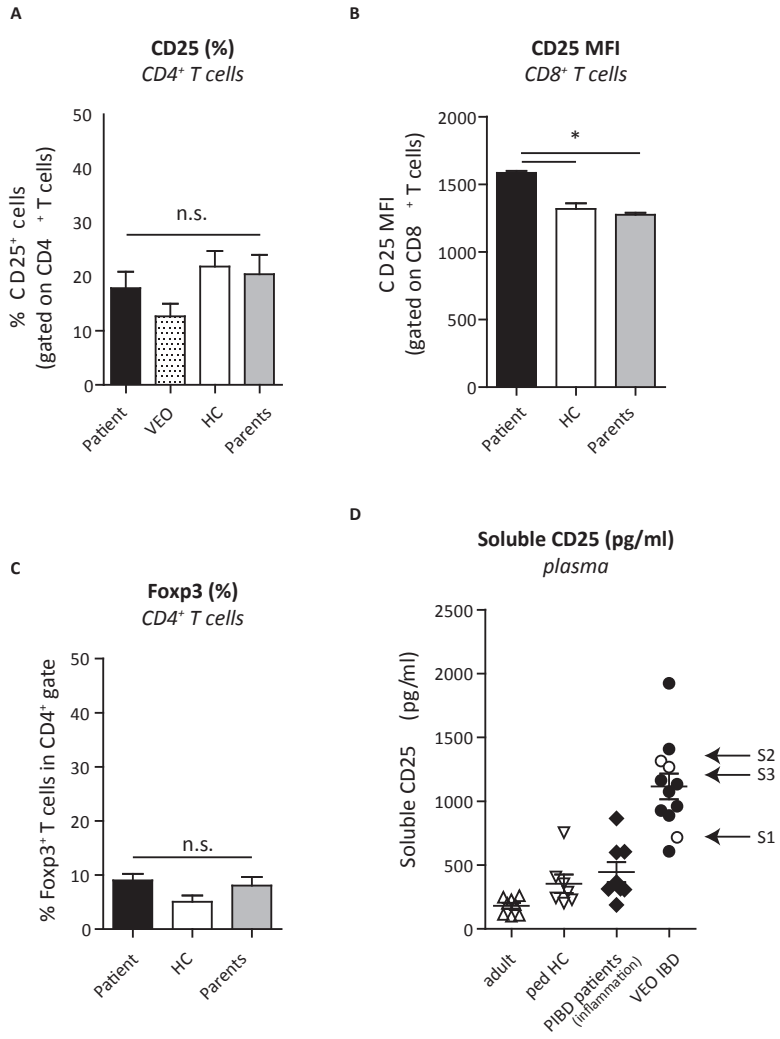


Figure S2. Frequencies of CD25⁺CD4⁺ T cells, Foxp3⁺CD4⁺ T cells and soluble CD25 concentrations in patient and controls. (A-C) Flow cytometric analysis of CD3, CD4, CD8, CD62L, CD38, CD25 and/or Foxp3 expression was performed on peripheral blood from adult healthy controls (HC, n=4), VEO-IBD patients (n=9), the patient and her parents (time points S3 and S4). **(A)** Frequencies of CD25⁺ cells gated on total circulating CD4⁺ T cells. **(B)** CD25 expression (MFI) on total circulating CD8⁺ T cells. **(C)** Frequencies of regulatory CD4⁺Foxp3⁺ T cells in peripheral blood. **(D)** Plasma concentrations of soluble CD25 in adult healthy controls, pediatric healthy controls, PIBD patients with intestinal inflammation (n=8) and VEO-IBD patients (n=9). N.s., not significant, *p<0.05, **p<0.01, ***p<0.001 using one-way ANOVA followed by the Bonferron's Multiple Comparison Test. PIBD, pediatric-onset IBD.

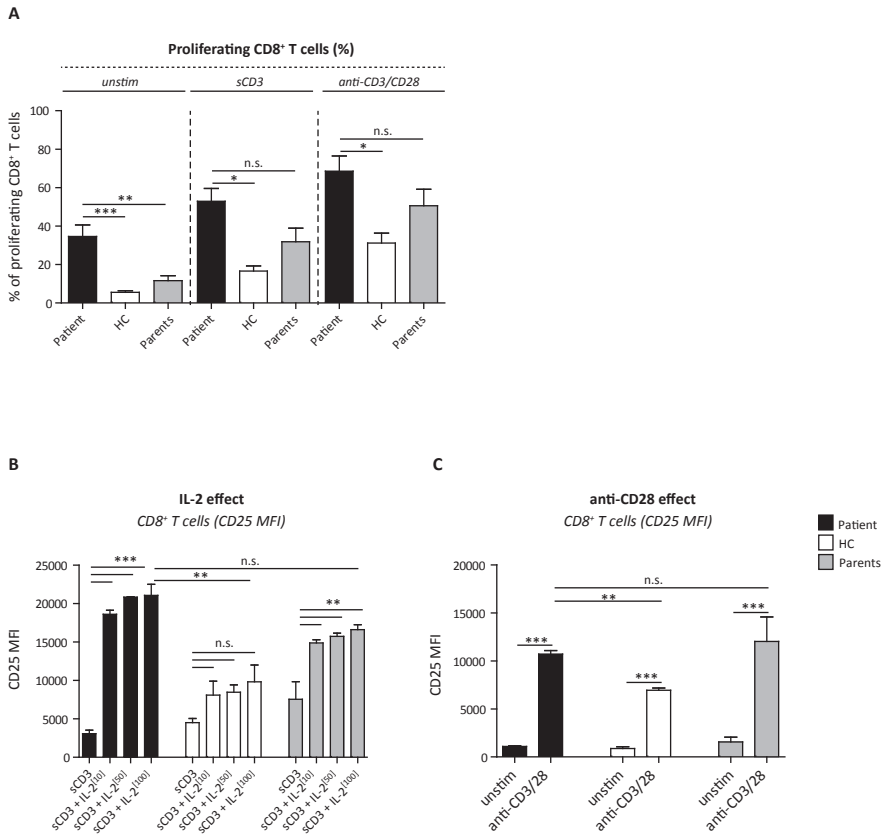
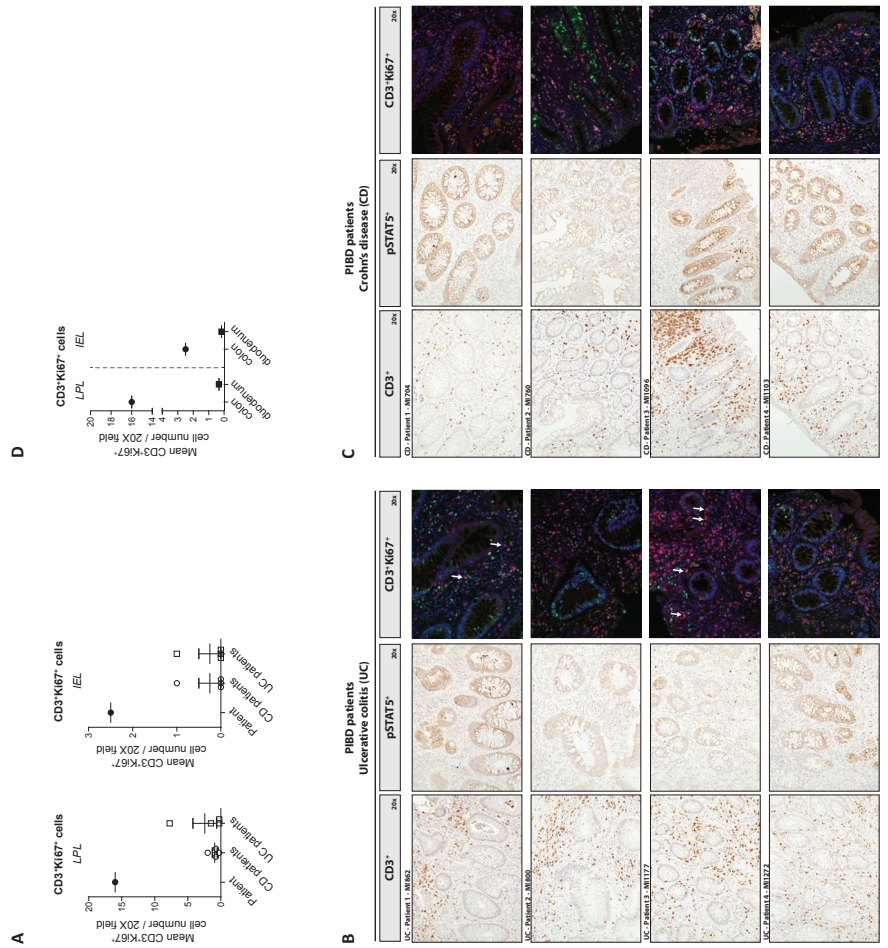


Figure S3. Frequencies of proliferating CD8⁺ T cells and CD25 expression on CD8⁺ T cells after TCR-ligation in combination with increasing doses of exogenous IL-2. (A) Healthy adult control and patient PBMCs (visits S3 and S4) were stimulated with anti-CD3 (500 ng/ml) or antiCD3/anti-CD28 beads (bead-to-cell ratio 1:2) for 48h. **(A)** Percentage of proliferating CD8⁺ T cells was analyzed by CellTrace Violet dilution. **(B)** PBMCs were stimulated with anti-CD3 (500 ng/ml) in the absence or presence of IL-2 (1, 50 or 100 IU/ml). After 48h, cells were stained for CD3, CD8 and CD25 and analyzed by flow cytometry. **(C)** PBMCs were stimulated with anti-CD3/CD28 beads (bead-to-cell ratio 1:2). CD25 expression on CD8⁺ T cells was analyzed at 48h. Data are mean ± SD (patient) or mean ± SEM (healthy adult controls, n=4). Representative of two independent experiments (time points S3 and S4); N.s., not significant, *p<0.05, **p<0.01, ***p<0.001 using one-way ANOVA followed by the Bonferron's Multiple Comparison Test.

Figure S4. Low frequency of proliferating CD3⁺Ki67⁺ cell infiltration and low STAT5 phosphorylation in resected colonic tissue sections of PIBD patients. (A) Numbers of CD3⁺Ki67⁺ cells in resected colonic tissues of the patient and control PIBD patients were quantified in blinded fashion by counting four 20X images. **(B-C)** Representative immunohistochemical staining for CD3 and pSTAT5 and immune fluorescence for CD3 and Ki67 in paraffin-embedded resected inflamed colonic tissue of treatment-resistant pediatric-onset UC patients (n=4) and treatment-resistant pediatric-onset CD patients (n=4). Green=Ki67, red=CD3, blue=4',6-diamidino-2-phenylindole (DAPI) nuclear staining. **(D)** Numbers of CD3⁺Ki67⁺ cells in the patient's colonic inflamed biopsies were compared to non-inflamed duodenal tissue obtained during initial assessment at diagnosis.



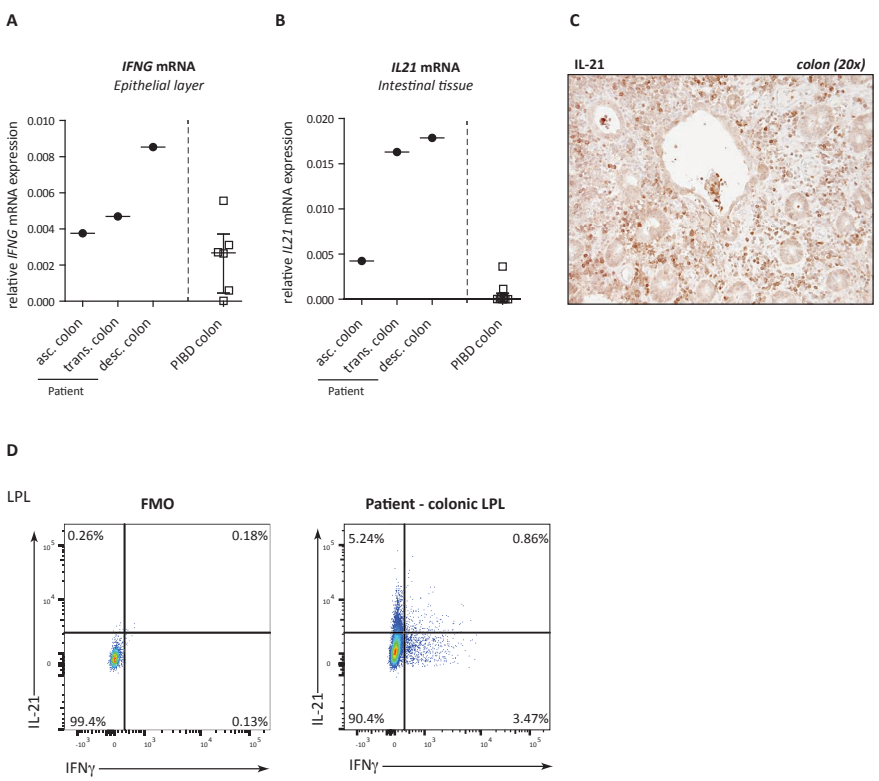


Figure S5. IL-21⁺ and IFNγ⁺ cells in inflamed patient colonic tissue. (A) *IFNG* mRNA expression in epithelial layers isolated form resected colonic tissue in the patient and PIBD patients. **(B)** *IL21* mRNA expression in total resected colonic tissue of the patient and PIBD patients. **(C)** Representative immunohistochemical staining for IL-21 in paraffin-embedded resected inflamed colonic tissue (visit S2). **(D)** LPL were isolated from patient inflamed colonic tissue and stimulated with PMA (0.02 µg/ml) and ionomycin (0.5 µg/ml). Frequencies of IL-21⁺ and IFNγ⁺ cell in CD3⁺ LPL were analyzed by flow cytometry.

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7

MALIGNANCY AND MORTALITY IN PEDIATRIC-ONSET INFLAMMATORY BOWEL DISEASE: A SYSTEMATIC REVIEW

Chapter 7

Inflamm. Bowel Dis. 2018 Mar 19;24(4):732-741.

ABSTRACT

Background: Cancer and death are the most severe outcomes that affect patients with IBD. These outcomes are even more severe if they occur at young age but are rare, even in the general population. We conducted a systematic review to provide an overview of all reported pediatric (PIBD) patients with severe outcome.

Methods: A literature search identified publications that reported development of cancer or fatal outcome in PIBD patients. Studies were eligible for inclusion when (1) article written in English, (2) original data, (3) individual patient information, (4) full text available, (5) study population consisting of patients diagnosed with IBD under the age of 19 years, who (6) developed malignancy or fatality at any point later in life.

Results: A total of 98 included studies comprised data of 271 PIBD patients who developed cancer and/or fatal outcome at any point later in life. Meta-analysis demonstrated an increased risk for cancer in PIBD patients (pooled standardized incidence ratio 2.23, 95% CI: 1.98 – 2.52). The most frequent type of non-fatal cancer was lymphoma, whereas colorectal carcinomas were the most frequently reported type of fatal cancer in PIBD patients and were particularly associated with PSC. The majority of patients with non-cancer related fatal outcomes were diagnosed with ulcerative colitis and most often died due to infectious complications or severe disease-associated complications.

Conclusions: The data in this review confirm that PIBD associated malignancy and mortality are rare and detailed clinical characteristics are limited. Prospective and international collaborations are needed to obtain more detailed patient-specific information, which is necessary to investigate the relationship between severe outcomes in PIBD patients and the currently used therapeutic strategies.

INTRODUCTION

The incidence of pediatric-onset inflammatory bowel disease (PIBD) has risen significantly in the past two decades in Europe and North America[1-3] and PIBD is becoming more prevalent in the rest of the world.[4] PIBD is characterized by extensive intestinal involvement and rapid disease progression.[5-7] Consequently, it is often accompanied by severe complications, such as growth failure and need for bowel surgery.[8] In addition, the longer disease duration, frequently combined with a more extensive and severe colitis, puts PIBD patients at a higher risk for developing colorectal cancer (CRC) than adult-onset IBD patients.[9] Even after adjusting for extent of disease, age at diagnosis was found to be an independent risk factor for CRC.[10, 11]

Despite their success in limiting persistent disease activity in IBD patients, immunosuppressant drugs can lead to serious and opportunistic infections and increase the risk of developing cancer.[12-14] The use of thiopurines has been shown to increase the risk for lymphoma[15, 16], non-melanoma skin cancers[17], and cervical cancer.[18] In addition, biological or non-biological immune suppression increases the risk of infectious complications, which may translate into an increased risk of mortality.[11, 19, 20] There is a growing concern that cancer and mortality will affect pediatric patients, as a growing number of PIBD patients are now being exposed to immunosuppressant drugs over longer periods of time.

Due to the rarity of cases, few studies have investigated cancer and mortality in patients with PIBD. Much of the literature on severe outcomes in PIBD includes anecdotal case reports and case-series but very few population-based studies. To date, there is only one systematic review focussed on lymphoma and infections related to biological therapy[21], hence, a coherent overview of PIBD patients that develop cancer or fatal outcome is lacking. Better insight in the characteristics of these patients will help to identify predictive factors of severe disease course, which are ultimately needed to further optimize our therapeutic strategies. In this review, we conducted a systematic literature search to provide an overview of patients with IBD diagnosed at pediatric age who developed cancer or suffered a fatal outcome at any point later in life.

METHODS

Literature search.

We conducted a literature search to identify all published studies that reported cancer or mortality in patients diagnosed with PIBD under the age of 19 years. A systematic search up to 1st of June 2017 was performed in the following databases: Medline, Ovid

MEDLINE, Embase, Cochrane Central, Web of Science and Google Scholar. The detailed search strategy was developed in consultation with a research librarian and is outlined in the Supplemental Search Strategy (provided online on the website of the *Inflammatory Bowel Diseases* journal). Reference lists of review articles and selected papers were also reviewed. In order to obtain full texts of potentially relevant papers both electronic databases and libraries were accessed.

Selection criteria.

All studies (prospective and retrospective cohort studies, case control studies, cross-sectional studies, case series and case reports) that fulfilled the following criteria were included: (1) article written in English, (2) original data available in article, (3) individual patient information available, (4) full text article available, (5) study population consisting of patients diagnosed with IBD under the age of 19 years, who (6) developed cancer or fatal outcome at any point later in life. No publication date restrictions were imposed. Animal studies, reviews, meta-analysis, editorials and practical summaries or guidelines were excluded.

Data extraction and quality assessment.

Systematic review has been performed according to PRISMA guidelines.[22] Two reviewers (MA and MEJ) independently conducted an initial screen of identified abstracts and titles. Abstracts were eliminated in this initial screen if they did not meet the criteria as described above. Inconsistencies on inclusions were resolved by consensus. Abstracts meeting these criteria were eligible for full-text review. The risk of bias was determined using the Newcastle-Ottawa Quality Assessment Scale for cohort studies. From the included articles the following information was retrieved using a standard data extraction form: gender, IBD type, age at IBD onset, IBD treatment, type of cancer or cause of mortality, age at cancer diagnosis and/or age at death. A random effects meta-analysis was performed to compute pooled standardized incidence ratios (SIR) for all types of cancer and CRC. Analyses were performed by a biostatistician using R version 3.4.2.

RESULTS

The electronic search yielded a total of 10,187 articles, of which 6,984 potentially relevant articles remained after duplicates were removed (Figure 1A). An additional 43 articles were manually selected from reference lists. All records were screened on the basis of title and abstract to identify studies reporting development of cancer or fatal outcome in patients with IBD diagnosed at pediatric age. After screening, a total of 6,820 were

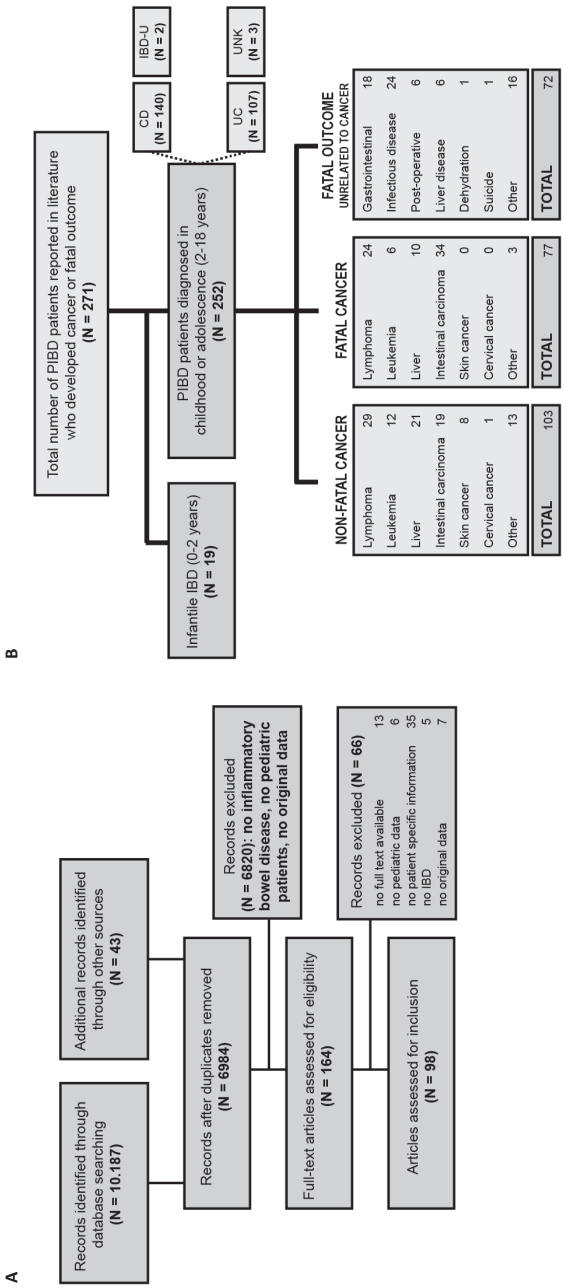

Figure 1. Flow diagram of study selection and overview of included patients. (A) PRISMA Flow diagram of study selection and exclusion stges. **(B)** Summary of total number of PIBD patientsreported in literature who developed cancer or suffered a fatal outcome.

excluded and 164 potential articles remained that were eligible for full-text assessment. Of these, 66 articles were excluded. Most important reasons for exclusion were the lack of patient specific information (n=35) and the unavailability of full text after extensive online and medical library search (n=13). Six studies did not report any pediatric data, 5 studies did not describe IBD patient population, 7 studies were either retrieved twice due to publication in different sources or discussed data of an original article already included in the review. A total of 98 articles were included with a high inter-observer agreement (Cohen's kappa κ= 0,92146).

Table 1 provides characteristics of the 98 included studies. Most identified studies were case reports (n=47), case series (n=20) and retrospective cohort studies (n=25). Only 4 prospective cohort studies, 1 cross-sectional study and 1 case control study were retrieved with our literature search. Most retrospective cohort studies were single-center (68%). There were 2 prospective studies published by Hyams *et al.* that were both multicenter cohort studies, with data on PIBD patients (both UC and CD) from the Pediatric Inflammatory Bowel Disease Collaborative Research Group[23] and the DEVELOP study.[24] Two prospective studies were single-center studies focusing on long-term outcomes in pediatric-onset CD patients.[25, 26] The follow-up period was reported in 18 studies of all cohort studies and case control studies (60%, Table S1). The median time of follow-up was 13.0 years (IQR 5.6-17.7).

Table 1. Characteristics of included studies.

	Type of study	Number
1	Retrospective study	**25**
2	Case report	**47**
3	Case series	**20**
4	Prospective study	**4**
5	Cross sectional	**1**
6	Case control study	**1**
	Total	**98**

A total of 4 population-based studies (Figure 2) describe SIR for all types of cancer in PIBD patients over the last 3 decades. Of these, 3 were pooled in a meta-analysis demonstrating an increased risk for cancer in PIBD patients (pooled SIR 2.23, 95% CI: 1.98-2.52). Five studies reported SIR for CRC. One study reported a SIR for CRC in a PIBD cohort of 69 patients with 1602 patient-years of follow-up (25.7, 95% CI: 3.1-92.7).[27] However, as development of CRC is associated with colonic disease, most studies reported SIRs for CRC in CD and UC separately. Meta-analysis showed a strongly increased risk for CRC in UC patients (pooled SIR 54.74, 95% CI: 25.98-115.35) and a lower but significantly increased

						Newcastle-Ottawa tool	
Study	Population	Size (N)	Period of diagnosis	Follow-up period	Malignancy (N)	Selection	Comparability	Outcome
Peneau (2013)	Pediatric IBD patients < 17 years	698	1988-2004	Median 11.5 years	9	****	*	**
Kappelman (2014)	Pediatric IBD patients < 20 years	NR	1978-2010	NR	NR	****	*	**
Hyams (2017)	Pediatric IBD patients < 17 years	5,766	2007-2016	24,543 patient-years (median 4.7 years)	15	****	*	**
Olen (2017)	Pediatric IBD patients < 18 years	9,405	1964-2014	148,682 patient-years	497	****	**	**

SIR for cancer	95% CI
IBD: 3.0	1.3 – 5.9
CD: 2.3 UC: 2.0	1.5 - 3.4 1.4 - 1.7
Drug exposed: 2.43 Non-drug exposed: 1.30	Drug exposed: 1.29-4.15 Non-drug exposed: 0.16-4.71
Total: 2.2 UC: 2.6 CD: 1.7	Total: 2.0 - 2.5 UC: 3.2 - 3.0 CD: 1.5 - 2.1

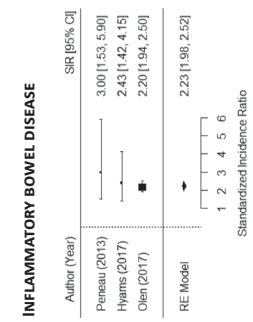

Figure 2. SIR for all types of cancer in PIBD patients over the last three decades. Table and graphs presenting SIRs by study including a pooled SIR for all types of cancer occurring in patients with pediatric-onset IBD following a Random effects (RE) model. Kappelman et al. (2014) was not included in the meta-analysis as a SIR in the total PIBD population was not described. SIR, Standardized incidence ratio; RE, random effects; CI confidence interval; IBD, inflammatory bowel disease; UC, ulcerative colitis; CD, Crohn's disease; CRC, colorectal cancer.

risk in CD patients (pooled SIR 6.32, 95% CI: 3.93- 10.15).

No statistically significant increased standardized mortality ratios (SMR) in PIBD patients compared to the background population have been reported in the last 2 decades. Peneau *et al.* observed a 1.4-fold increased risk of mortality (95% CI: 0.5-2.9) in PIBD patients but this did not significantly differ from the background population. Likewise, the SMR was not significantly increased in pediatric CD patients compared to the background population in the cohort studied by Jakobsen *et al* (2.1, 95% CI: 0.6-5.4).

Study population.

To gain further insight in the characteristics of PIBD patients that develop cancer or suffer a fatal outcome, detailed patient characteristics from all studies describing PIBD patients with cancer or fatal outcome were obtained. The 98 studies included in our review comprised detailed data of 271 PIBD patients who developed cancer and/or fatal outcome at any point later in life (Figure 1B). Eight reports described patients diagnosed with IBD under the age of 2 years; detailed characteristics of these infantile IBD patients are not further described in this manuscript. The remaining 92 studies comprised a total group of 252 PIBD patients with IBD diagnosed during childhood or adolescence. These patients were diagnosed with CD (65.2%, n=140), UC (43.0%, n=107) or IBD-U (0.8%, n=2) at a median age of 12.0 years (IQR 8.78-15.0; IBD type missing in 3 cases). The majority of those patients were male (63.4%, n=151). Median age at either diagnosis of cancer or occurrence of death was 18.00 years (IQR 15.0-25.0). All individual patient information of PIBD patients with IBD diagnosed during childhood or adolescence can be reviewed in Supplementary Table 1 (provided online on the website of the *Inflammatory Bowel Diseases* journal).

Malignancy.

Cases with cancer were reported in 80 of 92 studies (87.0%), comprising a total of 180 pediatric-onset IBD patients with a diagnosis of cancer. Of these patients, 77 (42.8%) were reported to have a fatal outcome. Patient characteristics of PIBD patients who developed non-fatal cancer or fatal cancer are described separately in Table 2 and Table 3, respectively.

Non-fatal cancer

A total of 103 patients with cancer but without a fatal outcome were reported in 42 studies. Of these studies 18 were case reports, 8 were cases series, 10 were retrospective studies, 4 were prospective studies, 1 was a case control study and 1 was a cross sectional study. Of the retrospective studies only 2 were multicenter.

In this patient group, patients were diagnosed with CD (63.7%, n=65) or UC (36.3%, n=37) at a median age of 12.00 years (IQR 10.00-15.00; IBD type missing in 1 case). The

median age at diagnosis of cancer was 17.50 years (IQR 15.00-25.00). The majority of patients (64.2%) was male.

Most frequently reported non-fatal cancers were lymphomas (n=29), cholangiocarcinomas (n=16) and CRC (n=19), as shown in Table 2. In the group of patients diagnosed with a lymphoma, 17 patients had a non-Hodgkin lymphoma, including 2 hepatosplenic T-cell lymphomas (HSTCL). The majority of patients with a lymphoma had been exposed to thiopurines (n=18, 62.1%), of which 4 patients had also used a biological. Two-third (68.8%) of the patients with a CRC was male and 84.2% of these cases (n=16) was diagnosed with UC. Most patients with CRC were exposed to monotherapy, either of 6-mercaptopurine (n=3), aminosalicylates (n=2) or steroids (n=1). One patient received no treatment (n=1). Treatment was not reported in the remaining cases (n=12, 63.2%). Of the 19 CRC cases, 9 patients had a documented concomitant diagnosis of primary sclerosing cholangitis (PSC). The median duration of PSC to CRC was 4.0 years (IQR 1.0-8.0; n=5). Two patients were diagnosed with CRC before PSC diagnosis and in 2 patients age at PSC diagnosis was unknown. PSC was not mentioned in the studies reporting the other 7 CRC cases. The youngest 2 patients diagnosed with CRC were 15 years old, and had an IBD duration of 10 and 2 years respectively. In these 2 patients, no information on the presence of PSC was provided. Three of the patients with CRC also had a cholangiocarcinoma (CCA). Of the group of patients with CCA, all but 1 patient had PSC (87.5%, n=14, not mentioned in 1 case) complementary to their UC (n=11) or CD (n=3). Of the 15 patients with CCA, 5 were male, 3 were female and in 7 patients the gender was not described, and 6 of the 15 (40.0%) developed CCA before 19 years of age. The youngest patient diagnosed with CCA was 8 years old and had been diagnosed with IBD since 3 years of age. All non-melanoma skin cancers in this group comprised basal cell carcinomas.

Fatal cancer

A total of 49 studies reported patients who developed cancer with a fatal outcome, including 19 case reports, 11 case series, 18 retrospective studies and 1 prospective study. Of the retrospective studies 4 were multicenter studies and the remaining 14 studies were performed in a single center. In total, the 49 studies comprised patient data of 77 PIBD patients who developed IBD at a median age of 13.00 years (IQR 10.00-16.00). In this patient group, 75.0% of patients were male. Most patients were diagnosed with CD (57.9%, n=44), followed by UC (39.5%, n= 30) and IBD-U (2.6%, n=2). In 1 patient the IBD type was unknown. The median age at death was 20.5 years (IQR 17.0-28.75). In 43 patients the duration from cancer to death was not mentioned. Of the remaining 34 patients, 20 died within one year after their cancer diagnosis and in 14 patients the median duration from cancer diagnosis to mortality was 2.00 years (IQR 1.00-15.00).

Intestinal carcinomas (n=34) and lymphomas (n=24) were most frequently reported

Table 2. Clinical characteristics of PIBD patients with non-fatal cancer.

	Type	**Subtype (number)**	**No.**	**Median age at IBD Dx** (IQR, min-max) [number missing]	**Median age at cancer** (IQR, min-max) [number missing]	**number male (%)** [number missing]	**number UC (%)** **number CD (%)** [number missing]
1	Lymphoma	1a NHL (17) 1b Hodgkin (11) 1c UNK (1)	29	12.00 (10.50-15.00, 2.00-17.00) [0]	16.00 (14.50-17.00, 9.00-49.00) [0]	19 (65.5%) [0]	4 (13.8%) 25 (86.2%) [0]
2	Leukemia	2a ALL (3) 2b AML (3) 2c chronic leukemia (3) 2d UNK (3)	12	13.50 (11.25-15.00, 6.00-18.00) [0]	15.50 (14.25-20.75, 11.00-24.00) [0]	10 (83.3%) [0]	4 (33.3%) 8 (66.7%) [0]
3	Liver	3a CCA (16) 3b HCC (4) 3c UNK (1)	21	12.00 (5.00-14.00, 2.00-17.00) [4]	22.00 (17.50-32.00, 8.00-52.00) [0]	8 (57.1%) [7]	12 (57.1%) 9 (42.9%) [0]
4	Intestinal carcinoma	4a CRC (16) 4b Small intestinal adenocarcinoma (1) 4c Carcinoid (2)	19	12.50 (10.00-15.25, 5.00-18.00) [1]	24.00 (16.00-33.50, 13.00-69.00) [0]	11 (61.1%) [1]	14 (73.7%) 5 (26.3%) [0]
5	Skin cancer	5a Non-melanoma skin cancer (6) 5b Melanoma (2)	8	12.00 (7.50-15.00, 7.00-16.00) [0]	17.00 (15.50-22.00, 14.00-27.00) [0]	5 (62.5%) [0]	1 (12.5%) 7 (87.5%) [0]
6	Cervical cancer		1	17.00 (NR) [0]	33.00 (NR) [0]	0 (0%) [0]	0 (0%) 1 (100%) [0]
7	Other		13	15.00 (11.50-16.50, 3.00-17.00) [0]	18.50 (15.00-34.75, 4.00-47.00) [1]	8 (61.5%) [0]	2 (16.7%) 10 (83.3%) [1]
	Total		**103**	**12.00** **(10.00-15.00, 2.00-18.00)** **[5]**	**17.50** **(15.00-25.00, 4.00-69.00)** **[1]**	**61 (64.2%)** **[8]**	**37 (36.3%)** **65 (63.7%)** **[1]**

NR, not relevant, UNK, unknown, NHL, non-Hodgkin lymphoma, ALL, acute lymphoblastic leukemia, AML, acute myeloid leukemia, CCA, cholangiocarcinoma, HCC, hepatocellular carcinoma, CRC, colorectal carcinoma.

Table 3. Clinical characteristics of PIBD patients with fatal cancer.

	Type	**Subtype (number)**	**No.**	**Median age at IBD Dx** (IQR, min-max) [number missing]	**Median age at mortality due to cancer** (IQR, min-max) [number missing]	**number male (%)** [number missing]	**number UC (%)** **number CD (%)** **number IBD-U (%)** [number missing]
1	Lymphoma	1a NHL (19) 1b Hodgkin (2) 1c UNK (3)	24	14.50 (11.25-16.00, 2.00-18.00) [0]	18.00 (15.25-19.75, 12.00-52.00) [0]	20 (87.0 %) [1]	4 (16.7 %) 18 (75.0%) 2 (8.3%) [0]
2	Leukemia	2a ALL (2) 2b AML (2) 2c chronic leukemia (0) 2d UNK (2)	6	7.50 (2.00-14.75, 2.00-17.00) [0]	18.00 (2.00-33.75, 2.00-51.00) [0]	4 (66.7%) [0]	2 (33.3%) 4 (66.7%) [0]
3	Liver	3a CCA (8) 3b HCC (2) 3c UNK (0)	10	11.00 (8.00-13.00, 7.00-17.00) [2]	22.50 (19.50-31.00, 17.90-33.00) [0]	7 (70.0%) [0]	5 (50.0%) 5 (50.0%) [0]
4	Intestinal carcinoma	4a CRC (25) 4b Small intestinal adenocarcinoma (9) 4c Carcinoid (0)	34	12.00 (10.75-15.00, 2.00-17.00) [0]	25.50 (20.00-30.50, 7.00-48.00) [0]	22 (71.0%) [3]	19 (55.9%) 15 (44.1%) [0]
5	Skin cancer	5a Non-melanoma skin cancer (0) 5b Melanoma (0)	0	NR	NR	NR	NR
6	Cervical cancer		0	NR	NR	NR	NR
7	Other		3	8.00 (NR) [2]	19.50 (14.00-25.00, 14.00-25.00) [1]	1 (50%) [1]	0 (0%) 2 (100% [1]
	Total		**77**	**13.00** **10.00-16.00, 2.00-18.00** **[4]**	**20.50** **(17.00-28.75, 2.00-52.00)** **[1]**	**54 (75.0%)** **[5]**	**30 (39.5%)** **44 (57.9%)** **2 (2.6%)** **[1]**

NR, not relevant, UNK, unknown, NHL, non-Hodgkin lymphoma, ALL, acute lymphoblastic leukemia, AML, acute myeloid leukemia, CCA, cholangiocarcinoma, HCC, hepatocellular carcinoma, CRC, colorectal carcinoma.

in PIBD patients who died of cancer. Most intestinal carcinomas were CRC (n=25, 73.5%). Treatment was not reported in 72.0% (n=18) of CRC cases (Table S1), whereas treatment information was available in the majority of patients who died due to a lymphoma (not reported in n=4, 16.7%). Two-third of the patients with a lymphoma had been exposed to thiopurines (n=17, 70.8%). In almost all patients diagnosed with lymphoma the duration of thiopurine use and the duration from diagnosis of lymphoma to moment of death was unknown (n=18, 75%). Non-Hodgkin lymphoma was diagnosed in 19 patients, of which the majority (84.2%, n=16) had a HSTCL. All of the patients diagnosed with a HSTCL had been exposed to thiopurines. Detailed data on the use of biologicals was lacking in this group of patients. A strong male predominance was observed among HSTCL cases (87.5%, n=14). Two male patients developed Hodgkin lymphoma and 1 of those was also diagnosed with CCA.

In total 8 patients with UC (n=5) or CD (n=3) were diagnosed with CCA (5 males). PSC was described in 5 of those patients (62.5%); 4 males and 1 female, who died at a median age of 21.0 years (IQ range 18.0-24.5). The female patient was known to have CD, the male patients both CD (n=2) and UC (n=2).

Most patients with a fatal intestinal carcinoma had a CRC (73.5%, n=25). This was most frequently observed in males (60.0%, n=15) and patients with UC (72%, n=18; gender not described in 3 cases). Seven of the 25 (28.0%) developed CRC before 19 years of age. Information on concomitant PSC was provided in only 4 of the fatal CRC reports (16.0%; 2 cases without PSC and 2 cases with a documented concomitant PSC diagnosis). The 2 patients in whom PSC was mentioned developed IBD at 3 and 15 years of age and CRC at 16 and 28 years of age, respectively, after a PSC disease duration of 3 and 12 years. In this group of fatal cancer, no patients with skin cancer or cervical cancer were described.

Non-cancer related deaths.

Cases with fatal outcome were reported in 63 of 87 studies (72.4%). Of these studies, 48 studies (76,2%) reported cases in which mortality was related to cancer (as described above), and 23 studies (36,5%) reported cases with fatal outcome due to other causes. Of these 23 studies, 8 were case reports, 5 were case series, and 10 were cohort studies, of which 7 were retrospective and 3 prospective. Most retrospective studies were single centre or multicentre but performed in a single country.

A total of 72 cases with fatal outcome unrelated to cancer were reported, of which 50.7% of patients was male (n=36, Table 4). Patients were diagnosed with UC (56.3%, n=40) or CD (43.7%, n=31) at a median age of 10.0 years (IQR 6.50-13.0; IBD type missing in 1 case). The median age at death was 15.00 years (IQR 13.0-20.0).

Infectious disease (n=24) and gastrointestinal complications (n=18) were the most common causes of non-cancer related deaths. A total of 16 patients died of sepsis; most

Table 4. Clinical characteristics of PIBD patients with fatal outcomes unrelated to cancer.

	Type	Subtype (number)	No.	Median age at IBD Dx (IQR, min-max) [number missing]	Median age at mortality (IQR, min-max) [number missing]	number male (%) [number missing]	number UC (%) number CD (%) [number missing]
1	Gastrointestinal	1a Acute severe colitis (3) 1b Toxic megacolon (2) 1c Perforation (5) 1d UNK (8)	18	10.00 (6.50-14.00, 2.00-15.50) [0]	14.00 (9.50-16.25, 5.00-28.00) [5]	8 (47.1%) [1]	14 (77.8%) 4 (22.2%) [0]
2	Infectious disease	2a Neurologic (1) 2b Pulmonary (4) 2c Sepsis (16) 2d Other (3)	24	9.00 (5.10-12.00, 2.00-14.00) [3]	16.00 (13.13-18.75, 8.00-31.00) [4]	13 (54.2%) [0]	11 (45.8%) 13 (54.2%) [0]
3	Post-operative		6	6.00 (4.20-10.45, 3.00-10.90) [1]	16.55 (12.35-30.00, 7.40-36.00) [0]	3 (33.3%) [0]	4 (66.7%) 2 (33.3%) [0]
4	Liver disease		6	15.00 (10.40-18.00, 10.40-19.00) [1]	27.00 (20.70-36.00, 19.40-44.00) [1]	5 (83.3%) [0]	5 (100%) [1]
5	Dehydration		1	2.00 (NR) [0]	5.00 (NR) [0]	1 (100%) [0]	1 (100%) [0]
6	Suicide		1	15.00 (NR) [0]	24.00 (NR) [0]	Not applicable	0 (0%) 1 (100%) [0]
7	Other	7a Accident (1) 7b Underlying disease (2) 7c Neurological disease (2) 7d Cardiovascular disease (5) 7e MOF (3) 7f UNK (3)	16	9.50 (7.00-13.25,4.00-17.00) [0]	14.50 (13.25-20.00, 2.00-27.00) [0]	8 (50.0%) [0]	5 (31.3%) 11 (68.7%) [0]
	Total		**72**	**10.00 6.50-13.00, 2.00-19.00 [5]**	**15.00 [13.00-20.00, 2.00-44.00) [9]**	**36 (50.7%) [1]**	**40 (56.3%) 31 (43.7%) [1]**

NR, not relevant, UNK, unknown, MOF, multi organ failure.

of these were male (62.50%, n=10) and diagnosed with CD (68.57%, n=11). The majority of the patients who died of sepsis was exposed to two immunosuppressants (n=7, 43.8%). The remaining were exposed to one immunosuppressant (n=4, 25.0%) or information on medication was not reported (n=5, 31.2%). A central line was present in 4 patients (26.7%) who developed sepsis. Other underlying causes of sepsis were bowel perforation (n=2), peritonitis (n=2), abdominal abscess (n=2), disseminated varicella zoster infection (n=1), fulminant *Campylobacter jejuni* infection (n=1), pneumonia (n=1) and unknown (n=3). In the 8 patients who died due to infectious causes other than sepsis no information on medication was reported.

Refusal of medical treatment was reported in 2 of the 18 cases (11%) who died due to a gastrointestinal complication. Medication refusal or non-adherence was not mentioned in any of the other groups. Intestinal surgery was the most frequently reported cause of post-operative death (83.3%, n=5). Of these 5 patients, 4 patients (80%) died after colectomy and 1 patient died after an ileal resection (20%). One patient was reported who died after a renal transplantation.[26] A total of 7 patients were reported who died due to concomitant liver disease. Of these patients, the majority (71.4%, n=5) was male and was diagnosed with UC (85.7%, n=6). Hepatic cirrhosis (n=2), PSC (n=1), liver failure (n=2) and non-infectious hepatitis (n=1) were mentioned as underlying causes for liver failure. PSC was reported in only one patient.

DISCUSSION

Over the last years the medical treatment of PIBD has changed considerably with a tendency for using more intensive immunosuppressive medications earlier in the disease course. There is a rising concern that these therapies may be associated with an increased risk of developing cancer, and subsequent mortality. Following our meta-analyses we demonstrate an increased risk for all types of cancer in PIBD patients. However, although long-term follow-up studies are important to calculate incidence rates, these studies also illustrate that the absolute number of PIBD patients with cancer and mortality is low, resulting in limited detailed clinical characteristics of the group of PIBD patients of interest. Better insight in the characteristics of PIBD patients who develop cancer or have a fatal outcome will help to identify predictive factors of severe disease course. Therefore, with this review we aimed to provide an overview of patients with IBD diagnosed at pediatric age who developed cancer or suffered a fatal outcome at any point later in life.

The studies identified by our literature search report a wide variety of cancer types but show a strong predominance of CRC, CCA and HSTCL cases, which are usually very uncommon among adolescents and young adults. Lymphomas were the most frequent type of non-fatal cancer in PIBD patients reported in the literature. The high frequency of lymphomas in this systematic review reflects the results of the retrospective study by de Ridder *et al.* who showed that 9 out of 18 PIBD patients with cancer were diagnosed with lymphoma.[28] In agreement, a study using the FDA's Adverse Events Reporting System to identify malignancies associated with the use of biologicals[29], reported that 15 of the 24 PIBD patients that developed a malignancy were diagnosed with a lymphoma (62.5%). Recently, the large prospective DEVELOP study by Hyams *et al.* reported that 8 of 15 patients with a malignancy had either leukemia (n=3) or lymphoma (n=5).[24] These patients were more likely to be on current thiopurine monotherapy or combination therapy with a biologic and thiopurine, as there was only one case reported with TNF monotherapy (no thiopurine exposure). However, in the population-based EPIMAD registry bowel-related carcinomas were most frequently observed and no lymphomas were reported after a median follow-up time of 11.5 years (IQR 7-15).[30] Possibly, these discrepancies might be due to differences in study population size or duration of follow-up. The DEVELOP study demonstrated that SIR for malignancy among 5766 patients was significantly elevated only for patients receiving combination therapy with thiopurine and biologic (SIR 3.06) but not with thiopurine or anti-TNF monotherapies.

The group with the highest apparent risk for intestinal carcinoma appeared to be UC patients with PSC. UC was more prevalent in patients diagnosed with CCA and CRC (75.0% and 81.3%, respectively). All but one patient with CCA had a concomitant diagnosis of PSC, emphasizing the increased risk of neoplasia in this subgroup of PIBD patients. A strikingly large number of those patients were diagnosed with CCA before 19 years of age. These results are in line with literature in adults, which shows increased risk of CRC development in UC patients[31-33] and both CRC and CCA development in PSC-IBD patients.[34, 35]

CRC was the most frequently reported type of fatal cancer in PIBD patients. The median age at diagnosis of intestinal carcinoma was 25.5 years (IQR 20.0-30.5), which is a strikingly young age to develop this type of cancer. In line with this, recent data from the National Patient Register in Sweden over a period of 50 years (1964-2014) show that even before their 18th birthday, PIBD patients have an increased risk of cancer (Hazard ratio=4.1; 95%CI=1.8-8.6) compared to the general population.[36] Gastrointestinal cancers were associated with the highest relative risks (Hazard ratio 9.7, 95% CI 0.4-246), although absolute risks were low. Our review demonstrates a lack of information on therapy in patients with CRC, which limits conclusions on undertreatment in this population. Remarkably, most cases of HSTCL were fatal, as 16 PIBD patients with HSTCL were reported to have a fatal outcome versus only 2 non-fatal HSTCL cases. Thiopurine exposure was reported in all patients with HSTCL, but details on current or previous thiopurine exposure and combination with biologicals were often limited. The duration from diagnosis of cancer to death was not reported in the majority of patients with fatal cancer. However,

the available data suggest a very short cancer duration at moment of death, as in 20 patients the median duration from cancer diagnosis to death was less than 1 year and in 14 patients the median duration was only 2.0 years (IQR 1.0-15.0). This could reflect the severity of cancers associated with IBD diagnosed at a pediatric age or might be caused by reporting bias.

In our review, mortality was most often due to infectious complications, followed by malignancy and disease associated complications. Disease associated fatal complications were more common in UC than in CD. The median age of death in patients with non-cancer related mortality was 15.0 years (IQR 13.0-20.0). The majority of patients with non-cancer related fatal outcome were diagnosed with UC (56.3%). This is consistent with recent data from an adult population-based IBD cohort[37] and the retrospective study by de Ridder *et al*. that showed increased mortality mainly among UC patients (61%). The outlier for these pooled data was the French population based study in which mortality occurred more commonly in CD patients.[30] Recent data by Olen *et al.* confirm that PIBD patients have an increased risk of mortality compared to the general population, with the highest hazard ratios observed in UC patients (Hazard ratio=4.0;95%CI=3.4-4.7). In our review, mortality was most often due to infectious complications of intestinal origin. Mortality primarily related to infections has been reported by de Ridder *et al.*[28] but was not observed in several large studies in pediatric[30, 38, 39] and adult IBD patients.[37, 40] Both in the pediatric[21] and adult IBD population[41], patients treated with steroids are more likely to develop serious infections. Most of the patients who died of sepsis reported in this review were exposed to at least two immunosuppressants, often including steroids, which suggests that much of the sepsis-related mortality in PIBD patients is drug-related and potentially avoidable. In addition, central venous lines were also a risk factor. Hence, it is important to closely monitor PIBD patients who develop fever and are using or have recently used more than one immunosuppressant drug. Surprisingly, no fatal cases due to hemophagocytic lymphohistiocytosis (HLH), a potentially fatal syndrome of pathologic immune activation that is associated with the use of immunosuppressants, were reported in the literature. It is plausible that certain cases of HLH in PIBD patients were not recognized as such due to diagnostic difficulties.[42] It is important to acknowledge that the association between thiopurine use and HLH in both adult and pediatric patients with IBD is well-established[16, 43, 44], and has recently been highlighted by the results from the DEVELOP study.[24]

Although this is the first systematic review to provide an overview of cases of PIBD patients who developed malignancy or fatality, it is impossible to ascertain that we captured all cases. It is likely that many cases are underreported. A second limitation is the quality of the reports. In order to be as inclusive as possible about these rare outcomes, we decided to include all types of studies with no restriction to the year the study was published in. Moreover, it is likely that severe cases are reported more frequently, which is illustrated by the larger number of cases with fatal cancer compared to cases with non-fatal cancer in our data. Limited patient specific information, in combination with heterogeneous study settings, precluded comparative analysis. Lastly, long-term follow-up of PIBD patients is difficult, as follow-up is often associated with transition to specialist adult care or private clinics. Studies reporting IBD patients who develop malignancy or mortality during adult care often do not provide individual patient data on age at IBD diagnosis. This leads to a risk of underreporting of malignancy and mortality in patients with a pediatric-onset of IBD. Moreover, the presenting age of cancer and/or death is likely to be biased towards a younger age due to limited long-term follow up in most cohorts.

Overall, PIBD associated malignancy and mortality is rare. To assess severe outcomes in PIBD patients long term follow-up and multicenter or population based collaborations are indispensable. Furthermore, more detailed patient specific information is necessary to investigate the relationship between severe outcomes in PIBD patients and the currently used therapeutic strategies. Obtaining this data is crucial in order to develop evidenced based strategies that incorporate long term risks as well as benefits.

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REFERENCES

1. Henderson P, Hansen R, Cameron FL, et al. Rising incidence of pediatric inflammatory bowel disease in Scotland. Inflamm Bowel Dis. 2012 Jun;18(6):999-1005.
2. Lehtinen P, Ashorn M, Iltanen S, et al. Incidence trends of pediatric inflammatory bowel disease in Finland, 1987-2003, a nationwide study. Inflamm Bowel Dis. 2011 Aug;17(8):1778-83.
3. Martin-de-Carpi J, Rodriguez A, Ramos E, et al. Increasing incidence of pediatric inflammatory bowel disease in Spain (1996-2009): the SPIRIT Registry. Inflamm Bowel Dis. 2013 Jan;19(1):73-80.
4. Ng SC, Tang W, Ching JY, et al. Incidence and phenotype of inflammatory bowel disease based on results from the Asia-pacific Crohn's and colitis epidemiology study. Gastroenterology. 2013 Jul;145(1):158-65 e2.
5. Van Limbergen J, Russell RK, Drummond HE, et al. Definition of phenotypic characteristics of childhood-onset inflammatory bowel disease. Gastroenterology. 2008 Oct;135(4):1114-22.
6. Aloi M, D'Arcangelo G, Pofi F, et al. Presenting features and disease course of pediatric ulcerative colitis. J Crohns Colitis. 2013 Dec;7(11):e509-15.
7. Vernier-Massouille G, Balde M, Salleron J, et al. Natural history of pediatric Crohn's disease: a population-based cohort study. Gastroenterology. 2008 Oct;135(4):1106-13.
8. Pigneur B, Seksik P, Viola S, et al. Natural history of Crohn's disease: comparison between childhood- and adult-onset disease. Inflamm Bowel Dis. 2010 Jun;16(6):953-61.
9. Gyde SN, Prior P, Allan RN, et al. Colorectal cancer in ulcerative colitis: a cohort study of primary referrals from three centres. Gut. 1988 Feb;29(2):206-17.
10. Ekbom A, Helmick C, Zack M, et al. Ulcerative colitis and colorectal cancer: A population-based study. NEW ENGL J MED. 1990;323(18):1228-33.
11. Jess T, Frisch M, Simonsen J. Trends in overall and cause-specific mortality among patients with inflammatory bowel disease from 1982 to 2010. Clin Gastroenterol Hepatol. 2013 Jan;11(1):43-8.
12. Osterman MT, Haynes K, Delzell E, et al. Effectiveness and Safety of Immunomodulators With Anti-Tumor Necrosis Factor Therapy in Crohn's Disease. Clin Gastroenterol Hepatol. 2015 Jul;13(7):1293-301 e5; quiz e70, e72.
13. Annese V, Duricova D, Gower-Rousseau C, et al. Impact of New Treatments on Hospitalisation, Surgery, Infection, and Mortality in IBD: a Focus Paper by the Epidemiology Committee of ECCO. J Crohns Colitis. 2016 Feb;10(2):216-25.
14. Toruner M, Loftus EV, Jr., Harmsen WS, et al. Risk factors for opportunistic infections in patients with inflammatory bowel disease. Gastroenterology. 2008 Apr;134(4):929-36.
15. Kotlyar DS, Lewis JD, Beaugerie L, et al. Risk of lymphoma in patients with inflammatory bowel disease treated with azathioprine and 6-mercaptopurine: a meta-analysis. Clin Gastroenterol Hepatol. 2015 May;13(5):847-58 e4; quiz e48-50.
16. Beaugerie L, Brousse N, Bouvier AM, et al. Lymphoproliferative disorders in patients receiving thiopurines for inflammatory bowel disease: a prospective observational cohort study. Lancet. 2009 Nov 07;374(9701):1617-25.
17. Ariyaratnam J, Subramanian V. Association between thiopurine use and nonmelanoma skin cancers in patients with inflammatory bowel disease: a meta-analysis. Am J Gastroenterol. 2014 Feb;109(2):163-9.
18. Allegretti JR, Barnes EL, Cameron A. Are patients with inflammatory bowel disease on chronic immunosuppressive therapy at increased risk of cervical high-grade dysplasia/cancer? A meta-analysis. Inflamm Bowel Dis. 2015 May;21(5):1089-97.
19. Cottone M, Kohn A, Daperno M, et al. Advanced age is an independent risk factor for severe infections and mortality in patients given anti-tumor necrosis factor therapy for inflammatory bowel disease. Clin Gastroenterol Hepatol. 2011 Jan;9(1):30-5.
20. O'Connor A, Qasim A, O'Morain CA. The long-term risk of continuous immunosuppression using thioguanides in inflammatory bowel disease. Ther Adv Chronic Dis. 2010 Jan;1(1):7-16.
21. Dulai PS, Thompson KD, Blunt HB, et al. Risks of Serious Infection or Lymphoma With Anti-Tumor NecrosisFactor Therapy for Pediatric Inflammatory Bowel Disease: ASystematic Review. Clin Gastroenterol Hepatol. 2014;12(9):1443-51.
22. Moher D, Liberati A, Tetzlaff J, et al. Preferred reporting items for systematic reviews and meta-analyses: the PRISMA statement. PLoS Med. 2009 Jul 21;6(7):e1000097.
23. Hyams JS, Lerer T, Griffiths A, et al. Long-term outcome of maintenance infliximab therapy in children with Crohn's disease. Inflammatory Bowel Dis. 2009;15(6):816-22.
24. Hyams JS, Dubinsky MC, Baldassano RN, et al. Infliximab Is Not Associated With Increased Risk of Malignancy or Hemophagocytic Lymphohistiocytosis in Pediatric Patients With Inflammatory Bowel Disease. Gastroenterology. 2017 Feb 11.
25. Freeman HJ. Long-term prognosis of early-onset Crohn's disease diagnosed in childhood or adolescence. Can J Gastroenterol. 2004;18(11):661-5.
26. Puntis J, McNeish AS, Allan RN. Long term prognosis of Crohn's disease with onset in childhood and adolescence. GUT. 1984;25(4):329-36.
27. Jakobsen C, Paerregaard A, Munkholm P, et al. Pediatric inflammatory bowel disease during a 44-year period in Copenhagen County: Occurrence, course and prognosis - A population-based study from the Danish Crohn Colitis Database. Eur J Gastroenterol Hepatol. 2009;21(11):1291-301.
28. De Ridder L, Turner D, Wilson DC, et al. Malignancy and mortality in pediatric patients with inflammatory bowel disease: A multinational study from the porto pediatric IBD group. Inflammatory Bowel Dis. 2014;20(2):291-300.
29. Diak P, Siegel J, La Grenade L, et al. Tumor necrosis factor α blockers and malignancy in children: Forty-eight cases reported to the food and drug administration. Arthritis Rheum. 2010;62(8):2517-24.
30. Peneau A, Savoye G, Turck D, et al. Mortality and cancer in pediatric-onset inflammatory bowel disease: A population-based study. Am J Gastroenterol. 2013;108(10):1647-53.
31. Herrinton LJ, Liu L, Levin TR, et al. Incidence and mortality of colorectal adenocarcinoma in persons with inflammatory bowel disease from 1998 to 2010. Gastroenterology. 2012 Aug;143(2):382-9.
32. Eaden JA, Abrams KR, Mayberry JF. The risk of colorectal cancer in ulcerative colitis: A meta-analysis. Gut. 2001;48(4):526-35.
33. Castano-Milla C, Chaparro M, Gisbert JP. Systematic review with meta-analysis: the declining risk of colorectal cancer in ulcerative colitis. Aliment Pharmacol Ther. 2014 Apr;39(7):645-59.
34. Gulamhusein AF, Eaton JE, Tabibian JH, et al. Duration of inflammatory bowel disease is associated with increased risk of cholangiocarcinoma in patients with primary sclerosing cholangitis and IBD. Am J Gastroenterol. 2016;111(5):705-11.
35. Boonstra K, Weersma RK, van Erpecum KJ, et al. Population-based epidemiology, malignancy risk, and outcome of primary sclerosing cholangitis. Hepatology. 2013 Dec;58(6):2045-55.
36. Olen O, Askling J, Sachs MC, et al. Childhood onset inflammatory bowel disease and risk of cancer: a Swedish nationwide cohort study 1964-2014. BMJ. 2017 Sep 20;358:j3951.
37. Caini S, Bagnoli S, Palli D, et al. Total and cancer mortality in a cohort of ulcerative colitis and Crohn's disease patients: The Florence inflammatory bowel disease study, 1978-2010. Dig Liver Dis. 2016 Oct;48(10):1162-7.
38. Michener WM, Farmer RG, Mortimer EA. Long-term prognosis of ulcerative colitis with onset in childhood or adolescence. J CLIN GASTROENTEROL. 1979;1(4):301-5.
39. Olén O. AJ, Sachs M.C., Frumento P., Neovius M., Smedby K.E., Ekbom A., Malmborg P., Ludvigsson J.F. Mortality in childhood-onset inflammatory bowel disease - a population-based cohort study spanning 50 years. ESPGHAN Annual Meeting 2017. 2017:G-eP-053.
40. Jess T, Gamborg M, Munkholm P, et al. Overall and cause-specific mortality in ulcerative colitis: Meta-analysis of population-based inception cohort studies. Am J Gastroenterol. 2007;102(3):609-17.
41. Lichtenstein GR, Rutgeerts P, Sandborn WJ, et al. A pooled analysis of infections, malignancy, and mortality in infliximab- and immunomodulator-treated adult patients with inflammatory bowel disease. Am J Gastroenterol. 2012 Jul;107(7):1051-63.
42. Henter JI, Horne A, Arico M, et al. HLH-2004: Diagnostic and therapeutic guidelines for hemophagocytic lymphohistiocytosis. Pediatr Blood Cancer. 2007 Feb;48(2):124-31.
43. Kandiel A, Fraser AG, Korelitz BI, et al. Increased risk of lymphoma among inflammatory bowel disease patients treated with azathioprine and 6-mercaptopurine. Gut. 2005 Aug;54(8):1121-5.
44. Vos AC, Bakkal N, Minnee RC, et al. Risk of malignant lymphoma in patients with inflammatory bowel diseases: a Dutch nationwide study. Inflamm Bowel Dis. 2011 Sep;17(9):1837-45.

SUPPLEMENTARY REFERENCE LIST OF INCLUDED STUDIES

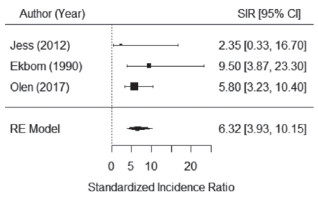
COMPLEMENTARY TO TABLE 1

1. Fabry TL, Sachar DB, Janowitz HD. Acute myelogenous leukemia in patients with ulcerative colitis. J Clin Gastroenterol. 1980 Sep;2(3):225-7.
2. Patterson JF, Norton RA, Schwartz RS. Azathioprine treatment of ulcerative colitis, granulomatous colitis and regional enteritis. Am J Dig Dis. 1971;16(4):327-32.
3. Mir Madjlessi SH, Farmer RG, Weick JK. Inflammatory bowel disease and leukemia. A report of seven cases of leukemia in ulcerative colitis and Crohn's disease and review of the literature. Dig Dis Sci. 1986 Oct;31(10):1025-31.
4. Fraser AG, Orchard TR, Robinson EM, et al. Long-term risk of malignancy after treatment of inflammatory bowel disease with azathioprine. Aliment Pharmacol Ther. 2002 Jul;16(7):1225-32.
5. Gyde SN, Prior P, Macartney JC, et al. Malignancy in Crohn's disease. Gut. 1980 Dec;21(12):1024-9.
6. Jess T, Loftus Jr EV, Velayos FS, et al. Risk of Intestinal Cancer in Inflammatory Bowel Disease: A Population-Based Study From Olmsted County, Minnesota. Gastroenterology. 2006;130(4):1039-46.
7. Karlén P, Löfberg R, Broström O, et al. Increased risk of cancer in ulcerative colitis: A population-based cohort study. Am J Gastroenterol. 1999;94(4):1047-52.
8. Winther KV, Jess T, Langholz E, et al. Long-term risk of cancer in ulcerative colitis: a population-based cohort study from Copenhagen County. Clin Gastroenterol Hepatol. 2004 Dec;2(12):1088-95.
9. Deneau M, Jensen MK, Holmen J, et al. Primary sclerosing cholangitis, autoimmune hepatitis, and overlap in utah children: Epidemiology and natural history. Hepatology. 2013;58(4):1392-400.
10. Ashworth LA, Billett A, Mitchell P, et al. Lymphoma risk in children and young adults with inflammatory bowel disease: Analysis of a large single-center cohort. Inflammatory Bowel Dis. 2012;18(5):838-43.
11. Diak P, Siegel J, Grenade LL, et al. Tumor necrosis factor α blockers and malignancy in children: Forty-eight cases reported to the food and drug administration: Wiley Online Library; 2010.
12. Jakobsen C, Paerregaard A, Munkholm P, et al. Pediatric inflammatory bowel disease during a 44-year period in Copenhagen County: Occurrence, course and prognosis - A population-based study from the Danish Crohn Colitis Database. Eur J Gastroenterol Hepatol. 2009;21(11):1291-301.
13. Lindberg J, Stenling R, Palmqvist R, et al. Early onset of ulcerative colitis: Long-term follow-up with special reference to colorectal cancer and primary sclerosing cholangitis. J Pediatr Gastroenterol Nutr. 2008;46(5):534-8.
14. Gurbuz AK, Giardiello FM, Bayless TM. Colorectal neoplasia in patients with ulcerative colitis and primary sclerosing cholangitis. DIS COLON RECTUM. 1995;38(1):37-41.
15. Gryboski JD. Crohn's disease in children 10 years old and younger: Comparison with ulcerative colitis. J PEDIATR GASTROENTEROL NUTR. 1994;18(2):174-82.
16. Barton JR, Ferguson A. Clinical features, morbidity and mortality of Scottish children with inflammatory bowel disease. Q J MED. 1990;75(277):423-39.
17. Kirchmann HMA, Bender SW. Intestinal obstruction in Crohn's disease in childhood. J PEDIATR GASTROENTEROL NUTR. 1987;6(1):79-83.
18. Goel KM, Shanks RA. Long-term prognosis of children with ulcerative colitis. Arch Dis Child. 1973;48(5):337-42.
19. Yoon J, Oh SH, Kim HJ, et al. Primary Sclerosing Cholangitis with Inflammatory Bowel Disease in Korean Children. Pediatr gastroenterol hepatol nutrition. 2015 Dec;18(4):268-75.
20. Cannioto Z, Berti I, Martelossi S, et al. IBD and IBD mimicking enterocolitis in children younger than 2 years of age. European Journal of Pediatrics. [Article]. 2009 Feb;168(2):149-55.
21. Korelitz BI, Mirsky FJ, Fleisher MR, et al. Malignant neoplasms subsequent to treatment of inflammatory bowel disease with 6-mercaptopurine. Am J Gastroenterol. 1999 Nov;94(11):3248-53.
22. Katsanos KH, Roda G, McBride RB, et al. Increased Risk of Oral Cancer in Patients With Inflammatory Bowel Diseases. Clin Gastroenterol Hepatol. 2016 Mar;14(3):413-20.
23. Kim J, Lee HS, Park SH, et al. Pathologic features of colorectal carcinomas associated with Crohn's disease in Korean population. Pathol Res Pract. 2017 Mar;213(3):250-5.
24. Madanchi M, Zeitz J, Barthel C, et al. Malignancies in Patients with Inflammatory Bowel Disease: A Single-Centre Experience. Digestion. 2016;94(1):1-8.
25. Peneau A, Savoye G, Turck D, et al. Mortality and cancer in pediatric-onset inflammatory bowel disease: A population-based study. Am J Gastroenterol. 2013;108(10):1647-53.
26. Shouval DS, Ebens CL, Murchie R, et al. Large B-cell lymphoma in an adolescent patient with interleukin-10 receptor deficiency and history of infantile inflammatory bowel disease. J Pediatr Gastroenterol Nutr. 2016;63(1):e15-e7.
27. Baumer FM, Ouahed J, Verhave M, et al. Fatal central nervous system disease following first infliximab infusion in a child with inflammatory bowel disease. Pediatr Neurol. 2016;57:91-4.
28. Terada T. Extranodal marginal zone B-cell lymphoma of Mucosa-Associated Lymphoid Tissue (MALT lymphoma) in ulcerative colitis. Saudi J Gastroenterol. 2014;20(5):319-22.
29. Rodriguez AA, Kerner J, Luna-Fineman S, et al. Hodgkin Lymphoma Following Adalimumab for the Treatment of Crohn's Disease in an Adolescent. Dig Dis Sci. 2014;59(10):2403-5.
30. Noh SY, Oh SY, Kim SH, et al. Fifteen-year-old colon cancer patient with a 10-year history of ulcerative colitis. World J Gastroenterol. 2013;19(15):2437-40.
31. Kondoh K, Morimoto M, Keino D, et al. T-cell large granular lymphocyte leukemia in a child with anemia and Crohn's disease. Pediatr Int. 2013;55(1):111-4.
32. Altaf S, Atreaga GM, Joshi AY, et al. Diffuse large B-cell lymphoma in an adolescent female presenting with Epstein-Barr virus-driven hemophagocytic lymphohistiocytosis: A case report. J Med Case Rep. 2012;6.
33. Jamali M, Raca G, Rubin CM, et al. γδ Hepatosplenic T-cell lymphoma in a pediatric patient with Crohn's disease on combined immunosuppressive and immunomodulator therapy. Pathol Case Rev. 2012;17(3):101-7.
34. Loganathan SS, Zaitoun A, Charlton CPJ. Lymphoma with central nervous system involvement in a young patient with crohn disease treated with azathioprine. J Pediatr Gastroenterol Nutr. 2010;51(6):790-2.
35. Deneau M, Wallentine J, Guthery S, et al. Natural killer cell lymphoma in a pediatric patient with inflammatory bowel disease. Pediatrics. 2010;126(4):e977-e81.
36. Aydoğan A, Çorapçioğlu F, Levent Elemen E, et al. Childhood non-Hodgkin's lymphoma arising as a complication early in the course of Crohn's disease. Turk J Pediatr. 2010;52(4):411-5.
37. Destombe S, Bouron-Dalsoglio D, Rougemont AL, et al. Lymphomatoid granulomatosis: A unique complication of crohn disease and its treatment in pediatrics. J Pediatr Gastroenterol Nutr. 2010;50(5):559-61.
38. Ochenrider MG, Patterson DJ, Aboulafia DM. Hepatosplenic T-Cell lymphoma in a young man with Crohn's disease: Case report and literature review. Clin Lymphoma Myeloma. 2010;10(2):144-8.
39. Van Biervliet S, Vande Velde S, De Bruyne R, et al. Epstein-Barr virus related lymphoma in inflammatory bowel disease. Acta Gastro-Enterol Belg. 2008;71(1):33-5.
40. Nahas S, Nahas C, Marques C, et al. Concurrent development of Crohn disease and myelodysplastic syndrome in a child: Case report and literature review. Pediatr Hematol Oncol. 2006;23(6):477-83.
41. Oliver JW, Farnsworth B, Tonk VS. Juvenile myelomonocytic leukemia in a child with Crohn disease. Cancer Genet Cytogenet. 2006;167(1):70-3.
42. Thayu M, Markowitz JE, Mamula P, et al. Hepatosplenic T-cell lymphoma in an adolescent patient after immunomodulator and biologic therapy for Crohn disease. J Pediatr Gastroenterol Nutr. 2005;40(2):220-2.
43. Ross Iv AM, Anupindi SA, Balis UJ. Case 11-2003: A 14-year-old boy with ulcerative colitis, primary sclerosing cholangitis, and partial duodenal obstruction. New Engl J Med. 2003;348(15):1464-76.
44. Limon J, Taylor DS, Haight M, et al. Crohn's disease and acute lymphoblastic leukemia in a two-year-old child. J Pediatr Gastroenterol Nutr. 2002;35(1):99-101.
45. Tiszlavicz L, Kapin M, Várkonyi A, et al. Adenocarcinoma of the colon developing on the basis of Crohn's disease in childhood. Eur J Pediatr. 2001;160(3):168-72.
46. Foster-Barber A, Magee J, Heyman MB. Rapidly progressive and fatal scleromyxedema in an adolescent with Crohn's disease. J PEDIATR GASTROENTEROL NUTR. 1995;20(1):107-11.
47. Jackson DL, Korbin J, Youngner S. Fatal outcome in untreated adolescent ulcerative colitis: An unusual case of child neglect. CRIT CARE MED. 1983;11(10):832-3.
48. Coffin CM, L'Heureaux P, Dehner LP. Campylobacter-associated enterocolitis in childhood. Report of a fatal case. AM J CLIN PATHOL. 1982;78(1):117-23.
49. Rosen RB, Teplitz RL. CHRONIC GRANULOCYTIC LEUKEMIA COMPLICATED BY ULCERATIVE COLITIS. Blood. 1965;26:148-56.
50. Brown CH, Diaz RJ, Michener WM. CARCINOMA OF THE COLON AND ILEUM IN CHRONIC ULCERATIVE COLITIS WITH REFLUX ILEITIS. REPORT OF A CASE OF A SIXTEEN-YEAR-OLD BOY. Gastroenterology. 1964;47:306-12.
51. Wilcox Jr HR, Beattie JL. Carcinoma complicating ulcerative colitis during childhood. Am J Clin Pathol. 1956;26(7):778-86.
52. Wetzler G, Tomer G. Carcinoid Tumor Complicating Crohn Disease in a Female Adolescent. J Pediatr Gastroenterol Nutr. 2016 Aug;63(2):e25-6.

53. Murakami A, Tanaka Y, Ueda M, et al. Hepatocellular carcinoma occurring in a young Crohn's disease patient. Pathol Int. 2009 Jul;59(7):492-6.
54. Cameron C, Greenbaum L, Sato T, et al. Renal cell carcinoma in a patient with cystinosis and inflammatory bowel disease: a case report. Pediatr Nephrol. 2008 Jul;23(7):1167-70.
55. van der Hoeven JG, de Koning J, Masclee AM, et al. Fatal pneumococcal septic shock in a patient with ulcerative colitis. Clin Infect Dis. 1996 May;22(5):860-1.
56. Keene JK, Lowe DK, Grosfeld JL, et al. Disseminated varicella complicating ulcerative colitis. JAMA. 1978 Jan 2;239(1):45-6.
57. Bisordi W, Lightdale CJ. Identical twins discordant for ulcerative colitis with colon cancer. Am J Dig Dis. 1976 Jan;21(1):71-3.
58. Mittal S, Milner BJ, Johnston PW, et al. A case of hepatosplenic gamma-delta T-cell lymphoma with a transient response to fludarabine and alemtuzumab. Eur J Haematol. 2006 Jun;76(6):531-4.
59. Bersack SR, Howe JS, Rehak EM. A unique case with roentgenologic evidence of regional enteritis of long duration and histologic evidence of diffuse adenocarcinoma. Gastroenterology. 1958 Apr;34(4):703-10.
60. Ginzburg L, Schneider KM, Dreizin DH, et al. Carcinoma of the jejunum occurring in a case of regional enteritis. Surgery. 1956 Feb;39(2):347-51.
61. Demarchi B, Bresso F, Novero D, et al. Hepatocellular carcinoma complicating primary sclerosing cholangitis in Crohn's disease: A case report. Minerva Gastroenterol Dietol. 2007;53(3):279-83.
62. Michener WM. Pulmonary emboli causing sudden death in ulcerative colitis. CLEVELAND CLIN Q. 1974;41(2):71-4.
63. Sanderson IR, Kingston JE, Khan S, et al. Hodgkin's disease complicating Crohn's disease in a child. J R Soc Med. 1985 Oct;78(10):860-1.
64. Weingarten B, Parker JG, Chazen EM, et al. Adenocarcinoma of the jejunum in nonspecific granulomatous enteritis. AMA Arch Surg. 1959 Mar;78(3):483-9.
65. Orii S, Sugai T, Nakano O, et al. Acute promyelocytic leukemia in Crohn's disease. Case report and review of the literature. J Clin Gastroenterol. 1991 Jun;13(3):325-7.
66. Borum ML. Unusual development of hepatocellular carcinoma in a patient with Crohn's disease. Dig Dis Sci. 2001 Oct;46(10):2199-200.
67. Hatake K, Tanaka M, Muroi K, et al. Leukaemia risk in Crohn's disease. Lancet. 1996 Apr 13;347(9007):1049-50.
68. Goldman LI, Bralow SP, Cox W, et al. Adenocarcinoma of the small bowel complicating Crohn's disease. Cancer. 1970;26(5):1119-25.
69. Cattan S, Wendum D, Chazouilleres O, et al. Hepatocellular carcinoma and focal hepatic glycogenosis after prolonged azathioprine therapy. Hum Pathol. 2000 Jul;31(7):874-6.
70. Chen SC, Cummings OW, Hartley MP, et al. Hepatocellular carcinoma occurring in a patient with Crohn's disease treated with both azathioprine and infliximab. Dig Dis Sci. 2006 May;51(5):952-5.
71. R.M. S. Chronic Idiopathic Ulcerative Colitis in Children. N Engl J Med. 1937;217:541-6.
72. Mallory T.B. CB, Towne V.W.,. Case records of the Massachusetts General Hospital; Case #36501. N Engl J Med. 1950;243(24):967-70.
73. Defilippis EM, Barfield E, Leifer D, et al. Cerebral venous thrombosis in inflammatory bowel disease. J Dig Dis. 2015;16(2):104-8.
74. Marx G, Seidman EG, Martin SR, et al. Outcome of Crohn's disease diagnosed before two years of age. J Pediatr. 2002;140(4):470-3.
75. Evans DJ, Pollock DJ. In-situ and invasive carcinoma of the colon in patients with ulcerative colitis. Gut. 1972;13(7):566-70.
76. Rankin JG, Skyring AP, Goulston SJ. Liver in ulcerative colitis: obstructive jaundice due to bile duct carcinoma. Gut. 1966;7(5):433-7.
77. Goldgraber MB, Kirsner JB. CARCINOMA OF COLON COMPLICATING ULCERATIVE COLITIS: REPORT OF TEN CASES. Dis Colon Rectum. 1964;19:336-44.
78. Hawker PC, Gyde SN, Thompson H, et al. Adenocarcinoma of the small intestine complicating Crohn's disease. Gut. 1982 Mar;23(3):188-93.
79. Neven B, Mamessier E, Bruneau J, et al. A Mendelian predisposition to B-cell lymphoma caused by IL-10R deficiency. Blood. 2013;122(23):3713-22.
80. Valdes-Dapena A, Rudolph I, Hidayat A, et al. Adenocarcinoma of the small bowel in association with regional enteritis. Four new cases. Cancer. 1976 Jun;37(6):2938-47.
81. Lagercrantz R. Follow-up investigation of children with ulcerative colitis; with special reference to indications for surgical therapy. Acta Paediatr. 1955 Jul;44(4):302-17.
82. Schofield PF. Intestinal malignancy and Crohn's disease. Proc R Soc Med. 1972 Sep;65(9):783-4.
83. Engelhardt KR, Shah N, Faizura-Yeop I, et al. Clinical outcome in IL-10- and IL-10 receptor-deficient patients with or without hematopoietic stem cell transplantation. J Allergy Clin Immunol. 2013 Mar;131(3):825-30.
84. Muraoka R, Tominaga K, Sai X, et al. Ulcerative Colitis-associated Cancer/Dysplasia Detected Using Surveillance Colonoscopy Performed in the Clinical Remission Phase: A Report of Five Cases. Intern Med. 2016;55(8):911-7.
85. de Ridder L, Escher JC, Bouquet J, et al. Infliximab therapy in 30 patients with refractory pediatric crohn disease with and without fistulas in The Netherlands. J Pediatr Gastroenterol Nutr. 2004;39(1):46-52.
86. Liu R, Cox K, Guthery SL, et al. Cholangiocarcinoma and High-Grade Dysplasia in Young Patients with Primary Sclerosing Cholangitis. Dig Dis Sci. 2014;59(9):2320-4.
87. De Ridder L, Turner D, Wilson DC, et al. Malignancy and mortality in pediatric patients with inflammatory bowel disease: A multinational study from the porto pediatric IBD group. Inflammatory Bowel Dis. 2014;20(2):291-300.
88. Russell RK, Wilson ML, Loganathan S, et al. A british society of pediatric gastroenterology, hepatology and nutrition survey of the effectiveness and safety of Adalimumab in children with inflammatory bowel disease. Aliment Pharmacol Ther. 2011;33(8):946-53.
89. Björnsson E, Angulo P. Cholangiocarcinoma in young individuals with and without primary sclerosing cholangitis. Am J Gastroenterol. 2007;102(8):1677-82.
90. Cuttner J. Increased incidence of acute promyelocytic leukemia in patients with ulcerative colitis. Ann Intern Med. 1982 Dec;97(6):864-5.
91. Dayharsh GA, Loftus EV, Jr., Sandborn WJ, et al. Epstein-Barr virus-positive lymphoma in patients with inflammatory bowel disease treated with azathioprine or 6-mercaptopurine. Gastroenterology. 2002 Jan;122(1):72-7.
92. Yabe M, Medeiros LJ, Daneshbod Y, et al. Hepatosplenic T-cell lymphoma arising in patients with immunodysregulatory disorders: a study of 7 patients who did not receive tumor necrosis factor-alpha inhibitor therapy and literature review. Ann Diagn Pathol. 2017 Feb;26:16-22.
93. Hyams JS, Dubinsky MC, Baldassano RN, et al. Infliximab Is Not Associated With Increased Risk of Malignancy or Hemophagocytic Lymphohistiocytosis in Pediatric Patients With Inflammatory Bowel Disease. Gastroenterology. 2017 Feb 11.
94. Hyams JS, Lerer T, Griffiths A, et al. Long-term outcome of maintenance infliximab therapy in children with Crohn's disease. Inflammatory Bowel Dis. 2009;15(6):816-22.
95. Freeman HJ. Long-term prognosis of early-onset Crohn's disease diagnosed in childhood or adolescence. Can J Gastroenterol. 2004;18(11):661-5.
96. Puntis J, McNeish AS, Allan RN. Long term prognosis of Crohn's disease with onset in childhood and adolescence. GUT. 1984;25(4):329-36.
97. Markowitz J, McKinley M, Kahn E, et al. Endoscopic screening for dysplasia and mucosal aneuploidy in adolescents and young adults with childhood onset colitis. AM J GASTROENTEROL. 1997;92(11):2001-6.
98. Broomé U, Löfberg R, Veress B, et al. Primary sclerosing cholangitis and ulcerative colitis: Evidence for increased neoplastic potential. HEPATOLOGY. 1995;22(5):1404-8.

								Newcastle-Ottawa tool		
Study	**Population**	**Size (N)**	**Period of diagnosis**	**Follow-up period**	**Malignancy (N)**	**SIR for CRC**	**95% CI**	**Selection**	**Comparability**	**Outcome**
Jakobsen (2009)	Pediatric IBD patients < 15 years	69	1962-1987	Total of 1,602 person-years	2	25.7	3.1–92.7	****	*	**
Ekbom (1990)	Pediatric UC patients < 15 years	266	1922-1983	4,220 person-years	13	118.3	63.0 - 202.3	****	*	*
Jess (2012)	Pediatric IBD patients 0-19 years	UC: 2,483 CD: 2,280	1979-2008	UC: 25,086 person-years CD: 27,014 person-years	UC: 17 CD: 1	UC: 43.8 CD: 2.35	UC: 27.2-70.7 CD: 0.33-16.7	****	*	**
Ekbom (1990)	Pediatric CD patients < 30 years	964	1983	12,025 person-years	5	9.5	3.1 - 23.3	****	*	*
Olen (2017)	Pediatric IBD patients < 18 years	9,405	1964-2014	148,682 patient-years	122	IBD: 19.5 UC: 33.3 CD: 5.8	IBD: 14.7 - 26.2 UC: 23.1 - 49.1 CD: 3.2 - 10.4	****	**	**

Crohn's disease



Ulcerative colitis

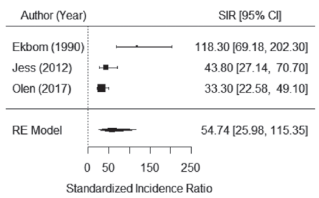


Figure S1. SIR for CRC in pediatric-onset CD and UC patients. Table and graphs presenting SIRs by study including a pooled SIR for CRC following a Random effects (RE) model in both CD and UC patients. Jakobsen et al. (2009) was not included in the meta-analysis as only a SIR for CRC in the total PIBD population was described. SIR, Standardized incidence ratio; RE, random effects; CI confidence interval; IBD, inflammatory bowel disease; UC, ulcerative colitis; CD, Crohn's disease; CRC, colorectal cancer.

SUPPLEMENTARY SEARCH STRATEGY

1ST OF JUNE 2017

Database	**Retrieved references**	**References after deduplication**
Embase.com	4578	4532
Medline Ovid	3660	1355
Web of Science	1706	900
Cochrane Central	43	22
Google Scholar	200	122
Total	**10187**	**6941**

Embase. ('inflammatory bowel disease'/exp OR enteritis/de OR ((inflammator* NEAR/3 bowel NEAR/3 diseas*) OR crohn* OR (ulcer* NEAR/3 colit*) OR IBD OR PIBD):ab,ti) AND (neoplasm/exp OR 'oncological parameters'/exp OR mortality/exp OR (neoplas* OR tumo* OR cancer* OR lymphoma* OR carcinoma* OR malign* OR adenoma* OR oncolog* OR melanoma* OR sarcom* OR leukem* OR leukaem* OR mortalit* OR death* OR fatal*):ab,ti) AND (child/exp OR adolescent/exp OR adolescence/exp OR pediatrics/exp OR childhood/exp OR 'child development'/de OR 'child growth'/de OR 'child health'/de OR 'child health care'/exp OR 'child care'/exp OR 'childhood disease'/exp OR 'pediatric ward'/de OR 'pediatric hospital'/de OR (adolescen* OR infan* OR newborn* OR (new NEXT/1 born*) OR baby OR babies OR neonat* OR child* OR kid OR kids OR toddler* OR teen* OR boy* OR girl* OR minors OR underag* OR (under NEXT/1 age*) OR aging)) OR juvenil* OR youth* OR kindergar* OR puber* OR pubescen* OR prepubescen* OR prepubert* OR pediatric* OR school* OR preschool* OR highschool*):ab,ti) NOT ([Conference Abstract]/lim OR [Letter]/lim OR [Note]/lim OR [Editorial]/lim) AND [english]/lim

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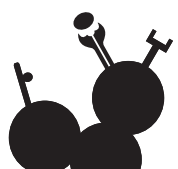
Google scholar. "inflammatory bowel disease" | crohn | "ulcerative colitis" cancer | neoplasms | tumor | tumour | mortality | death infants | children | child.



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On behalf of the Pediatric IBD Porto group of ESPGHAN

8

MALIGNANCY AND MORTALITY IN PEDIATRIC-ONSET INFLAMMATORY BOWEL DISEASE: A 3-YEAR PROSPECTIVE, MULTINATIONAL STUDY FROM THE PEDIATRIC IBD PORTO GROUP OF ESPGHAN

Chapter 8

Aliment. Pharmacol. Ther. 2018 Sep;48(5):523-537.

8

MALIGNANCY AND MORTALITY IN PEDIATRIC-ONSET INFLAMMATORY BOWEL DISEASE: A 3-YEAR PROSPECTIVE, MULTINATIONAL STUDY FROM THE PEDIATRIC IBD PORTO GROUP OF ESPGHAN

ABSTRACT

Background: Risk benefit strategies in managing inflammatory bowel diseases (IBD) are dependent upon understanding the risks of uncontrolled inflammation versus those of treatments. Malignancy and mortality in IBD have been associated with disease-related inflammation and immune suppression, but data are limited due to their rare occurrence.
Aim: The aim of this study was to identify and describe the most common causes of mortality, types of cancer and previous or current therapy among children and young adults with pediatric-onset IBD.
Methods: Information on pediatric-onset IBD patients diagnosed with malignancy or mortality was prospectively collected via a survey in 25 countries over a 42-months period. Patients were included if death or malignancy occurred after IBD diagnosis but before the age of 26 years.
Results: In total 60 patients were identified including 43 malignancies and 26 fatal cases (9 due to cancer). Main causes of fatality were malignancies (n=9), IBD or IBD-therapy related non-malignant causes (n=10; including 5 infections), and suicides (n=3). Three cases, all fatal, of hepatosplenic T-cell lymphoma were identified, all were biologic naive but thiopurine exposed. No other haematological malignancies were fatal. The six other fatal cancer cases included 3 colorectal adenocarcinomas and 3 cholangiocarcinomas. Primary sclerosing cholangitis was present in five (56%) of fatal cancers (1 colorectal carcinomas, 3 cholangiocarcinomas, 1 hepatosplenic T-cell lymphomas).
Conclusions: We report the largest number of pediatric-onset IBD patients with cancer and/or fatal outcomes to date. Malignancies followed by infections were the major causes of mortality. We identified primary sclerosing cholangitis as a significant risk factor for cancer-associated mortality. Disease-related adenocarcinomas were a commoner cause of death than lymphomas.

INTRODUCTION

Inflammatory bowel diseases (IBD) are associated with a long list of both treatment and disease-related complications and cancer. Pediatric-onset IBD is characterized by more extensive and aggressive disease, a longer disease duration, and a higher need for immune suppression early in the disease[1, 2], all of which may be risk factors for complications or cancer. Although, immune suppression may be associated with malignancy, hemophagocytic lymphohistiocytosis and opportunistic infections, it may reduce the risk of fibrostricturing disease, surgery and disease-associated tumors such as bowel adenocarcinoma.[3-8] Combination therapy with other immunosuppressive medications may increase the treatment success of biologics but is associated with an increased risk of adverse events. Hyams *et al.*[7] demonstrated that the standardized incidence ratio (SIR) for malignancy among 5766 pediatric-onset IBD patients included in the DEVELOP registry was significantly elevated for patients receiving combination therapy with thiopurine and biologics (SIR 3.06) but not with thiopurine or anti-tumor necrosis factor alpha (TNFα) as monotherapy. Several studies in adult IBD patients have shown that current exposure to thiopurines appeared to be more significantly associated with increased incidence of thiopurine-related malignancies than past exposure to thiopurines[9], while the DEVELOP registry did not delineate treatment as current or past.[7]

Given the rarity of some of these events, more detailed information on pediatric-onset IBD patients who develop cancer or have a fatal outcome is needed to obtain more insight in predictive factors of severe outcomes and to be able to optimize evidence-based treatment guidelines. The aim of this study was to identify and describe the most common causes of mortality, types of cancer and previous or current therapy among children with pediatric-onset IBD. We also aimed to describe the patient-specific and disease-specific characteristics of these groups and investigate the relationship between severe outcomes in pediatric-onset IBD patients and treatment exposure.

METHODS

Study design.
This was a prospective multinational observational study performed in collaboration with the European Society for Pediatric Gastroenterology, Hepatology and Nutrition (ESPGHAN). We collected patients at all sites in 25 countries for 42 months from June 2013 to December 2016 (Table S1 tabulates all participating countries). The study was conducted in all sites according to the instructions of the local ethical committees. Some committees waived the need for informed consent due to the anonymous and non-

interventional fashion of the study.

In each participating country, both a pediatric gastroenterologist and an adult gastroenterologist were appointed as national representatives. The representative pediatric gastroenterologist contacted pediatric gastroenterologists in each country through e-mail every 6 months throughout the study period in attempt to obtain all new cases of malignancy and/or mortality in patients with pediatric-onset IBD over the previous 6 month period. An explicit case report form (provided online on the website of the *Alimentary Pharmacology & Therapeutics* journal) was completed by the reporting pediatric gastroenterologist for all newly reported cases, using data from individual patient records. Cases that occurred prior to the study period were not included in our study. Existing IBD- or cancer registries were not used to identify cases. In order to assess response rates, pediatric gastroenterologists were asked to actively respond negatively if no case was identified. When data were unclear the pediatric gastroenterologist was queried further by e-mail. In order to identify cases that may have transitioned to adult care or developed malignancies after age 18 as a result of pediatric disease, the representative adult gastroenterologists followed the same procedures. Cases were ascertained by comparing and cross-checking all reported cases on country, year of reporting, sex and exact diagnosis.

Survey response rates per country were monitored throughout the study period to obtain insight into coverage (Table S1). Response rate was calculated as the number of physicians replying to the e-mail call out of the total number of registered pediatric or adult gastroenterologists contacted in that country. Additional e-mails were sent out by ESPGHAN to increase awareness among gastroenterologists. The European Crohn's and Colitis Organisation (ECCO) provided logistical support and invited national representatives to nominate national coordinators. In addition, national patient organizations were informed on the study and every year investigators meetings were organized with national representatives from all participating countries.

Patient selection.

Inclusion criteria for reported cases were patients with pediatric-onset IBD diagnosed according to the revised Porto criteria before 19 years of age[10], who died or were diagnosed with a malignancy after IBD diagnosis but before the age of 26 years. Patients with IBD-like inflammation due to proven monogenetic defects were excluded. Although infections are usually due to current immune suppression, malignancies may develop later after transition to adult care. In almost all European countries, pediatric-onset IBD patients < 16 years are cared for by pediatric gastroenterologists, and transition to adult care occurs after this age. As current guidelines for cancer surveillance in children and adults recommend surveillance starting from 8-10 years after disease-onset of pediatric-onset IBD, we extended follow up to 10 years after the age of 16 years to capture pediatric-onset IBD patients who developed cancer or mortality after transition to adult care.

Data collection.

Data were collected by means of a case report form, which included eight domains and 42 questions. The first three domains were divided into demographics, patient characteristics and disease characteristics, including questions on reporting physician, country, sex, IBD type, age at IBD diagnosis and comorbidities. The following domains of characteristics of malignancy and/or mortality included questions on type of malignancy and/or cause of death, age at malignancy and/or death, and IBD disease duration. The last domains contained questions on current and past therapy exposure, including thiopurines, biologics (agents blocking TNFα) or other immunosuppressant drugs (steroids, methotrexate and calcineurin inhibitors), as well as exposure to combination therapies and duration of exposure. Current exposure was defined as exposure in the three months prior to malignancy diagnosis or fatal outcome. Past exposure was defined as exposure previous to the last three months. "Ever exposed" was defined as exposure at any time prior to the malignancy or fatal outcome. Data was stored in a central database in the Erasmus Medical Centre in Rotterdam, The Netherlands.

Number of pediatric-onset IBD patients at risk (denominator).

Representative pediatric gastroenterologists of all European participating countries were requested to complete a survey that collected data stating which regions in their country as defined by the Nomenclature of Territorial Units for Statistics (NUTS) were covered during the years of data collection in this study (Table S2). Coverage was calculated for all European countries responding to the survey (Table S2). Full coverage was assumed for adults (20-26 years).

Eurostat most recent census data (2016) were used to obtain the total number of individuals covered in the general population (0-26 years) per country. This comprised the population from which the reported pediatric-onset IBD cases with cancer and/or mortality in our study were derived. In literature, pediatric-onset IBD prevalence among children (0-19 years) is around 30 per 100,0000.[11-15] An estimated pediatric-onset IBD prevalence < 26 years of 60 per 100,000 was used to calculate the number of pediatric-onset IBD patients at risk for all countries. As this prevalence is likely an overestimation of the real prevalence[11-15], this is a conservative approach ensuring that the incidences of cancer and mortality in the pediatric-onset IBD patient population are not overestimated.

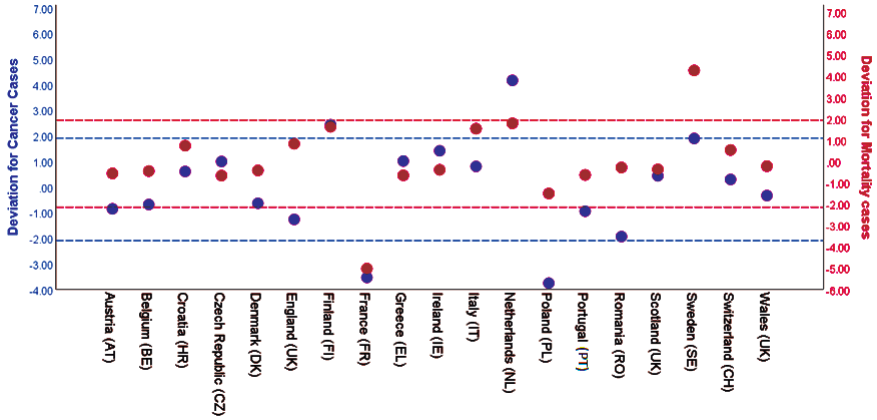
The total covered population (0-26 years) was multiplied by the estimated pediatric-onset IBD prevalence for this age group, resulting in an estimation of the true population that has the disease (number of pediatric-onset IBD patients 0-26 years at risk). As the

study was conducted over 42 months, the number of pediatric-onset IBD patients at risk per year was multiplied by the years of exposure (3.5 patient-years).

Calculation of cancer and mortality incidence.

Cancer and mortality incidences for pediatric-onset IBD patients < 26 years were calculated based on the number of patient-years (as described above) and the number of reported cases per country and in total (Table 1 and 2). Confidence intervals were calculated using

A



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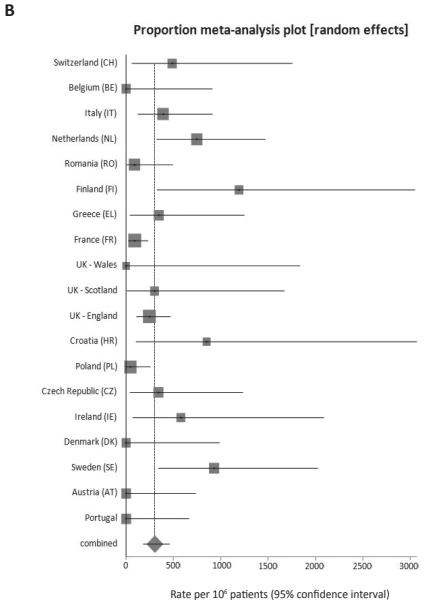

Figure 1. European incidence maps of malignancy and mortality in pediatric-onset IBD patients < 26 years. (A) The average incidence for all countries was used to calculate the expected number of cases per country according to their covered population. Deviation of the number of reported cancer and mortality cases from the expected number of cases is shown for European countries. **(B)** Forest plot with 95% confidence intervals for each country. Random-effects model for meta-analysis was used due to high heterogeneity in the results to investigate the incidences of cancer and/or mortality.

Byar's approximation based on a Poisson distribution. The relative risk (RR) and its 95% confidence interval (CI) were calculated according to Altman, 1991, in order to compare cancer incidence in pediatric-onset IBD patients with the general population. The average incidence for all countries was used to calculate the expected number of cases per country according to their covered population (Table 1 and 2, Figure 1). Poisson analysis was used to investigate the extent of variation in the number reported cases per country with statistical inference. Random-effects model for meta-analysis was used due to high heterogeneity in the results to investigate the incidences of cancer and/or mortality. The rare nature of the examined cases precluded comparisons between smaller regions and/or comparisons between sub-categories of the cases.

Statistics.

Data are presented as median and interquartile range (IQR) or percentages. Data analyses were performed using IBM SPSS version 24 and GraphPad Prism version 5.0. Baseline demographic and disease characteristics were evaluated for the entire cohort using descriptive statistics, including means and standard deviations (SD) or median and IQR for continuous variables, and frequencies and percentages for categorical outcomes. For comparison between three groups, the Fisher's exact test was used for categorical outcomes and the Kruskal Wallis H test was used for continuous variables. If there was a statistically significant difference between the three groups, the Mann-Whitney test (continuous variables) or Fisher's exact test (categorical variables) was used to test differences between two groups as a follow up analysis. P values <0.05 were considered to present a statistically significant difference. In case of multiple comparisons, an adjusted significance level was used according to the Bonferroni correction (significance level = 0.05/number of comparisons).

RESULTS

Coverage and participation response rates per country.

Except for three countries, all European countries responding to the survey claimed full coverage (Table S2). Response rates differed between the 25 countries (Table S1). Data collection among pediatric gastroenterologists was high in 15 out of 25 participating countries, with ≥ 70% of pediatric gastroenterologists responding to the semi-annual e-mails throughout the study period with a valid reply. Data collection from adult gastroenterologists had a ≥ 70% response rate in nine out of 25 countries, respectively (Table S1).

Patient Characteristics.

A total of 60 patients with either fatalities or cancer were identified during the study period (UC, n=21; CD, n=33; IBD-U, n=6). Of the 60 patients, 43 were diagnosed with malignancy and in 26 a fatality occurred; in nine (35%) of the latter the cause of death was cancer.

Malignancy and mortality incidence.

The final estimated number of pediatric-onset IBD patients aged 0-26 years at risk in Europe was 192,625 patient-years. Since 33 cancer cases were reported in 192,625 patient-years (Table 1), the cancer incidence in pediatric-onset IBD patients aged 0-26 years was 171 per 1,000,000 (95% CI 120 – 238). Based on literature from national cancer registries, the cancer incidence in the general population aged 0-26 years is estimated at 210 per 1,000,000.[16, 17] Overall, the cancer incidence among pediatric-onset IBD patients was not found to be significantly different compared to the general population for this age group. The relative risk for malignancy in the pediatric-onset IBD population compared to the general population was found to be 0.816 (95% CI 0.57 – 1.18, p=0.277). However, cancer incidences among

Table 1. Malignancy incidence in pediatric-onset IBD patients < 26 years in European countries.

Country	No. malignancy cases reported	Pediatric-onset IBD patients in 3.5 years DENOMINATOR DATA‡	Annual incidence per 1.000.000 patients†	No. of expected cases§	Variation from expected
Austria (AT)	0	5,021	0	0.86	-0.86
Belgium (BE)	0	4,056	0	0.69	-0.69
Croatia (HR)	1	2,353	425	0.40	0.60
Czech Republic (CZ)	2	5,866	341	1.01	0.99
Denmark (DK)	0	3,737	0	0.64	-0.64
Finland (FI)	3	3,356	894	0.57	2.43
France (FR)	4	44,016	91	7.54	-3.54
Greece (EL)	2	5,800	345	0.99	1.01
Ireland (IE)	2	3,460	578	0.59	1.41
Italy (IT)	3	12,834	234	2.20	0.80
Poland (PL)	0	21,957	0	3.76	-3.76
Portugal (PT)	0	5,565	0	0.95	-0.95
Romania (RO)	0	11,332	0	1.94	-1.94
Sweden (SE)	3	6,461	464	1.11	1.89
Switzerland (CH)	1	4,118	243	0.71	0.29
Netherlands (NL)	6	10,722	560	1.84	4.16
UK - England	5	36,624	137	6.27	-1.27
UK - Scotland	1	3,338	300	0.57	0.43
UK - Wales	0	2,010	0	0.34	-0.34
Total	**33**	**192,625**	**171**	**33**	**NR**

‡See table S2 for calculation of denominator data. †Incidence calculation based on the number of patient-years and the number of reported cases. Incidence is reported in patient-years. §The average incidence for all countries was used to calculate expected number of cases per country. Abbreviations: IBD = inflammatory bowel disease, No = number of, NR = not relevant, UK = United Kingdom.

Table 2. Mortality incidence in pediatric-onset IBD patients < 26 years in European countries.

Country	No. fatal cases reported¶	Pediatric-onset IBD patients in 3.5 years DENOMINATOR DATA‡	Annual incidence per 1.000.000 patients†	No. of expected cases§	Variation from expected
Austria (AT)	0	5,021	0	0.57	-0.57
Belgium (BE)	0	4,056	0	0.46	-0.46
Croatia (HR)	1	2,353	425	0.27	0.73
Czech Republic (CZ)	0	5,866	0	0.67	-0.67
Denmark (DK)	0	3,737	0	0.43	-0.43
Finland (FI)	2	3,356	596	0.38	1.62
France (FR)	0	44,016	0	5.03	-5.03
Greece (EL)	0	5,800	0	0.66	-0.66
Ireland (IE)	0	3,460	0	0.40	-0.40
Italy (IT)	3	12,834	234	1.47	1.53
Poland (PL)	1	21,957	46	2.51	-1.51
Portugal (PT)	0	5,565	0	0.64	-0.64
Romania (RO)	1	11,332	88	1.29	-0.29
Sweden (SE)	5	6,461	774	0.74	4.26
Switzerland (CH)	1	4,118	243	0.47	0.53
Netherlands (NL)	3	10,722	280	1.22	1.78
UK - England	5	36,624	137	4.18	0.82
UK - Scotland	0	3,338	0	0.38	-0.38
UK - Wales	0	2,010	0	0.23	-0.23
Total	**22**	**192,625**	**114**	**22**	**NR**

¶Including fatal cancer and mortality due to other causes. ‡See table S2 for calculation of denominator data. †Incidence calculation based on the number of patient-years and the number of reported cases. Incidence is reported in patient-years. §The average incidence for all countries was used to calculate expected number of cases per country. Abbreviations: IBD = inflammatory bowel disease, No = number of, NR = not relevant, UK = United Kingdom.

pediatric-onset IBD patients in specific countries, including the Netherlands, Finland and Sweden, were higher compared to the general population (Table 1).

To obtain insight in under-reporting per country, reported number of cases were compared to the average rate of reported rates (Table 1 and 2, Figure 1A). Five countries were found to have significantly different (lower or higher) number of reported cases compared to the average rate of reported rates (Figure 1B). A negative difference in variation was seen in France, Poland and Romania. Particularly, France and Poland reported a significantly lower number of cases than expected for both cancer and mortality (p=0.013 and 0.024 respectively, based on a Poisson distribution) which indicated potential under-reporting. When excluding these three countries, the cancer incidence in pediatric-onset IBD patients aged 0-26 years increased to 230 per 1,000,000 (95% CI 157 – 326). Sweden,

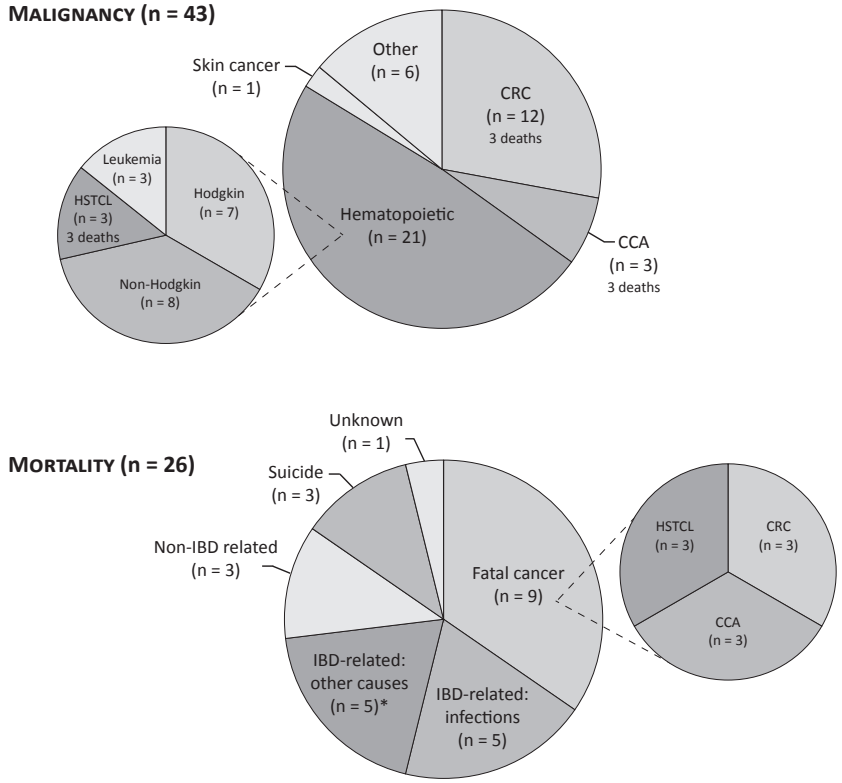

Figure 2. Causes of malignancy and mortality in pediatric-onset IBD patients (total cohort, n=60). A total of 43 malignancies and 26 mortalities were included in our cohort. Patients who died due to cancer were diagnosed with CRC (n=3), CCA (n=3) and HSTCL (n=3).

Table 3. Patient characteristics of pediatric-onset IBD patients who developed a malignancy and/or had a fatal outcome.

	Non-fatal cancer	Fatal cancer	Mortality due to other causes	Total	p value
Total patients, N	34	9	17	60	
Sex					
Male, N (%)	18 (52.9)	6 (66.7)	6 (35.3)	30 (50)	NS
Female, N (%)	16 (47.1)	3 (33.3)	11 (64.7)	30 (50)	
IBD diagnosis					
CD, N (%)	20 (58.9)	4 (54.4)	9 (52.9)	33 (55.0)	NS
UC, N (%)	11 (32.4)	5 (55.6)	5 (29.4)	21 (35.0)	
IBD-U, N (%)	3 (8.7)	NA	3 (17.7)	6 (10.0)	
Age at IBD diagnosis					
Mean (SD)	12.3 (4.1)	12.1 (4.3)	10.3 (4.2)	11.7 (4.2)	NS
Median (IQR)	13.5 (10.6 - 15.4)	12.9 (8.3 - 15.4)	11.8 (7.7 - 13.5)	12.7 (9.0 - 14.8)	
Duration of disease to cancer, y					
Mean (SD)	5.5 (4.0)	9.1 (3.5)	NA	6.2 (4.1)	**0.019**[g]
Median (IQR)	4.3 (2.0 - 9.0)	9.1 (5.8 - 12.2)	NA	5.5 (2.8 - 9.5)	
Duration of disease to death, y					
Mean (SD)	NA	9.5 (3.4)	5.3 (4.8)	6.7 (4.8)	**0.018**[h]
Median (IQR)	NA	9.9 (6.4 - 13.0)	3.6 (1.6 - 8.0)	7.0 (2.1 - 10.0)	
Age at cancer, y					
Mean (SD)	17.7 (4.2)	21.0 (2.4)	NA	18.4 (4.1)	**0.012**[g]
Median (IQR)	16.6 (15.0 - 21.9)	20.0 (19.0 - 23.5)	NA	17.4 (16.0 - 22.0)	
Age at death, y					
Mean (SD)	NA	21.9 (2.8)	15.2 (5.4)	17.5 (5.7)	**0.002**[h]
Median (IQR)	NA	20.0 (19.5 - 24.7)	15.1 (12.9 - 18.8)	17.0 (14.0 - 21.8)	
Comorbidities					
PSC, N (%)	2 (5.9)	5 (55.6)	2 (11.8)	9 (15.0)	**0.002**[a]; NS[b]; NS[c]
Perianal disease, N (%)	9 (26.5)[d]	1 (11.1)[§]	6 (35.3)[e]	16 (26.7)[f]	NS

For comparison between 3 groups, the Fisher's exact test was used for categorical outcomes and the Kruskal Wallis H test was used for continuous variables. If there was a statistically significant difference between the 3 groups, the Mann-Whitney test (continuous variables) or Fisher's exact test (categorical variables) was used to test differences between 2 groups. Abbreviations: NA, not applicable; PSC, primary sclerosing cholangitis; CD, Crohn's disease; UC, Ulcerative colitis; IBD-U, IBD unclassified; IQR, interquartile range; SD, standard deviation; N, number; y, year.

[a] P value calculated with Fisher's exact test comparing nonfatal cancer vs fatal cancer.
[b] Nonfatal cancer vs mortality.
[c] Fatal cancer vs mortality.
[d] One missing value.
[e] Two missing values.
[f] Three missing values.
[g] P value calculated with Mann-Whitney test comparing nonfatal cancer vs fatal cancer.
[h] P value calculated with Mann-Whitney test comparing fatal cancer vs mortality.

Finland and the Netherlands presented significantly more cases than expected (p=0.007, 0.011 and 0.007 respectively).

Malignancy.

We identified 43 malignancies during the study period of which 24 (56%) occurred in patients with CD (Table 3). Patients who developed a fatal outcome due to their malignancy (6 males, 3 females) had been diagnosed with IBD at a median age of 12.9 year (IQR 8.3 – 15.4). They had significantly longer IBD disease duration to cancer than those with a non-fatal outcome (9.1 years [IQR 5.8 – 12.2] versus 4.3 years [IQR 2.0 – 9.0], p=0.019) and developed cancer at a higher median age of 20.0 years (IQR 19.0 – 23.5) versus 16.6 years (IQR 15.0 – 21.9) in patients with non-fatal cancer (p=0.012).

Hematopoietic tumors (n=21, 49%) were the most frequently reported type of malignancy (Figure 2). The median age at development of a hematopoietic tumor was 17.0 years (IQR 14.9 – 20.5), which was slightly lower compared to the other types of cancer (19.0 years, IQR 16.2 – 24.0, p=0.19). The majority of hematopoietic tumors included Hodgkin and non-Hodgkin lymphomas (n=7 and n=8, respectively). Two patients with non-Hodgkin lymphomas were Epstein-Barr-virus (EBV) positive (25%), four patients were EBV negative (50%) and the EBV status was unknown in two patients. None of the patients with Hodgkin lymphoma or NHL died. Three male patients (1 UC; 2 CD) were diagnosed with a hepatosplenic T-cell lymphoma (HSTCL, n=3, 14.3%). All of the patients with HSTCL died. These three patients developed HSTCL at the age of 20.0, 18.0 and 23.0 years, after an IBD

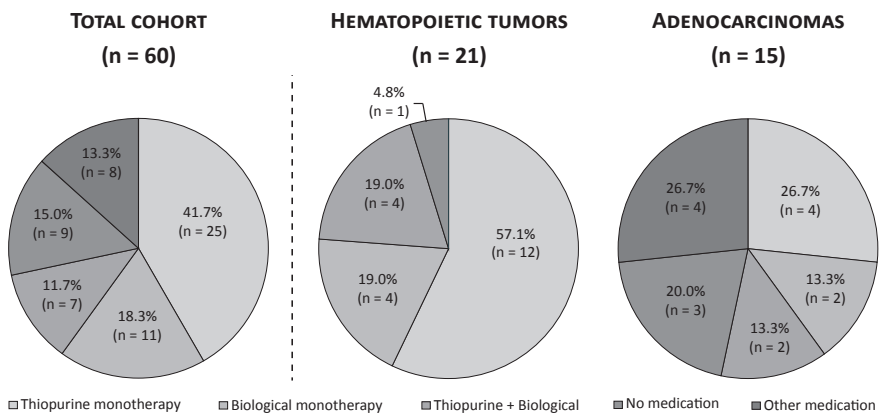

Figure 3. Medication exposure in pediatric-onset IBD patients in the 3 months prior to malignancy or mortality diagnosis. Current exposure (in the 3 months prior to severe outcome) was divided in 1) thiopurine monotherapy, 2) biologic monotherapy, 3) combination therapy of thiopurine with a biologic, 4) no medication or 5) other medication consisting of steroids, methotrexate, calcineurin inhibitors. Numbers and frequencies for the total cohort and patients who developed a hematopoietic malignancy or adenocarcinoma are shown.

disease duration of 5.2, 6.0 and 5.3 years, respectively. In all cases death occurred within a year after the HSTCL diagnosis at the age of 20.3, 19.0 and 23.5 years.

In patients diagnosed with colorectal carcinoma (CRC, n=12, 29%), UC was the most frequent type of IBD (UC, n=6; CD, n=4; IBD-U, n=2). The four CD patients who developed CRC all had disease confined to the colon (L2; Paris classification[18]). IBD disease duration to cancer in patients with CRC was significantly longer than in patients who developed a hematopoietic tumor, with a median duration of 9.3 years (IQR 4.3 – 11.8) versus 3.7 years (IQR 2.0 – 7.8, p=0.034, Table S3). The youngest reported CRC patient was a female who developed CRC at the age of 14.5 years, four years after her initial IBD diagnosis. CRC was fatal in 25% of cases (n=3), always within one year after cancer diagnosis. The youngest patient with a fatal outcome due to CRC was 19.5 years old when CRC was diagnosed, 9.0 years after her IBD disease diagnosis, and died after a period of six months.

Cholangiocarcinoma (CCA) was the third group of most frequently reported malignancies (n=3, 7%, all male UC patients). Notably, all CCA cases were fatal and all patients had a concomitant diagnosis of primary sclerosing cholangitis (PSC). CCA was diagnosed after a median IBD duration of 12.9 years (IQR 5.0 – 16.0). The youngest reported CCA patient was 19 years old when his cancer was diagnosed and died within the same year, 14 years after his IBD diagnosis and 11 years after his PSC diagnosis. Of all patients who died due to an adenocarcinoma (CRC, n=3, and CCA, n=3), 67% (n=4) had concomitant PSC.

Other causes of cancer were melanoma (Clark's level 1) at the age of 12.4 years (n=1), myeloid sarcoma (n=1), neuroendocrine tumor with liver metastasis (n=1), alveolar rhabdomyosarcoma (n=1), thyroid carcinoma (n=1), brain glioblastoma grade IV (n=1) and renal cell carcinoma (n=1).

Malignancy and Treatment: current exposure

Overall, virtually all patients who developed a hematopoietic malignancy (n=21) had ever been exposed to thiopurines (n=20, 95%, Table 4). With regard to the three months prior to their cancer diagnosis, the majority of these patients were exposed to thiopurine monotherapy (n=12, 57%). Four patients with a hematopoietic malignancy used thiopurines in combination with a biologic (n=4, 19%), while others were exposed to biologic monotherapy (n=4, 19%) or were not using any medication (n=1, 5%) in the three months prior to their cancer diagnosis (Figure 3).

Analysis of subgroups of hematopoietic tumors demonstrated that all patients who developed a HSTCL were exposed to thiopurines in the three months prior to their cancer diagnosis without concurrent or prior biologic exposure. We observed a numerically lower frequency of current exposure to thiopurine monotherapy in patients diagnosed with an adenocarcinoma compared to patients with a hematopoietic tumor (n=4, 27% vs. n=12,

Table 4. Medication exposure in pediatric-onset IBD patients who developed a malignancy

	Total (n=60)	Hematopoietic (n=21)	HSTCL (n=3)	Non-HSTCL (n=18)	Adenocarcinoma (n=15)	CRC (n=12)	CCA (n=3)	p value
CURRENT EXPOSURE								
Thiopurine monotherapy, N (%)	25 (41.7)	12 (57.1)	3 (100)	9 (50.0)	4 (26.7)	4 (33.3)	0 (0)	0.096
Biologic monotherapy, N (%)	11 (18.3)	4 (19.0)	0 (0)	4 (22.2)	2 (13.3)	2 (16.7)	0 (0)	1.00
Combination: Thiopurine + Biologic, N (%)	7 (11.7)	4 (19.0)	0 (0)	4 (22.2)	2 (13.3)	2 (16.7)	0 (0)	1.00
No medication, N (%)	9 (15.0)	1 (4.8)	0 (0)	1 (5.6)	3 (20.0)	1 (8.3)	2 (66.7)	0.287
Other medication, N (%)	8 (13.3)	0 (0.0)	0 (0)	0 (0)	4 (26.7)	3 (25.0)	1 (33.3)	0.023
PAST EXPOSURE								
Thiopurine monotherapy, N (%)	25 (41.7)	9 (42.9)	3 (100)	6 (33.3)	5 (33.3)	4 (33.3)	1 (33.3)	0.732
Biologic monotherapy, N (%)	2 (3.3)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	NA
Combination: Thiopurine + Biologic, N (%)	17 (28.3)	8 (38.1)	0 (0)	8 (44.4)	4 (26.7)	4 (33.3)	0 (0)	0.721
No medication, N (%)	8 (13.3)	3 (14.3)	0 (0)	3 (16.7)	3 (20.0)	2 (16.7)	1 (33.3)	0.287
Other medication, N (%)	9 (15.0)	1 (4.8)	0 (0)	1 (5.6)	3 (20.0)	2 (16.7)	1 (33.3)	0.677
TOTAL EXPOSURE								
Thiopurines (ever exposed, N (%)	49 (81.6)	20 (95.2)	3 (100)	17 (94.4)	10 (66.6)	9 (75.0)	1 (33.3)	0.063
Biologics (ever exposed), N (%)	22 (36.7)	9 (42.8)	0 (0)	9 (50.0)	5 (33.3)	5 (41.7)	0 (0)	0.732
Methotrexate (ever exposed), N (%)	9 (15.0)	3 (14.2)	0 (0)	3 (16.7)	2 (13.3)	2 (16.7)	0 (0)	1.00
Steroids (ever exposed), N (%)	44 (73.3)	16 (76.2)	3 (100)	13 (72.2)	11 (73.3)	9 (75.0)	2 (66.6)	1.00
Calcineurin inhibitor (ever exposed), N (%)	5 (8.3)	2 (9.5)	0 (0)	2 (11.1)	1 (6.7)	1 (8.3)	0 (0)	1.00
DURATION OF TOTAL EXPOSURE								
Duration thiopurine (y, median + IQR)	2.5 (0.9-5.7)†	2.6 (0.9-4.8)	4.2 (4.0-5.0)	1.9 (0.9-4.5)	6.0 (1.5-8.6)	6.0 (1.3-8.8)	6.0 (NA)	0.13
Duration biologic (y, median + IQR)	2.2 (0.6-4.0)†	3.0 (2.1-4.8)	NA	3.0 (2.1-4.8)	2.0 (0.3-2.5)	2.0 (0.3-2.5)	NA	0.083

P values are from Fisher's Exact Test for categorical variables. Definitions: Thiopurine monotherapy, exposure to thiopurines without any biologic exposure; Biological monotherapy, exposure to biologics without any thiopurine exposure; Combination therapy, combined exposure to thiopurines or biologics, either at the same time (n=18 for the total group) or in consecutive fashion (only in 1 patient for the total group); Other medications, consisting of MTX, CAI and steroids; No medication, not using thiopurines, biologicals, MTX, CAI or steroids; Current exposure, exposure in the 3 months prior to malignancy diagnosis or fatal outcome; Past exposure, exposure previous to the last 3 months; Ever exposed, exposure at any time prior to the malignancy or fatal outcome. †two missing values. Abbreviations: CAI, calcineurin inhibitors, MTX, methotrexate, NA, not applicable; CD, Crohn's disease; UC, Ulcerative colitis; IBD-U, IBD unclassified; IQR, interquartile range; SD, standard deviation; N, number; y, year.

57%, respectively, p=0.096). Curiously, none of the three patients who developed a CCA were receiving a thiopurine or biologic.

Malignancy and Treatment: Past exposure

There were no significant differences in percentage of patients with past exposure when comparing patients with hematopoietic cancer to patients who developed an adenocarcinoma (Table 4). Total duration of thiopurine exposure was numerically higher in the group of patients with an adenocarcinoma compared to patients who developed a hematopoietic tumor (median duration of 6.0 years (IQR 1.5 – 8.6) and 2.6 years (IQR 0.9 – 4.8), respectively; p=0.13). This observation can be explained by the significantly longer IBD disease duration in patients diagnosed with an adenocarcinoma compared to patients with a hematopoietic tumor (9.5 years [IQR 5.0 – 12.0] versus 3.7 years [IQR 2.0 – 7.8], respectively, p=0.0064).

All three patients with a HSTCL had been previously exposed to thiopurines. The median duration of thiopurine exposure in the three HSTCL patients was 4.2 years (IQR 4.0 – 5.0) compared with 1.9 years (IQR 0.9 – 4.5) in the patients with other hematopoietic malignancies.

Other risk factors

Fatal cancers consisted of CRC (n=3), CCA (n=3) and HSTCL (n=3). In this total group of fatal cancers (n=9), 56% of patients (n=5) had a concomitant diagnosis of PSC, compared to 6% in the group of patients with non-fatal cancer (n=2, p=0.002; Table 3). The majority of patients with fatal cancer and a concomitant PSC diagnosis died due to adenocarcinomas (CCA, n=3, CRC, n=1, HSTCL, n=1). CCA was diagnosed at a median duration of 4.9 years after the initial PSC diagnosis (IQR 0.04 – 8.9 years). The PSC patient who died of CRC was a male patient who developed IBD at the age of 14 years. He was diagnosed with both PSC and CRC at the age of 24 years.

Only a small number of patients who developed a malignancy had a positive family history for cancer (n=5, 12%), consisting of three patients with a hematopoietic tumor (1 Hodgkin lymphoma, 1 non-Hodgkin lymphoma, 1 leukaemia) and two CRC patients. None of the patients who developed a malignancy had had a previous cancer diagnosis.

Mortality.

A total of 17 non-cancer related deaths were reported during the study period (28%, Table 3). Patients who died due to non-cancer related causes were significantly younger than patients who died due to cancer (15.1 years [IQR 12.9 – 18.8] versus 20.0 years [IQR 19.0 – 23.5], p=0.002, Table 3). Infections (29%, n=5) were the main cause of non-cancer related deaths. These included four patients with sepsis and one patient who developed

Table 5. Medication exposure of pediatric-onset IBD patients who developed a fatal outcome.

							Current exposure			Previous exposure		
	Cause of mortality	**Total (n=26)**	**Sex (M/F)**	**IBD type**	**Age at IBD diagnosis, y**	**Age at death, y**	***Thiopurines***	***Biologics***	***Steroids***	***Thiopurines***	***Biologics***	***Steroids***
	Fatal cancer, N (%)	**9 (35)**										
1	*CRC*		F	CD	10.5	19.9	yes	no	no	yes	yes	yes
2	*CRC*		F	CD	6	20	yes	no	yes	yes	no	no
3	*CRC*		F	UC	14.1	25.4	yes	no	no	yes	no	no
4	*CCA*		M	UC	12.9	26	no	no	yes	no	no	yes
5	*CCA*		M	UC	16	24	no	no	no	no	no	no
6	*CCA*		M	UC	5	19	no	no	yes	yes	no	no
7	*HSTCL*		M	CD	14.8	20	yes	no	no	yes	no	yes
8	*HSTCL*		M	UC	12.0	19.0	yes	no	no	yes	no	yes
9	*HSTCL*		M	CD	17.7	23.5	yes	no	no	no	no	yes
	IBD-related deaths: infectious causes, N (%)	**5 (19)**										
10	*Sepsis due to unknown infectious agent*		F	CD	14	21	no	yes	no	yes	yes	yes
11	*Sepsis due to unknown infectious agent*		F	CD	13.3	22.6	no	no	no	yes	yes	no
12	*Disseminated tuberculosis on IFX treatment*		F	CD	11.8	12.8	no	yes	yes	yes	yes	yes
13	*Central line sepsis while on TPN*		F	IBDU	1.9	2.2	yes	no	yes	no	no	yes
14	*Joubert Syndrome with renal dysfunction and sepsis*		F	UC	12.4	15.1	yes	no	no	yes	no	yes
15	*Post operatively*		M	CD	13.5	15.4	yes	no	no	yes	no	no
16	*Liver failure (in patient with concomitant PSC)*		F	UC	14.9	16	no	no	yes	yes	no	Yes
17	*Necrotizing encephalitis*		M	IBDU	5	7.7	yes	no	no	yes	no	no
18	*Acute necrotizing encephalopathy*		F	UC	13	13	no	no	yes	no	no	yes
19	*Multi organ failure while on TPN*		F	CD	13.5	20.5	no	no	no	yes	no	yes
20	*Suicide*		M	UC	2	12	no	no	yes	no	no	yes
21	*Suicide*		M	CD	7.9	25	no	no	no	yes	yes	yes
22	*Suicide*		F	CD	9	15	no	yes	yes	no	yes	yes
	Non-IBD related deaths, N (%)	**3 (12)**										
23	*Traffic accident*		M	CD	9.1	17	no	yes	no	yes	yes	yes
24	*Traffic accident*		M	CD	11.6	13	no	yes	no	no	yes	yes
25	*Cardiac asthma caused by an aortic stenosis*		F	IBDU	7.6	15	yes	no	no	yes	no	yes
	Unknown, N (%)	**1 (4)**										
26	*Unknown*		F	UC	14.3	15.9	yes	no	yes	no	no	yes

Abbreviations: CD, Crohn's disease; UC, Ulcerative colitis; IBD-U, IBD unclassified; CRC, colorectal carcinoma; TPN, total parental nutrition; CCA, cholangiocarcinoma; HSTCL, hepatosplenic T-cell lymphoma.

disseminated tuberculosis on anti-TNFα therapy (Figure 2 and Table 5). In addition to infectious causes, another five patients died due to IBD- or IBD-therapy related causes that were of non-infectious origin. Death due to liver failure occurred in a 16 year old female with UC and PSC at 14.9 years of age. One patient died post operatively, two days after a right hemi-colectomy for CD. One patient died of multi organ failure complicating total parental nutrition. An additional two patients developed encephalopathy, but an infectious origin was not proven in either case.

Three patients committed apparent suicide. All three patients had severely complicated disease. One patient had PSC and failed liver transplantation. The second had severe perianal disease and enterocutaneous fistulas, and committed suicide after several years of inpatient care for repeated surgeries and severe postoperative complications. The third was a 15 year-old patient who underwent multiple procedures and had been surgically treated for perianal disease. Based on literature from national registries, the European incidence rate of suicide ranges between 10 to 40 per 1,000,000 in people aged 0-26 years.[19, 20] Overall, the suicide incidence among pediatric-onset IBD patients was not found to be significantly different compared to the general population for this age group (RR = 0.45, 95% CI 0.14 – 1.45). Other causes of death were unrelated to disease or treatment (n=3) or unknown (n=1) and are shown in Table 5.

Risk factors and associated therapy for mortality

Four of the five (80%) patients who died due to an infectious cause were receiving immune suppression, of whom two were on dual immune suppressive agents and the rest on a single drug (Table 5). Only two of those four patients were exposed to steroids at the moment of death. The remaining patient was not taking any medication. She had been previously exposed to combination therapy but refused all immunomodulatory or biological therapy one year before her death.

Risks of malignancy and mortality in subpopulations.

The relative risk of malignancy or mortality was not found to be significantly different between CD and UC patients from this cohort (RR = 0.99; 95% CI 0.40 – 2.46). When comparing patients with concomitant PSC to patients without PSC, a higher relative risk for fatal malignancy was found (RR = 7.08, 95% CI 2.34 – 21.44, p = 0.0005).

DISCUSSION

In this first prospective multinational pediatric study to characterize malignancies and mortalities, we report the largest series of pediatric-onset IBD cases with these outcomes to date. Among the 26 identified fatalities over a three year period, nine were due to malignancies, ten were due to IBD or IBD-therapy related non-malignant causes (including 5 infections), and suicide was the third leading cause. The most common identified risk factor for cancer-associated mortality was presence of PSC (50% of cases).

In our study, the cancer incidence among pediatric-onset IBD patients was not found to be significantly different compared to the general population for this age group. We used the higher estimates for pan-European pediatric-onset IBD prevalence in order to be conservative with estimates for malignancy and mortality. Despite our conservative approach, incidences among pediatric-onset IBD patients were higher compared to the general population in some countries, including Sweden, Finland and the Netherlands (p=0.007, 0.011 and 0.007 respectively, Figure 1). As this study is unlikely to have bias from over-reporting, the data from these countries should raise some concern. When excluding countries with large negative deviation from the expected number of cases (i.e. Poland and France), the cancer incidence in pediatric-onset IBD patients aged 0-26 years increased to 230 per 1,000,000 (95% CI 157 - 326), demonstrating that these countries lowered the total reported incidence.

An increased relative risk for malignancy in pediatric-onset IBD patients has been established by several previous studies. Peneau *et al.* identified nine patients with cancer in 698 pediatric-onset IBD patients over a median follow-up period of 15 years, which translated into an increased risk of cancer in pediatric-onset IBD patients when compared to the background population (SIR 3.0 [95%CI 1.3–5.9]; p-value <0.02).[8] More recently, data from a Swedish cohort also demonstrated a significantly increased adjusted hazard ratio for cancer in pediatric-onset IBD patients (2.2; 95% CI 2.0 – 2.5) compared to a matched general population over a 25 year follow up period.[21, 22] Gastrointestinal cancers had the highest relative risks, with a hazard ratio of 134 (95% CI 59.6 – 382) for liver cancers and 19.5 (95% CI 14.7 – 26.2) for CRC.

CCA and CRC were the most common type of neoplastic fatalities in our cohort (CCA, n=3, CRC, n=3). This is in line with the results reported in the EPIMAD study, where CRC was the only cause of neoplastic fatality among 698 pediatric-onset IBD patients followed over a course of 15 years.[8] In fact, in our cohort, HTSCL was only the third most common type of neoplastic fatality occurring in only three cases over three years. Fatal adenocarcinomas, which are highly likely to be associated with disease rather than treatment, were usually detected after >8 years of disease with the earliest occurrence at age 19. This suggests that current guidelines for surveillance in children and adults which recommend surveillance starting from eight or ten years of disease based on risk factors for CRC are adequate except in very rare cases.[23] Unfortunately, data on adherence to surveillance guidelines in our current cohort was not available. Pediatric and adult gastroenterologists treating patients with IBD should have increased awareness that these fatal malignant outcomes

might occur in the second and third decades of life with early onset disease, and that patients with PSC should be followed closely including screening for CCA. It is interesting to note that all three patients with PSC-associated CCA were not receiving thiopurines or biologics. Thiopurines have been associated with a significant decrease in IBD associated CRCs by van Schaik *et al*, while 5-ASA did not lead to a significant protective effect.[4] However, a recent French study found that 5-ASA was effective but questioned the efficacy of thiopurines.[24] Data regarding chemoprevention in IBD associated PSC is a current research gap, highlighted by the increased risk for CRC and CCA in these patients at an early age.[24-26] In order to identify gaps and guide future research, details on all collected data for individual patients with PSC-related cancer and mortality in this cohort is provided in Table S4.

Despite large number of patients, previous population-based studies on pediatric-onset IBD patients are underpowered with regard to drug exposure in patients who develop malignancy, and included patients that were older than in our series. A case series like this cannot truly determine causality between drug use and development of malignancy, but several observations from this study are important. Development of HSTCL is still a rare event, documented in only three patients over a three year period in the numerous countries surveyed. The three new cases identified by this prospective study occurred in patients that had been on current thiopurines without current or previous biologic exposure. This could suggest that current thiopurine exposure can be associated with these rare lymphomas in the absence of anti-TNF therapy. Similar to previous reports[6, 27-30], all patients were males. The recently published DEVELOP study did not evaluate the association between current exposure to drugs and cancer[7], though current exposure and not previous exposure to thiopurines seems to be more important for development of lymphomas.[9] However, the DEVELOP study did demonstrate that exposure to combination therapy, but not to thiopurines or infliximab monotherapy, was associated with an increase in risk for malignancy, with an adjusted SIR of 3.06 for developing a cancer if combination therapy of a biologic and a thiopurine was used. Another interesting observation in our study is that the majority of patients who developed a hematopoietic malignancy had been exposed to thiopurines (n=20, 95%). In fact 71% (n=15) were exposed to thiopurines in the last three months prior to their diagnosis; which is significantly higher compared to patients who developed adenocarcinomas (p=0.041). Altogether this suggests that current exposure and not previous exposure to thiopurines seems to be more important for development of lymphomas in pediatric-onset IBD patients, which is in line with the findings in adults patients by the CESAME study group.

The second leading cause of fatalities in young patients with IBD was likely associated with therapy. Five patients died from presumed or proven infections, and 4/5 were receiving concomitant immune suppression. Two patients died from necrotizing encephalopathy of unknown origin, which could have been infectious. In a previous retrospective study by the Porto group, combination therapy with any combination of two immune suppressive drugs was associated with infection-associated mortality.[6] In that study 86% of patients developing a fatal infection or sepsis had received two or more immune suppressive agents. The current study identified fewer cases of infectious mortality than the previous study, but also covered a shorter period of time (3 vs. 5 years).

Surprisingly, suicides were the third most common reason for fatalities, surpassing procedural complications (n=2), thromboembolic events (n=0) and liver disease (n=1). It is important to be cautious when interpreting suicides because data regarding previous mental health and other comorbidities are not available. The three cases reported, all involved patients with a severe complicated course, including patients with repeated liver- and stem cell transplantations for refractory disease. In fact, one suicide occurred during a hospital admission. Although suicide incidence was not significantly increased compared to the general population, the suicide cases may be indirectly considered disease-related deaths. This emphasizes the importance of psychological support in addition to medical treatment in pediatric-onset IBD patients.

Our study provides important insight regarding severe outcomes of pediatric-onset IBD but is not without limitations. First, our results suggest underreporting in several countries. To avoid underestimation of incidence rates in a multinational set-up, it is pivotal to obtain registry-based denominator data in future studies. In addition, lower response rates among adult gastroenterologists and differences in practice between the participating countries may have contributed to underreporting of malignancies after transition to adult care and may have added bias to the reported data. Secondly, the use of thiopurines has become increasingly contentious, since the link between thiopurines and lymphomas[9, 31] and skin cancers[32] has become available. It would be very easy to over-interpret data and assign risk to therapy in the cases with hematopoietic malignancies. However, a case series cannot adjust for underlying age-adjusted population risk. With the exception of HSTCL, hematopoietic malignancies do occur in healthy adolescents without underlying disease or exposure to drugs. Since pediatric-onset IBD tends to be aggressive and extensive, thiopurines are used very early in the disease in a large proportion of patients. Thus, clear associations between therapy and malignancies will require analysis adjusted for underlying risk of IBD and cancer in populations being explored. Current studies that have limited analysis to past exposure are insufficient. We therefore need long-term, prospective data on all children and adults via fully consented (international) registers to provide perspective regarding risk from disease and from therapy.

In conclusion, data from this multinational cohort with the largest number of pediatric-onset IBD patients with cancer and/or fatal outcome to date, suggests that fatal outcomes and malignancies are still rare events. However, PSC appears to be a significant

risk factor. While current guidelines for surveillance in children appear to be adequate, our data raises the question whether chemoprevention in IBD patients with concomitant PSC should be instituted. Future analysis of databases in participating countries may allow us to better evaluate the relative risks for pediatric-onset IBD patients compared to the background population.

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REFERENCES

1. Van Limbergen J, Russell RK, Drummond HE, et al. Definition of phenotypic characteristics of childhood-onset inflammatory bowel disease. *Gastroenterology* 2008;**135**(4):1114-22.
2. Shaoul R, Karban A, Reif S, et al. Disease behavior in children with Crohn's disease: the effect of disease duration, ethnicity, genotype, and phenotype. *Dig Dis Sci* 2009;**54**(1):142-50.
3. Peyrin-Biroulet L, Oussalah A, Williet N, Pillot C, Bresler L, Bigard MA. Impact of azathioprine and tumour necrosis factor antagonists on the need for surgery in newly diagnosed Crohn's disease. *Gut* 2011;**60**(7):930-936.
4. van Schaik FD, van Oijen MG, Smeets HM, van der Heijden GJ, Siersema PD, Oldenburg B. Thiopurines prevent advanced colorectal neoplasia in patients with inflammatory bowel disease. *Gut* 2012;**61**(2):235-40.
5. Cleynen I, Gonzalez JR, Figueroa C, et al. Genetic factors conferring an increased susceptibility to develop Crohn's disease also influence disease phenotype: results from the IBDchip European Project. *Gut* 2013;**62**(11):1556-65.
6. De Ridder L, Turner D, Wilson DC, et al. Malignancy and mortality in pediatric patients with inflammatory bowel disease: A multinational study from the porto pediatric IBD group. *Inflammatory Bowel Dis* 2014;**20**(2):291-300.
7. Hyams JS, Dubinsky MC, Baldassano RN, et al. Infliximab Is Not Associated With Increased Risk of Malignancy or Hemophagocytic Lymphohistiocytosis in Pediatric Patients With Inflammatory Bowel Disease. *Gastroenterology* 2017.
8. Peneau A, Savoye G, Turck D, et al. Mortality and cancer in pediatric-onset inflammatory bowel disease: A population-based study. *Am J Gastroenterol* 2013;**108**(10):1647-1653.
9. Beaugerie L, Brousse N, Bouvier AM, et al. Lymphoproliferative disorders in patients receiving thiopurines for inflammatory bowel disease: a prospective observational cohort study. *Lancet* 2009;**374**(9701):1617-25.
10. Levine A, Koletzko S, Turner D, et al. ESPGHAN revised porto criteria for the diagnosis of inflammatory bowel disease in children and adolescents. *J Pediatr Gastroenterol Nutr* 2014;**58**(6):795-806.
11. Lakatos L, Lakatos PL. Is the incidence and prevalence of inflammatory bowel diseases increasing in Eastern Europe? *Postgrad Med J* 2006;**82**(967):332-7.
12. Hildebrand H, Brydolf M, Holmquist L, Krantz I, Kristiansson B. Incidence and prevalence of inflammatory bowel disease in children in south-western Sweden. *Acta Paediatr* 1994;**83**(6):640-5.
13. Ludvigsson JF, Busch K, Olen O, et al. Prevalence of pediatric inflammatory bowel disease in Sweden: a nationwide population-based register study. *BMC Gastroenterol* 2017;**17**(1):23.
14. Benchimol EI, Bernstein CN, Bitton A, et al. Trends in Epidemiology of Pediatric Inflammatory Bowel Disease in Canada: Distributed Network Analysis of Multiple Population-Based Provincial Health Administrative Databases. *Am J Gastroenterol* 2017;**112**(7):1120-1134.
15. Molodecky NA, Soon IS, Rabi DM, et al. Increasing incidence and prevalence of the inflammatory bowel diseases with time, based on systematic review. *Gastroenterology* 2012;**142**(1):46-54 e42; quiz e30.
16. Noone AM HN, Krapcho M, Miller D, Brest A, Yu M, Ruhl J, Tatalovich Z, Mariotto A, Lewis DR, Chen HS, Feuer EJ, Cronin KA SEER Cancer Statistics Review, 1975-2015, National Cancer Institute Bethesda, MD, https://seer.cancer.gov/csr/1975_2015/.**Accessed April 1, 2018**(Based on November 2017 SEER data submission, posted to the SEER web site, April 2018.).
17. Steliarova-Foucher E, Colombet M, Ries LAG, et al. International incidence of childhood cancer, 2001-10: a population-based registry study. *Lancet Oncol* 2017;**18**(6):719-731.
18. Levine A, Griffiths A, Markowitz J, et al. Pediatric Modification of the Montreal Classification for Inflammatory Bowel Disease: The Paris Classification. *Inflammatory Bowel Diseases* 2011;**17**(6):1314-1321.
19. Office for national Statistics, London, UK, https://www.ons.gov.uk/peoplepopulationandcommunity/birthsdeathsandmarriages/deaths/bulletins/suicidesintheunitedkingdom/2016registrations#suicides-in-the-uk-by-age. Accessed at April 1, 2018.
20. World Health Organization, Geneva, Switzerland, http://www.who.int/mental_health/media/swed.pdf. Accessed at April 1, 2018.
21. Olen O, Askling J, Sachs MC, et al. Childhood onset inflammatory bowel disease and risk of cancer: a Swedish nationwide cohort study 1964-2014. *BMJ* 2017;**358**:j3951.
22. Olen. Increased risk of cancer in children with inflammatory bowel disease. *BMJ* 2017;**358**:j4285.
23. Annese V, Daperno M, Rutter MD, et al. European evidence based consensus for endoscopy in inflammatory bowel disease. *J Crohns Colitis* 2013;**7**(12):982-1018.

24. Carrat F, Seksik P, Colombel JF, Peyrin-Biroulet L, Beaugerie L, Group CS. The effects of aminosalicylates or thiopurines on the risk of colorectal cancer in inflammatory bowel disease. *Aliment Pharmacol Ther* 2017;**45**(4):533-541.
25. Ekbom A, Helmick C, Zack M, Adami HO. Ulcerative colitis and colorectal cancer: A population-based study. *NEW ENGL J MED* 1990;**323**(18):1228-1233.
26. Jess T, Frisch M, Simonsen J. Trends in overall and cause-specific mortality among patients with inflammatory bowel disease from 1982 to 2010. *Clin Gastroenterol Hepatol* 2013;**11**(1):43-48.
27. Diak P, Siegel J, La Grenade L, Choi L, Lemery S, McMahon A. Tumor necrosis factor α blockers and malignancy in children: Forty-eight cases reported to the food and drug administration. *Arthritis Rheum* 2010;**62**(8):2517-2524.
28. Jamali M, Raca G, Rubin CM, Anastasi J. γδ Hepatosplenic T-cell lymphoma in a pediatric patient with Crohn's disease on combined immunosuppressive and immunomodulator therapy. *Pathol Case Rev* 2012;**17**(3):101-107.
29. Ochenrider MG, Patterson DJ, Aboulafia DM. Hepatosplenic T-Cell lymphoma in a young man with Crohn's disease: Case report and literature review. *Clin Lymphoma Myeloma* 2010;**10**(2):144-148.
30. Yabe M, Medeiros LJ, Daneshbod Y, et al. Hepatosplenic T-cell lymphoma arising in patients with immunodysregulatory disorders: a study of 7 patients who did not receive tumor necrosis factor-alpha inhibitor therapy and literature review. *Ann Diagn Pathol* 2017;**26**:16-22.
31. Kotlyar DS, Lewis JD, Beaugerie L, et al. Risk of lymphoma in patients with inflammatory bowel disease treated with azathioprine and 6-mercaptopurine: a meta-analysis. *Clin Gastroenterol Hepatol* 2015;**13**(5):847-58 e4; quiz e48-50.
32. Ariyaratnam J, Subramanian V. Association between thiopurine use and nonmelanoma skin cancers in patients with inflammatory bowel disease: a meta-analysis. *Am J Gastroenterol* 2014;**109**(2):163-9.

SUPPLEMENTARY DATA

Table S1. National pediatric and adult gastroenterologists, percentage of active response and number of cases per country.

		Pediatric representatives		Adult representatives		
	Country	**Response rate**	**Representative**	**Response rate**	**Representative**	**Reported cases**
1	Australia	UNK	P. Lewindon	UNK	NA	0
2	Austria	>90%	A.C. Hauer	>90%	C. Hoegenauer-Graz	0
3	Belgium	100%	G. Veereman	100%	S. Vermiere	0
4	Canada	UNK	A. Griffiths	UNK	NA	1
5	Croatia	100%	I. Hojsak	80%	B. Mijandrušić Sinčić	2
6	Czech Republic	100%	J. Bronsky	35%	M. Lukas	2
7	Denmark	100%	V. Wewer	UNK	E. Langholz	0
8	Finland	100%	K. Kolho	80%	T. Sipponen	4
9	France	53%	H. Garnier-Lengline	65%	H. Sokol	4
10	Germany	UNK	S. Koletzko	UNK	B. Brokemeyer	5
11	Greece	100%	E. Roma-Giannikou	95%	E. Tsianos	2
12	Hungary	100%	G. Veres	UNK	T. Molnar	1
13	Ireland	100%	S. Hussey	80%	G. Doherty	2
14	Israel	30-100%	R. Shaoul	20%	B. Bassat	2
15	Italy	UNK	P. Lionetti	92%	M. Lia Scribano	5
16	New Zealand	UNK	A. Day	UNK	NA	1
17	Norway	UNK	G. Perminov	UNK	M.L. Høivik	0
18	Poland	100%	M. Sladek	UNK	M. Klopocka	1
19	Portugal	100%	E. Trindade	UNK	F. Magro	0
20	Romania	100%	D. Serban	100%	M. Diculescu	1
21	Spain	UNK	F. Javier Martin Carpi	UNK	V. Garcia-Sanchez	1
22	Sweden	UNK	U. Fagerberg	UNK	S. Almer	6
23	Switzerland	100%	C. Braegger	UNK	G. Rogler	2
24	Netherlands	100%	L. de Ridder	70%	J. van de Woude	8
25	United Kingdom					10
	England (8)	UNK	C. Spray	UNK	A. Hart	
	Scotland (2)	100%	D. Wilson	55%	J. Satsangi	
	Wales (0)	UNK	I. Davies	UNK	NA	
	Total					**60**

UNK, unknown; NA, not applicable.

Table S2. Denominator data for PIBD patients < 26 years in European countries (denominator data)

Country	PAED coverage[‡]	PAED (0-19)[†]	ADULT (20-26)[†]	PAED covered[§]	ADULT covered[§]	Total covered[¶]	Hypothesized PIBD prevalence (0-26)[‡‡]	No. PIBD patients (0-26)	No. PIBD patients years studied DENOMINATOR DATA[§§]
Austria (AT)	100%	1,618,961	772,055	1,618,961	772,055	2,391,016	60/100.000	**1435**	**5,021**
Belgium (BE)	40%	2,431,351	959,118	972,540	959,118	1,931,658	60/100.000	**1159**	**4,056**
Croatia (HR)	100%	778,697	341,688	778,697	341,688	1,120,385	60/100.000	**672**	**2,353**
Czech Republic (CZ)	100%	2,012,817	780,667	2,012,817	780,667	2,793,484	60/100.000	**1676**	**5,866**
Denmark (DK)	100%	1,238,230	541,501	1,238,230	541,501	1,779,731	60/100.000	**1068**	**3,737**
Finland (FI)	100%	1,131,328	466,649	1,131,328	466,649	1,597,977	60/100.000	**959**	**3,356**
France (FR)	100%	15,626,143	5,333,802	15,626,143	5,333,802	20,959,945	60/100.000	**12576**	**44,016**
Greece (EL)	100%	1,985,281	776,569	1,985,281	776,569	2,761,850	60/100.000	**1657**	**5,800**
Ireland (IE)	100%	1,255,079	392,755	1,255,079	392,755	1,647,834	60/100.000	**989**	**3,460**
Italy (IT)	18%	10,489,520	4,223,265	1,888,114	4,223,265	6,111,379	60/100.000	**3667**	**12,834**
Poland (PL)	100%	7,233,823	3,221,678	7,233,823	3,221,678	10,455,501	60/100.000	**6273**	**21,957**
Portugal (PT)	100%	1,890,717	759,111	1,890,717	759,111	2,649,828	60/100.000	**1590**	**5,565**
Romania (RO)	100%	3,918,293	1,477,708	3,918,293	1,477,708	5,396,001	60/100.000	**3238**	**11,332**
Sweden (SE)	100%	2,183,338	893,132	2,183,338	893,132	3,076,470	60/100.000	**1846**	**6,461**
Switzerland (CH)	79%	1,599,086	697,507	1,263,278	697,507	1,960,785	60/100.000	**1176**	**4,118**
Netherlands (NL)	100%	3,611,877	1,493,725	3,611,877	1,493,725	5,105,602	60/100.000	**3063**	**10,722**
UK - England	100%	12,464,034	4,976,080	12,464,034	4,976,080	17,440,114	60/100.000	**10464**	**36,624**
UK - Scotland	100%	1,088,924	500,524	1,088,924	500,524	1,589,448	60/100.000	**954**	**3,338**
UK - Wales	100%	664,388	292,688	664,388	292,688	957,076	60/100.000	**574**	**2,010**
Summary	-	**73,221,887**	**28,900,222**	**62,825,862**	**28,900,222**	**91,726,084**	-	**55,036**	**192,625**

[‡]Coverage was based on data obtained by surveys that collected region coverage per country. We assumed full coverage for adults (20-26 years). [†]Numbers were retrieved from 2016 Eurostat census data. [§]Covered populations between 0-19 and 20-26 years were calculated by using the coverage per country and population numbers retrieved from 2016 Eurostat census data. [¶]Corresponding populations covered under the age of 26. [‡‡]An IBD prevalence of 60 per 10.000 was assumed (see methods section). [§§]Based on the percentage of population covered, European census data, IBD prevalence under the age of 26 years and the study duration. Abbreviations: PIBD = pediatric-onset IBD, PAED = pediatric, No = number of, UK = United Kingdom.

Table S3. Patient characteristics of PIBD patients who developed hematopoietic cancer, CRC, or CCA.

		Hematopoietic	CRC	CCA
Total patients, N (%)		21 (48.8)	12 (27.9)	3 (7.0)
Gender				
	Male, N (%)	10 (47.6)	7 (58.3)	3 (100)
	Female, N (%)	11 (52.4)	5 (41.7)	NA
IBD diagnosis				
	CD, N (%)	15 (71.4)	4 (33.3)	NA
	UC, N (%)	5 (23.8)	6 (50.0)	3 (100)
	IBD-U, N (%)	1 (4.8)	2 (16.7)	NA
Age at IBD diagnosis				
	Mean (SD)	13.1 (3.8)	12.2 (3.9)	11.3 (5.7)
	Median (IQR)	14.1 (11.0-15.6)	12.2 (10.1-14.9)	12.9 (5.0-16.0)
IBD disease duration to cancer, y				
	Mean (SD)	4.7 (3.6)	8.2 (4.1)	10.4 (4.1)
	Median (IQR)	3.7 (2.0-7.8)	9.3 (4.3-11.8)	11.3 (6.0-14.0)
Age at cancer, y				
	Mean (SD)	17.6 (3.5)	20.5 (4.0)	21.7 (2.5)
	Median (IQR)	17.0 (14.9-20.5)	20.7 (16.5-24.4)	22.0 (19.0-24.0)
Comorbidities				
	PSC, N (%)	2 (9.5)	2 (16.7)	3 (100)
	Perianal disease, N (%)	6 (30.0)	2 (18.2)*	0 (0)*
Mortality, N (%)		3 (14.3)	3 (25.0)	3 (100)

*One missing value; CRC, colorectal carcinoma; CCA, cholangiocarcinoma; NA, not applicable; CD, Crohn's disease; UC, Ulcerative colitis; IBD-U, IBD unclassified; IQR, interquartile range; SD, standard deviation; N, number; y, year.

Table S4. Patient characteristics of PSC patients with concomintant IBD who developed malignancy or a fatal outcome.

Outcome	Sex	IBD type	Paris	Age at IBD diagnosis, y	Age at PSC diagnosis, y	Age at cancer, y	Type of cancer	Age at death, y	Cause of death	Treatment
Fatal cancer										
	M	UC	UNK	13	14	14	Biliary cancer	6 months after cancer diagnosis	Cancer	14 months steroids
	M	UC	UNK	16	17	22	Biliary cancer	2 years after cancer diagnosis	Cancer	No medication
	M	UC	E4	5	8	19	Biliary cancer	same year as cancer diagnosis	Cancer	No medication
	F	UC	UNK	14	23	24	CRC	same year as cancer diagnosis	Cancer	thiopurines, duration unknown
	M	UC	UNK	12	13	18	HSTCL	8 months after cancer diagnosis	Cancer	5 years steroids 6 years thiopurines
Non-fatal cancer										
	M	IBDU	UNK	19	17	24	CRC	NR	NR	6.5 years tacrolimus
	F	UC	E4	7	15	16	MDS with secondary AML	NR	NR	0,5 years steroids 4.5 years thiopurines
Non-malignant deaths										
	M	UC	UNK	4	14	NR	NR	25	Suicide	steroids, duration unknown 5 years tacrolimus
	F	UC	UNK	UNK	UNK	16	Liver failure	NR	NR	5 months steroids 3 months thiopurines

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9

DISEASE PROGRESSION IN PEDIATRIC- AND ADULT-ONSET SCLEROSING CHOLANGITIS: RESULTS FROM TWO INDEPENDENT DUTCH REGISTRIES

Chapter 9

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ABSTRACT

Background: Sclerosing cholangitis (SC) is a severe liver disease leading to destruction of bile ducts. It is believed to run a milder course in children than in adults. To test this assumption, we evaluated time-to-complication curves in two independent pediatric-onset cohorts from the same geographical area.

Methods: Short-term disease outcomes were evaluated with an online clinical registry that was filled with data on children with SC diagnosed between 2000 and 2017 and who were followed bi-annually thereafter. Long-term disease outcomes were evaluated in a pediatric-onset subcohort derived from a previously published population-based study from the Netherlands. Time-to-complication in the first cohort was defined as the time from diagnosis until portal hypertension, biliary obstructions and infections, development of malignancy, or liver transplantation, whichever came first. In the second cohort time-to-complication was defined as the time until liver transplantation or PSC-related death.

Results: Median age at diagnosis in the first cohort (n=86) was 12.3 years. In the first 5 years post-diagnosis 23% of patients developed complications. The patients in the population-based study (n=683) were stratified into those diagnosed before the age of 18 years ("pediatric-onset" subcohort, n=43) and those diagnosed after the age of 18 years ("adult-onset" subcohort, n=640). Median age at diagnosis was 14.6 and 40.2 years, respectively. Median time-to-complication in the pediatric-onset and adult-onset subcohorts were not statistically different.

Conclusions: Pediatric and adult-onset SC run a similar long-term disease course. Pediatricians who treat children with SC should monitor them closely to recognize early complications and control long-term sequelae.

DISEASE PROGRESSION IN PEDIATRIC- AND ADULT-ONSET SCLEROSING CHOLANGITIS: RESULTS FROM TWO INDEPENDENT DUTCH REGISTRIES

INTRODUCTION

Sclerosing cholangitis (SC) is a rare cholestatic disease characterized by fibrosis of the intra- and/or extrahepatic bile ducts and is strongly associated with inflammatory bowel disease (IBD). The disease presents in most patients between the age of 25 and 40 years[1, 2], though it is recognized as an important cause of chronic liver disease in children. Patients with SC carry an ongoing and disproportionate high clinical need because of the association with poor clinical outcomes including end-stage biliary cirrhosis and hepatopancreatobiliary and colorectal malignancies.[3, 4] In the early stages bile duct disease may be easily overlooked, as symptoms are initially nonspecific and intestinal disease is frequently more prominent in patients with concomitant IBD.[5]

It is suggested by several authors that pediatric-onset SC runs a milder course and has a more favourable outcome compared to adult-onset SC.[6, 7] The time horizon in these papers could have been restricted by transfer of patients to adult-oriented care. To test the assumption of a relatively benign disease course in pediatric-onset SC, we evaluated time-to-complication curves in two independent Dutch cohorts. The first cohort contained data of children with SC who were followed bi-annually until transfer to adult-oriented care, the second cohort consisted of adults with pediatric-onset SC derived from a previously published population-based study from the Netherlands.

METHODS

Objectives.

Our objectives were to (1) describe short-term outcomes, for which we created an online registry filled with closely followed data on pediatric-onset SC patients in the Netherlands during the first five years after diagnosis, and (2) compare long-term disease outcomes between pediatric- and adulthood-onset SC patients, for which we used data from a second, independent, previously published population-based study from the Netherlands.[2]

Short-term disease outcomes (cohort 1).

Setting

We used longitudinal data on pediatric-onset SC patients who were diagnosed between 2000 and 2017 at five tertiary hospitals in the Netherlands, including one referral pediatric liver transplant center. The data were derived from local clinical databases and International Classification of Diseases code searches.

We assessed clinical, biochemical, radiological and histological parameters at

SC diagnosis, and followed the patients at least bi-annually until transfer to adult-oriented care. We created an online clinical registry using Castor Electronic Data Capture (Amsterdam, The Netherlands[8]), which was filled with retrospective data for this specific research project. After this project, the registry will be maintained and regularly updated to serve as a prospective registry.

The ethical committee of the Erasmus MC in Rotterdam reviewed the study protocol and waived the need for informed consent due to the anonymous and non-interventional fashion of the study (MEC-2016-736). Secondary approval was obtained from all other participating centers.

Participants

Pediatric-onset SC is classified as primary SC (PSC) or autoimmune SC (ASC). The latter is also known as PSC-autoimmune hepatitis (AIH) overlap syndrome, due to concurrence of increased levels of transaminases, hypergammaglobulinaemia and autoantibodies. We included patients diagnosed with SC before the age of 18 years. Patients with cholangiopathies secondary to surgical complications or other liver diseases were excluded. We reviewed immunological, radiological and/or histological features to determine if patients were appropriately assigned a diagnosis of PSC, ASC or AIH (Figure S1). The diagnosis of SC was based on a cholestatic biochemical profile (raised conjugated bilirubin levels and/or elevation of alkaline phosphatase (ALP) and gamma glutamyltransferase (GGT)), in combination with bile duct irregularities on endoscopic retrograde cholangiography (ERC) or magnetic resonance cholangiography (MRC), and/or ductular reactions on liver histology. SC patients with at least one of the appropriate auto-antibodies in their serum (anti-nuclear antibodies (ANA); anti-smooth muscle antibodies (anti-SMA); anti-liver kidney microsome type 1 (anti-LKM-1); or antibody to liver cytosol (anti-LC-1)) were classified as ASC. SC patients with negative auto-antibodies were classified as PSC.[9, 10] Patients were excluded from further analysis when they had isolated AIH,[11, 12] or when both cholangiography (ERC or MRC) and liver biopsy were missing in the diagnostic work-up (Figure S1).

Variables

Baseline data included patient demographics, associated immune disorders and presence and type of inflammatory bowel disease (IBD), signs, symptoms and age at SC diagnosis; and detailed information on diagnostic work up. We used age- and sex-adjusted cut points for elevated ALP. Cut-offs were 424 U/L for boys and girls younger than 13 years, 454 U/L for boys 13 to 17 years, and 254 U/L for girls between 13 and 17 years.[13] Hepatomegaly was defined as a liver length measured in the midclavicular line exceeding the upper limit of normal for height and age.[14] Splenomegaly was defined as a splenic length measured in coronal section (passing through the splenic hilum) exceeding the upper limit of normal for age and gender.[14]

Follow-up data included the clinical endpoints (1) portal hypertensive complications, (2) biliary complications, (3) hepatobiliary malignancy, (4) liver transplantation, or (5) death from liver disease. Portal hypertensive complications included thrombocytopenia ($<150 \times 10^9$/l), bleeding oesophageal varices and the need for placing a transjugular intrahepatic portosystemic shunt (TIPS). Biliary complications included cholangitis or bile duct obstruction, whether or not requiring endoscopic intervention. The composite outcome short-term disease progression was defined as occurrence of at least one of five clinical endpoints within the first five years following diagnosis of SC.

Predictors of short-term disease progression

To construct a prognostic model for short-term disease progression we carried out a backward stepwise elimination. Candidate predictors with $p<0.10$ in bivariate analysis were selected for use in the multivariate analysis. This level was chosen because of the limited number of patients in the analysis. For patients with an incomplete follow-up (i.e. less than five years postdiagnosis) we calculated the Follow-Up Index (FUI), which is defined as the ratio between the actual observed follow-up period and the minimally preferred follow-up period.[15] The FUI ranges from near 1.0 (almost 5 years follow-up) to near 0 (diagnosed just before the study closing date). The FUI is a simple measure to critically appraise the credibility of the prognostic model. Patients with isolated AIH were excluded from the survival and logistic regression analysis.

Long-term disease outcomes (cohort 2).

To obtain insight in long-term disease course of pediatric-onset SC patients after transition to adult care, we used a large comprehensive SC cohort that has been described before, and which came from the same geographical area of the Netherlands as cohort 1.[2] The difference between cohort 1 and cohort 2 is the moment of data collection: data from cohort 1 was collected at pediatric age, whereas cohort 2 was collected at adult age. Between January 2008 and December 2011, the researchers in this study identified all adult PSC patients in 44 hospitals in the Netherlands, that were diagnosed from 2000 onward. Data collection included patient demographics, disease characteristics at diagnosis and follow-up of clinical endpoints, as liver transplantation and SC-related death.

For our study, we performed an additional analyses of this cohort and stratified for age at diagnosis before 18 years ("pediatric-onset" cohort, n=43) or after 18 years ("adult-onset" cohort, n=640). We compared frequencies of liver transplantations, median time-to-complication, and SC-related death. Detailed information on the study design, participants, data collection and variables can be found in the original publication.[2]

Statistics.

Data analyses were performed using IBM SPSS version 23 Baseline demographic and disease characteristics were evaluated for both SC registries using descriptive statistics. We summarized continuous variables as medians and interquartile ranges (IQR: 25th percentile, 75th percentile). For discrete variables, we calculated the 95% confidence interval in OpenEpi, Version 3, using the Wilson method for calculating confidence intervals for proportions. Differences in groups were compared by the Mann Whitney U test for continuous variables; for categorical outcomes the Chi square test and Fisher's exact test were used. P values <0.05 were considered statistically significant.

In cohort 1, we estimated the cumulative incidence of any of the above-mentioned clinical endpoints, by performing Kaplan-Meier survival analysis. Time was defined as the moment of SC diagnosis until appearance of the first sign of early hepatic deterioration or until five years after SC diagnosis. Patients who did not have a complete follow-up of 5 years and did not develop signs of disease progression were censored. In cohort 2, a Kaplan-Meier survival analysis with Log rank test was performed to compare long-term disease outcomes between pediatric-onset and adult-onset SC patients. Event was defined as liver transplantation or death from liver disease. Time was defined as the moment of SC diagnosis until appearance of the event. Patients who were lost to follow-up without experiencing an event were censored.

Table 1. Patient characteristics, clinical presentation at time of diagnosis and association with IBD.

	PSC (n = 32)	ASC (n = 54)
Median age at diagnosis in years (IQR)	13.3 (10.2-15.2)	11.4 (8.2-14.3)
Male gender	75% (58-87)	61% (48-73)
Liver-related symptoms at diagnosis		
Jaundice	16% (7-32)	13% (7-24)
Hepatomegaly	6% (2-21)	11% (5-22)
Splenomegaly	3% (1-16)	7% (3-18)
Ascites	0% (0-11)	0% (0-7)
Fatigue	34% (21-52)	50% (37-63)
Pruritus	22% (11-39)	17% (21-45)
Coagulopathy	0% (0-11)	4% (1-13)
Liver disease in first degree relatives	3% (1-16)	4% (1-13)
Associated autoimmune disease	3% (1-16)	11% (5-22)
Association with IBD	84% (68-93)	76% (63-85)
Median age at IBD diagnosis in years (IQR)	12.2 (9.2-15.2)	11.5 (8.6-14.4)
Type of IBD, % (n)		
CD	11% (4-28)	22% (12-37)
IBD-U	4% (1-18)	5% (1-16)
UC	85% (68-94)	73% (58-84)
Pancolitis	70% (49-84)	83% (66-93)
Timing of diagnosis of liver disease, % (n)		
Simultaneous with diagnosis of IBD	41% (25-59)	73% (58-84)
Before diagnosis of IBD	11% (4-28)	15% (7-28)
During follow-up of IBD	48% (31-66)	12% (5-26)

Values are percentages (95% confidence interval) unless otherwise stated. Abbreviations: PSC, primary sclerosing cholangitis; ASC, autoimmune sclerosing cholangitis; CD, Crohn's disease; IBD, inflammatory bowel disease; IBD-U, IBD unclassified; IQR, interquartile range; PSC, primary sclerosing cholangitis; UC, Ulcerative colitis.

Table 2. List of clinical endpoints in patients with pediatric-onset SC in the first five years postdiagnosis.

Complications	PSC (n = 32)	ASC (n = 54)
Median follow-up time in years (range)	4.7 (0.2-12.3)	5.5 (0.7-14.5)
Portal hypertensive complications	16% (6.9-31.8)	17% (9.0-28.7)
Thrombocytopenia	9% (3-24)	11% (5-22)
Bleeding oesophageal varices	6% (2-20)	6% (2-15)
Need for TIPS placement	3% (1-16)	4% (1-12)
Biliary complications	16% (6.9-31.8)	17% (9.0-28.7)
Episodes of cholangitis	9% (3-24)	13% (6-24)
Need for ERC	6% (2-20)	11% (5-22)
Liver transplantation	3% (0.6-15.7)	4% (1.0-12.5)
Hepatobiliary malignancy	0% (0-11)	0% (0-7)
Death from liver disease	3% (0.6-15.7)	2% (0.3-9.8)

Values are percentages (95% confidence interval) unless otherwise stated. Abbreviations: ASC, autoimmune sclerosing cholangitis; AIH, autoimmune hepatitis; IQR, interquartile range; ERC, endoscopic retrograde cholangiography; PSC, primary sclerosing cholangitis; TIPS, transjugular intrahepatic portosystemic shunt.

RESULTS

Short-term disease outcomes in pediatric-onset SC.

Patient demographics and characteristics

We identified 160 patients who were diagnosed between 2000 and 2017. A total of 17

children were excluded because of an incomplete diagnostic work-up, and 57 for isolated AIH. Thirty-two patients were classified as PSC and 54 as ASC (Table 1). Patients were diagnosed at a median age of 12.4 years (IQR 9.1-14.8) and the gender distribution was predominantly male (PSC 75% and ASC 61%). Comorbidities in patients with ASC included insulin-dependent diabetes mellitus (n=2), celiac disease (n=1), autoimmune hemolytic anemia (n=1), rheumatoid arthritis (n=1) and idiopathic thrombocytopenic purpura (n=1). Only one PSC patient had an associated autoimmune disease (celiac disease). Both PSC and ASC were strongly associated with IBD (respectively in 84% and 76% of cases). UC was the predominant type of IBD in both PSC and ASC with a high proportion of pancolitis (PSC, 70%; ASC, 83%).

Characterization of SC at diagnosis

Among the 54 patients with ASC, 25 were positive for ANA alone, 9 for both SMA and ANA, 19 for SMA alone and 1 for LKM1 alone. Diagnostic liver ultrasonography was performed in 65 of the 86 patients (76%). Splenomegaly was present at ultrasonography in 19 patients (26%) and hepatomegaly in 31 patients (43%). MRC or ERC was performed in 73 patients (85%) and liver biopsy in 75 patients (87%). All PSC patients received ursodeoxycholic acid (UDCA); 94% of ASC patients received UDCA. Steroid tapering dose was given to 59% of PSC patients (n=19) and 85% of ASC patients (n=46), in the majority of cases (80%) as induction therapy for concomitant IBD. Thiopurines were used by 59% of PSC patients (n=19), of which 89% had concomitant IBD. 76% of the ASC patients (n=41) used thiopurines.

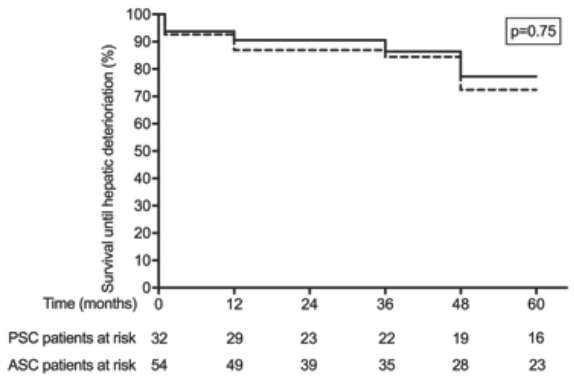

Figure 1. Short-term disease progression in children with SC. Kaplan-Meier plot demonstrating the percentage of patients with survival until disease progression after SC diagnosis. Adolescents with PSC (solid line) are compared with adolescents with ASC (dotted line). Event is defined as development of portal hypertensive complications, biliary complications, hepatobiliary malignancy, liver transplantation, or death from liver disease. Time is defined as the moment of SC diagnosis until the first complication, or five years postdiagnosis. Patients with no complete 5-year follow-up and who did not develop signs of disease progression were censored. The numbers on the lowest line indicate the number of patients being represented at that point in time.

Short-term disease progression

Individual patients were followed for a median of 5.1 years (IQR 2.6-7.8) after SC diagnosis (Table 2). Figure 1 shows that the interval to hepatic deterioration was similar for patients with PSC and ASC (p=0.752, log-rank test, Figure 1). Portal hypertensive complications developed in 16% of PSC patients (n=5) and 17% of ASC patients (n=9). Biliary complications developed in 16% of PSC patients (n=5) and 17% of ASC patients (n=9). Four ASC patients (7%) developed both portal hypertensive as well as biliary complications. Liver transplantation was performed in three patients after a mean disease duration of 9.3 years (range 5.9-9.8). Two patients died; one patient with ASC and end-stage liver disease from massive upper gastrointestinal hemorrhage and one patient with PSC died in a traffic accident. No patients developed cancer within the follow-up period.

Predictors of short-term disease progression

Twenty patients (23%) developed disease progression in the first 5 years after diagnosis. Fifty-eight of 86 patients (67%) had a complete 5-year follow-up. The remaining 28 patients with an incomplete follow-up had a mean (SD) FUI of 0.41 (0.21). We measured candidate factors to construct a prognostic model for short-term disease progression. Table 3 shows the results of the bivariate logistic regression analysis and the multivariate model, selected with the maximum likelihood approach. Treatment with thiopurines, steroids or UDCA did not influence disease course. Elevated ALP, fibrosis in liver biopsy, hepatomegaly and splenomegaly on ultrasonography had a P-value <0.10 and were selected for use in the multivariate analysis. In the multivariate model elevated ALP (odds ratio [OR] 5, 95%CI 1-21) and hepatomegaly on ultrasonography (OR 9, 95%CI 2-47) remained significant predictors of short-term disease progression. The logistic regression coefficients (β) in

Table 3. Predictors of short-term disease progression in the first five years after diagnosing SC.

Prognostic markers at diagnosis of liver disease	Bivariate			Multivariate		
	β	P	Odds Ratio	β	P	Odds Ratio (95%CI)
ALP elevation (age and sex adjusted)*	1.0	0.057	2.8	1.6	0.034	4.8 (1.1 – 20.6)
Fibrosis in liver biopsy*	1.0	0.037	2.8			
Hepatomegaly at ultrasonography*	1.9	0.019	6.9	2.1	0.014	8.5 (1.5 – 46.5)
Splenomegaly at ultrasonography*	1.2	0.067	3.2			
Constant				-3.4	0.000	0.033

* Binary variables are coded 0 for no or 1 for yes.
Cox and Snell R = 0.203, Nagelkerke R (Max rescaled R) = 0.305.
Risk for early hepatic deterioration can be calculated from the following standard formula:
Risk score = -3.4 + 1.6 (ALP elevation) + 2.1 (Hepatomegaly at ultrasonography)
Predicted risk = $1/(1 + e^{-\text{risk score}})$

Abbreviations: ALP, alkaline phosphatase.

Table 4. Comparison of long-term outcomes in pediatric-onset and adult-onset PSC.

	Pediatric cohort (N = 43)	Adult cohort (N = 640)	P value (Pediatric vs Adult)
Median age at diagnosis, yr (IQR)	16.0 (14-18)	40.0 (30-50)	-
Male, n (%)	67% (52-80)	65% (61-68)	NS
AIH overlap, n (%)	9% (4-22)	3% (2-5)	NS
Small duct SC, n (%)	9% (4-22)	9% (7-11)	NS
Association with IBD, n (%)	79% (62-87)	69% (64-72)	NS
Mortality, n (%)	9% (4-22)	17% (14-20)	NS
Median age at SC Related death, yr (IQR)	31.0 (1.0)	53.0 (20.25)	**0.03**
PSC-related death, n (%)	5% (1-15)	9% (7-12)	NS
CCA	1	26	NS
CRC	1	6	NS
Liver failure	0	16	NS
LTx related complications	0	9	NS
Gallbladder carcinoma	0	1	NS
Median transplant free survival, yr	21	23	NS
Liver transplantation, n (%)	23% (13-38)	17% (14-20)	NS
Median disease duration until LTx, months (IQR)	112.5 (155.25)	90.5 (107.75)	NS
Median age at LTx, yr (IQR)	22.5 (15-30)	48 (40-56)	**<0.001**

Data on a total of 697 SC patients is currently included in the Boonstra cohort. Fourteen patients were excluded from the analyses because the date of diagnosis was missing. Mann-Whitney U test (continuous variables) or Fisher's exact test (categorical variables) was used to test differences between 2 groups. Log rank test was used to compare transplant free survival. Abbreviations: SC, sclerosing cholangitis; AIH, autoimmune hepatitis; IBD, inflammatory bowel disease; CCA, cholangiocarcinoma; CRC, colorectal carcinoma; LTx, liver transplantation; yr, year; n, number of patients; NS, not significant; IQR, interquartile range.

the multivariate model allowed to construct a forecast for short-term disease progression in children at diagnosis of SC. The equation beneath table 3 indicates the mutually adjusted relative contribution of the factors to the risk score. As an example of the use of this equation, consider a teenager with the SC phenotype, with elevated ALP and hepatomegaly. The total risk score of this patient amounts to 0.3, which corresponds to a probability of short-term disease progression of 57%, compared to a pre-test probability of 23%.

Long-term disease outcomes in pediatric-onset versus adult-onset SC.

The large SC cohort by Boonstra *et al.* included a total of 697 SC patients. Fourteen patients were excluded from the analyses because the date of diagnosis was missing (Table 4).

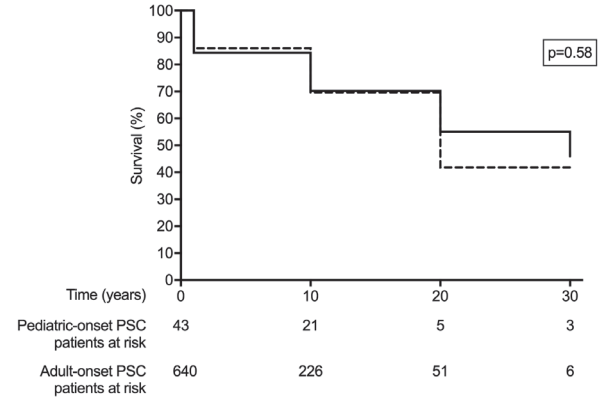

Figure 2. Time-to-transplantation or SC-related death. Kaplan-Meier plot demonstrating the percentage of patients with time-to-liver transplantation or SC-related death. Patients with adult-onset SC (solid line) are compared with patients with pediatric-onset SC (dotted line). Event is defined as liver transplantation or death from liver disease. Time is defined as the moment of SC diagnosis until appearance of the event. Patients who were lost to follow-up without experiencing an event were censored. The numbers on the lowest line indicate the number of patients being represented at that point in time.

Mean age of liver disease diagnosis was 14.6 years in the pediatric-onset cohort (n=43) and 40.2 years in the adult-onset cohort (n=640). There were no baseline differences in sex, presence of AIH overlap, small duct disease, or concomitant diagnosis with IBD between the pediatric-onset and adult-onset SC patients. Median follow up was 10 years (IQR 7-17) for the pediatric-onset cohort and 8 years (IQR 4-13.8) for the adult-onset cohort. During follow-up two pediatric-onset SC patients (5%) and 59 adult-onset SC patients (9%) died. Median transplant-free survival was 21 and 23 years in pediatric-onset and adult-onset SC patients, respectively. Frequencies of liver transplantations (23% vs 17%) and mean disease duration until liver transplantation (11 years vs 9.3 years) were not statistically different between the pediatric-onset and adult-onset cohort, respectively. Figure 2 shows that there is no difference in time-to-liver transplantation or SC-related death between both sub cohorts (p=0.58, Log-rank test).

DISCUSSION

Key findings.

We described clinical outcomes in two independent Dutch pediatric-onset SC registries. In cohort 1, which was ideal for studying short-term disease progression, twenty-three percent of patients developed portal hypertension, biliary complications, or progressed

to liver transplantation within 5 years after SC diagnosis. In cohort 2, which allowed us to study long-term disease outcomes beyond the age of transfer to adult-oriented care, we observed that pediatric and adult-onset SC run a similar disease course regarding time-to-transplantation and SC related death. Our findings contradict the current view that pediatric-onset SC runs a relatively benign disease course as compared to adult-onset SC.

Comparison with other studies.

Disease progression

We identified ten observational studies from MEDLINE and EMBASE that described pediatric cohorts with SC.[6, 7, 16-22] A recently published multicenter, international cohort study of children with SC (n=781) reported portal hypertensive and biliary complications in 30% of cases in the first five years postdiagnosis, and 12% of patients required a liver transplantation within 5 years after SC diagnosis.[6] Similarly, 11% of children included in a single center cohort of pediatric PSC patients from the United States (n=120) was transplanted in the first five years postdiagnosis.[7] Our data from cohort 1 demonstrates that 23% of patients develop short-term disease progression within 5 years after SC diagnosis. This is possibly an underestimation as indicated by the low FUI. Taken together, our data and earlier published information show that a quarter to a third of pediatric-onset SC patients have a progressively worsening liver condition before transfer to adult-oriented care. In contrast, a recently published Italian two-center study describing 45 pediatric patients with SC suggested that pediatric SC is a milder phenotype than adult-onset SC.[20] At the same time the authors explain that part of the patients in their cohort were diagnosed at a very early stage of SC, i.e. when they were still symptom-free and only had biochemical abnormalities. This illustrates the importance of multicenter prospective studies for defining SC phenotype and exploring pathogenic mechanisms.

Prognostic biomarkers

Low platelet count, prolonged pro-thrombin time and higher values of bilirubin and GGT have previously been identified as markers for progressive liver disease in children with SC.[6, 16, 17] In our multivariate model that was based on cohort 1 these laboratory markers had no prognostic value. Instead we identified hepatomegaly on ultrasonography and elevated ALP at diagnosis as significant and independent predictors of short-term disease progression. Elevated ALP has not been identified as a prognostic marker in previous pediatric research, but is consistently associated with poor prognosis in adult SC literature.[23-27] Some may argue that GGT is a more accurate diagnostic marker of SC in children than ALP, as ALP is also dependent on bone growth.[16, 17] However, in our patient cohort GGT was already elevated in 94% cases at diagnosis, which explains its poor specificity to use it as an indicator for future disease progression.

Phenotype of pediatric- versus adult-onset SC

Some groups have suggested that pediatric-onset SC has a milder phenotype and therefore a more favourable outcome compared to adult-onset SC.[6, 7] The international collaboration group that recently published the results of a large cohort of children with SC (n=781) reported a transplantation-free survival of 88% and 70% at 5 and 10 years respectively.[6] In a large international cohort of adult patients with SC (n=7121) transplantation-free survival was 80%, 63% and 48% after 5, 10 and 15 years postdiagnosis.[28] Variation in geographical backgrounds of these two large cohorts may have hampered a reliable comparison of transplantation-free survival. As far as we know this is the first time that long-term outcomes of pediatric-onset and adult-onset SC patients coming from the same geographical area were compared.[2] The time-to-complication analysis showed that there was no difference between pediatric- and adult-onset SC. We therefore argue that pediatric-onset SC follows the same disease course as adult-onset SC.

Implications for pediatric practice.

Despite the lack of current therapies that cure or halt disease progression, targeting and selecting children with a likelihood of short-term disease progression has several benefits, including appropriate counselling of the patient and family, close monitoring for potential severe complications (including hepatobiliary and colorectal malignancies) and timely referral to a liver transplantation center. The prognostic biomarkers described in cohort 1 (hepatomegaly on ultrasonography and elevated ALP) may allow physicians involved in the care of children to more accurately predict disease progression in the early stages of SC. The robustness of these biomarkers needs to be evaluated in a validation cohort of patients with pediatric-onset SC.

Our findings provide an evidence base that the time-to-complication in pediatric-onset SC is not different from the adult-onset type. We call for a more rigorous follow-up of children with SC, including monitoring for symptoms from dominant strictures such as cholangitis, jaundice, pruritus, right upper quadrant pain or worsening cholestatic biochemical profile. Additionally, in those without concurrent IBD annual fecal calprotectin screening is warranted. A detailed handover letter including a clear timeline of diagnostic and therapeutic procedures should be written by the pediatric team prior to the transfer to adult-oriented care.

Strengths and limitations.

The diagnostic criteria for pediatric-onset SC are not univocal. There is a need to develop an evidence-based guideline that brings more uniformity in the diagnostic criteria for

SC in children. One of the strengths of this study was the confirmation of the diagnosis of PSC or ASC with a detailed record review and diagnostic decision matrix with strict definitions for PSC and ASC (Figure S1), instead of merely relying on the administration of the treating doctor or on coding data. This strategy reduced the risk of misclassification and misdiagnosis, and consequently type I errors. A limitation was that not all patients in cohort 1 had a complete 5-year follow-up. Although our study provides important insight regarding disease progression in pediatric-onset SC, potential drawbacks of this study relate to the retrospective nature of the study and the limited sample size.

CONCLUSION

In conclusion, our data provide new insights into the course of disease in pediatric-onset SC. The results strongly suggest that pediatric- and adult-onset SC run a similar short- and long-term disease course. Both pediatricians as well as adult-oriented specialists who treat patients with pediatric-onset SC should monitor them closely to recognize early complications and control long-term sequelae. Finally, our results emphasize the importance of follow-up of young patients with rare diseases, whose disease course is blurred by transition to adult-oriented care.

REFERENCES

1. Molodecky NA, Kareemi H, Parab R, et al. Incidence of Primary Sclerosing Cholangitis: a Systematic Review and Meta-Analysis. Hepatology 2011;53:1590-1599.
2. Boonstra K, Weersma RK, van Erpecum KJ, et al. Population-based epidemiology, malignancy risk, and outcome of primary sclerosing cholangitis. Hepatology 2013;58:2045-55.
3. Deneau M, Jensen MK, Holmen J, et al. Primary sclerosing cholangitis, autoimmune hepatitis, and overlap in utah children: Epidemiology and natural history. Hepatology 2013;58:1392-1400.
4. Schrumpf E, Boberg KM. Hepatic and extrahepatic malignancies and primary sclerosing cholangitis. Gut 2003;52:165.
5. Karlsen TH, Folseraas T, Thorburn D, et al. Primary sclerosing cholangitis - a comprehensive review. J Hepatol 2017;67:1298-1323.
6. Deneau MR, El-Matary W, Valentino PL, et al. The natural history of primary sclerosing cholangitis in 781 children: A multicenter, international collaboration. Hepatology 2017;66:518-527.
7. Valentino PL, Wiggins S, Harney S, et al. The Natural History of Primary Sclerosing Cholangitis in Children: A Large Single-Center Longitudinal Cohort Study. J Pediatr Gastroenterol Nutr 2016;63:603-609.
8. Castor Electronic Data Capture CB, Amsterdam, The Netherlands, 2018.
9. Liberal R, Vergani D, Mieli-Vergani G. Pediatric Autoimmune Liver Disease. Dig Dis 2015;33 Suppl 2:36-46.
10. Lindor KD, Kowdley KV, Harrison ME, et al. ACG Clinical Guideline: Primary Sclerosing Cholangitis. Am J Gastroenterol 2015;110:646-59; quiz 660.
11. Mileti E, Rosenthal P, Peters MG. Validation and modification of simplified diagnostic criteria for autoimmune hepatitis in children. Clin Gastroenterol Hepatol 2012;10:417-21 e1-2.
12. Manns MP, Czaja AJ, Gorham JD, et al. Diagnosis and management of autoimmune hepatitis. Hepatology 2010;51:2193-213.
13. Derksen-Lubsen G MHA, Oudesluys-Murphy H M, Sprij A. Compendium kindergeneeskunde. Diagnostiek en behandeling, 5th edn. Stafleu van Loghum, 2018.
14. Beek E. avR. Diagnostic Pediatric Ultrasound. ISBN 9783131697318 August 2015.
15. von Allmen RS, Weiss S, Tevaearai HT, et al. Completeness of Follow-Up Determines Validity of Study Findings: Results of a Prospective Repeated Measures Cohort Study. PLoS One 2015;10:e0140817.
16. Feldstein AE, Perrault J, El-Youssif M, et al. Primary sclerosing cholangitis in children: a long-term follow-up study. Hepatology 2003;38:210-7.
17. Wilschanski M, Chait P, Wade JA, et al. Primary sclerosing cholangitis in 32 children: Clinical, laboratory, and radiographic features, with survival analysis. Hepatology 1995;22:1415-1422.
18. Miloh T, Arnon R, Shneider B, et al. A retrospective single-center review of primary sclerosing cholangitis in children. Clin Gastroenterol Hepatol 2009;7:239-45.
19. Rojas CP, Bodicharla R, Campuzano-Zuluaga G, et al. Autoimmune hepatitis and primary sclerosing cholangitis in children and adolescents. Fetal Pediatr Pathol 2014;33:202-9.
20. Ferrari F, Civitelli F, Di Nardo G, et al. Pediatric sclerosing cholangitis: A descriptive report of two italian referral centers. Digestive and Liver Disease 2013;45:e308.
21. Debray D, Pariente D, Urvoas E, et al. Sclerosing cholangitis in children. J Pediatr 1994;124:49-56.
22. Batres LA, Russo P, Mathews M, et al. Primary sclerosing cholangitis in children: a histologic follow-up study. Pediatr Dev Pathol 2005;8:568-76.
23. Watanabe T, Hirano K, Tada M, et al. Short-term prognostic factors for primary sclerosing cholangitis. J Hepatobiliary Pancreat Sci 2015;22:486-90.
24. de Vries EM, Wang J, Leeflang MM, et al. Alkaline phosphatase at diagnosis of primary sclerosing cholangitis and 1 year later: evaluation of prognostic value. Liver Int 2016;36:1867-1875.
25. Stanich PP, Bjornsson E, Gossard AA, et al. Alkaline phosphatase normalization is associated with better prognosis in primary sclerosing cholangitis. Dig Liver Dis 2011;43:309-13.
26. Al Mamari S, Djordjevic J, Halliday JS, et al. Improvement of serum alkaline phosphatase to <1.5 upper limit of normal predicts better outcome and reduced risk of cholangiocarcinoma in primary sclerosing cholangitis. J Hepatol 2013;58:329-34.
27. Angulo P, Maor-Kendler Y, Lindor KD. Small-duct primary sclerosing cholangitis: a long-term follow-up study. Hepatology 2002;35:1494-500.
28. Weismuller TJ, Trivedi PJ, Bergquist A, et al. Patient Age, Sex, and Inflammatory Bowel Disease Phenotype Associate With Course of Primary Sclerosing Cholangitis. Gastroenterology 2017;152:1975-1984.e8.

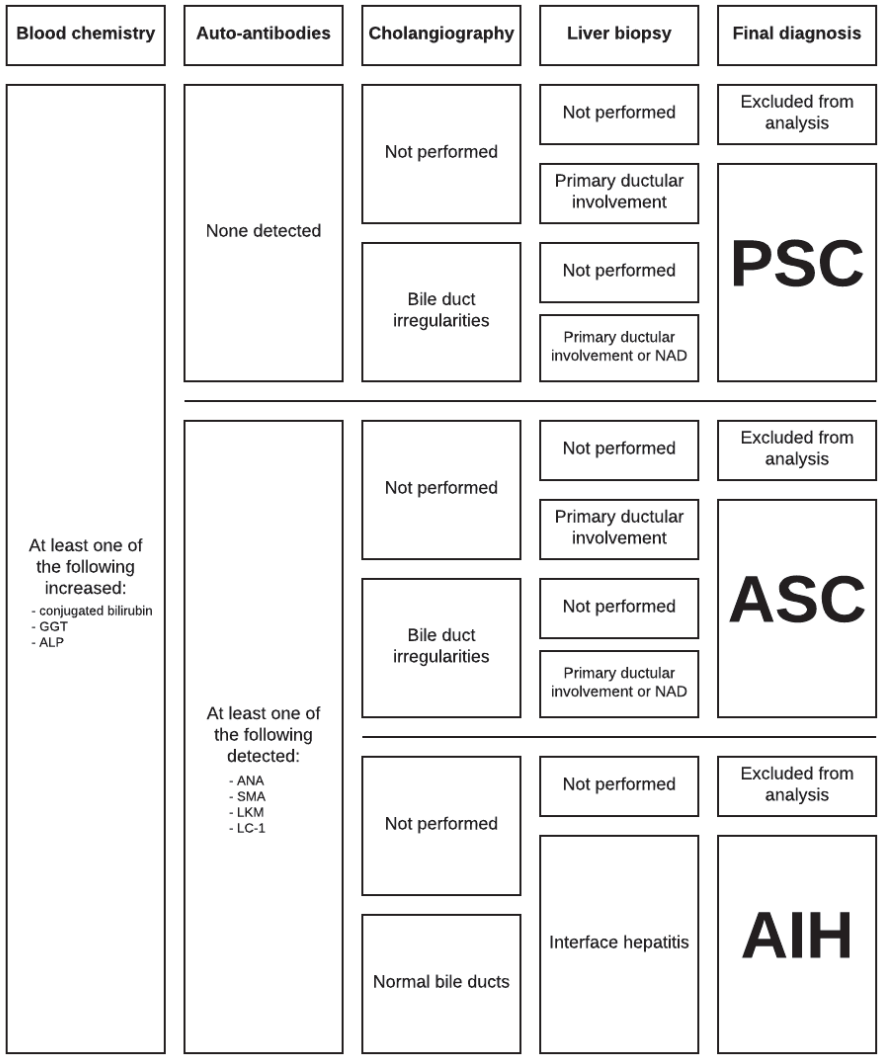

Figure S1. Diagnostic decision matrix. Immunological, radiological and/or histological features were reviewed to determine if patients were appropriately assigned a diagnosis of PSC, ASC or AIH. Patients were excluded from further analysis when they had isolated AIH, or when both cholangiography and liver biopsy were missing in the diagnostic work-up.

Chapter 10

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GENERAL DISCUSSION AND CONCLUSION

The aim of this thesis was to identify immune regulatory processes that are pivotal for intestinal homeostasis in order to yield parameters that classify immunological disease in patients with inflammatory bowel disease (IBD). In addition, by combining immunological disease profiling with extensive clinical characterization of each patient, the research presented in this thesis aimed to identify which clinical and immunological factors can be used to predict disease course and response to therapy. In this chapter, the results of this thesis are put into perspective and results that are of specific interest for future research are highlighted.

Disease heterogeneity in IBD.

IBD, including Crohn's disease (CD) and ulcerative colitis (UC), is a chronic inflammation of the gastrointestinal tract that is characterized by alternating phases of clinical remission and disease relapse. CD can develop in any part of the gastrointestinal tract, but tends to affect the terminal ileum and colon and is therefore classified into ileal, colonic or ileocolonic disease.[1-3] Several clinical subtypes of CD are distinguished based on the age at disease onset, the location of disease and disease behavior defined by the development of stricturing and/or fistulating complications. UC is mainly affects the colon and is classified based on the extent of disease: proctitis affecting only the rectum, left-sided colitis or pancolitis affecting the entire colon.[1-3] Histologic examination of endoscopic biopsies is a key step in the diagnosis of IBD, especially in differentiating between UC, CD and non-IBD intestinal inflammation.[4] In CD, the inflammation is often transmural and discontinuous and consists of a mononuclear cell infiltrate with non-caseating granulomas.[4] Deep fistulizing ulcers can penetrate from the intestinal lumen into the submucosa. Fibrosis in the deeper layers of the mucosa may contribute to the development of strictures. In contrast to CD, pathology in UC is restricted to the mucosa and submucosal layers.[4] The inflammation is often continuous, beginning from the rectum and extending proximally throughout the colon. The inflammatory infiltrate is dominated by neutrophils causing cryptitis and crypt abscesses, resulting in widespread crypt architectural distortion and epithelial damage.[4] Despite the detailed histologic criteria used to differentiate CD from UC, accurate discrimination between CD and UC is not always possible.[4] Moreover, as the composition of the inflammatory infiltrate and the localization of the inflammation can be strikingly different between individuals, the clinical classification of IBD into CD and UC likely does not capture the immunological heterogeneity and concomitant disease-causing mechanisms.

In addition to heterogeneity in disease phenotypes, the course of disease in IBD is also highly variable. Some patients have a quiescent course with long periods of clinical remission whereas others suffer from persistent chronic disease activity.[5] Disease course is often described in terms of disease activity scores, number of disease relapses, need for steroids, need for surgery and hospitalizations, and development of irreversible penetrating and/or stricturing lesions.[6] In both CD and UC, there can be local (i.e. cancer, see **Chapter 7** and **Chapter 8**) and extra-intestinal complications (i.e. sclerosing cholangitis, see **Chapter 9**) that further influence the disease course. The major goal of medical therapy in IBD is to modify or alter the disease course by achieving mucosal healing and steroid-free remission, thereby preventing disease-associated complications and reducing disease progression. Unfortunately, the disease course and therapy responsiveness of an individual IBD patient is difficult to predict. Clinical manifestations at diagnosis, such as age of disease onset, gender and disease phenotype, only moderately predict the disease course.[5, 7] Nevertheless, predicting the course of disease is important, not only to manage patient expectations, but also to individualize patient care. Knowing which patients are likely to have the worst outcomes could guide the choice of the optimal therapy and thereby lead to more efficient use of resources by preventing patient under- and overtreatment. Furthermore, selecting the most appropriate treatment for each individual patient would facilitate control of inflammation, achievement of mucosal healing, and prevention of irreversible bowel damage that necessitates surgical intervention.

The evidence that dysfunction of the immune system is involved for the development of IBD has become stronger over the past decade. First of all, genome-wide association studies (GWAS) have identified IBD-associated susceptibility loci containing genes involved in immune pathways.[8] Generally speaking, the main genetic associations in IBD can be divided into genes that contribute to the innate immune response (I.e. *NOD2*) and genes that contribute to the adaptive immune response (i.e. *IL23R*).[9] In addition, several genetic deficiencies in immune genes have been shown to cause IBD-like intestinal inflammation. For example, patients with mutations in the *IL10*, *IL10R* and *Foxp3* genes present with severe intestinal inflammation at infant age.[10-12] Lastly, the success of immunosuppressive therapies to decrease intestinal disease further emphasizes the important role of dysfunctional immune responses in IBD.[13] Together, this argues that classification on the basis of immune dysfunction could identify subgroups of IBD patients with similar immune pathways involved in their disease pathogenesis. To achieve such classification, an in-depth understanding of innate and adaptive intestinal immune responses and the inter-patient heterogeneity of these responses is required. In the following, Part I particularly focusses on the novel insights obtained through detailed characterization of $CD4^+$ T-cell responses in IBD patients that are described in this thesis. Part II discusses how patient stratification based on underlying disease-causing immune mechanisms may advance disease course prediction and the development of disease subtype-specific therapies.

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Immune mechanisms in IBD.

The intestinal immune system faces a unique challenge as it continuously encounters a diverse antigenic load derived from dietary components, commensal bacteria and infectious pathogens. Thus, gaining insight in microbiota-immune system interactions in the intestine, involving both innate and adaptive immune responses, has become of particular interest in understanding the pathogenesis of IBD. Both the innate and adaptive arms of the immune system contribute to IBD pathology, however, there is strong evidence that a balance between regulatory and inflammatory CD4$^+$ T-cell responses is crucial for the host's mutualism with its commensal microbiota.[14] In this thesis, we have used biological material derived from various cohorts of IBD patients to study CD4$^+$ T-cell reactivity, phenotype and function. By focusing our analyses on a circulating CD4$^+$ T-cell population known to be strongly enriched in gut-homing CD4$^+$ T cells[15, 16], we investigated whether changes in regulatory and inflammatory CD4$^+$ T-cell responses would enable IBD patient stratification prior to treatment (**Chapter 3**). In addition, we have characterized CD4$^+$ T-cell responses to bacterial flagellin, a bacterial antigen present on both commensal and pathogenic bacteria, in treatment-naive CD patients with high anti-microbial IgG titers (**Chapter 4 and 5**). The analysis of peripheral blood lymphocytes and intestinal tissue derived from a very early onset IBD patient with a genetic duplication of the *IL2RA* locus, allowed us to investigate the requirements for intestinal inflammation in the context of a hyperinflammatory CD4$^+$ T-cell defect (**Chapter 6**). When combining the original data presented in the various chapters of this thesis, a recurrent observation is that, when compared to healthy individuals, IBD patients have an altered balance between regulatory and inflammatory CD4$^+$ T-cell responses. We provide evidence for both reduced regulatory (**Chapter 3 and 5**) as well as increased inflammatory features (**Chapter 3 and 6**). In contrast to the widely held hypothesis that IBD may be caused by a defect in regulatory Foxp3$^+$ T cells (Tregs), we detected no differences in the frequencies of Foxp3$^+$ Tregs between IBD patients and healthy individuals. Instead, we demonstrate that characterization of inhibitory receptor expression on CD4$^+$ T cells allows IBD patient stratification prior to treatment. Thus, the data in this thesis substantiate the idea that microbial-host mutualism is lost in IBD due to a predominating inflammatory CD4$^+$ T-cell responses to commensal microbial antigens, promoting tissue destruction and chronic inflammation in the intestine.

Monitoring the inflammatory status of CD4$^+$ T cells in peripheral blood.

After the recognition of cognate antigen in secondary lymphoid organs, antigen-experienced effector CD4$^+$ T cells exit the lymph node, enter the blood and migrate to the intestinal lamina propria or enter the epithelial layer where they reside as long-lived memory CD4$^+$ T cells. Both regulatory and inflammatory CD4$^+$ T cells are imprinted to home

to the intestine (**Chapter 1**). As inflammatory CD4$^+$ T cells drive intestinal pathology in IBD, monitoring the inflammatory status of CD4$^+$ T cells in peripheral blood is highly desired to differentiate patients with a severe versus a mild disease course. Analysis of CD4$^+$ T cells in peripheral blood reflects ongoing T-cell priming and differentiation, but a large majority of circulating CD4$^+$ T cells does not home to the intestine. As a consequence, monitoring the phenotype of total CD4$^+$ T cells in peripheral blood of IBD patients has not yielded consistent changes in regulatory and inflammatory CD4$^+$ T-cell populations. For example, reduced frequencies of circulating CD4$^+$ T cells expressing the regulatory transcription factor Foxp3 have been reported in some IBD studies[17-20] but not in others.[21, 22] Moreover, frequencies of Foxp3$^+$ Tregs have been reported to increase in peripheral blood of IBD patients after anti-tumor necrosis factor alpha (TNFα) treatment[23-25] but this was not observed in a more recent study.[26] These variable results may reflect that monitoring the total circulating CD4$^+$ T-cell population is not sensitive enough to detect transient changes in regulatory and inflammatory mucosally-imprinted CD4$^+$ T-cell responses.

Phenotype of circulating gut-homing CD38$^+$ effector T cells in IBD is shifted towards inflammation

Circulating CD38$^+$ effector T cells comprise 4-10% of the total CD4$^+$ T-cell pool and are enriched in cells expressing the gut-homing chemokine receptor C-C chemokine receptor type 9 (CCR9) and β7-integrin compared to CD38^{neg} effector T cells.[15] In **Chapter 3**, we demonstrate that analysis of circulating CD38$^+$ effector T cells (CD4$^+$CD38$^+$CD62L^{neg}), instead of total CD4$^+$ T cells, lowers the threshold for detection of changes in regulatory versus inflammatory CD4$^+$ T-cell responses. We show that, in healthy individuals as well as IBD patients, the circulating CD38$^+$ effector T-cell population contained both regulatory and inflammatory cell populations. This is in line with our previously published murine data showing that imprinting of the CD62L^{neg}CD38$^+$ T-cell phenotype is induced regardless of regulatory or inflammatory T-cell function.[15] In healthy individuals, who have a balanced mucosal tolerance to commensal microbiota, CD38$^+$ effector T cells had a bias towards regulation with low frequencies of activated CD25$^+$CD45RA^{neg} and high frequencies of T cell Ig and ITIM domain (TIGIT)$^+$ cells (**Chapter 3**). TIGIT is a inhibitory receptor that is strongly associated with IL-10 expression in CD4$^+$ T cells and is known to modify DC function via bi-directional signaling through the ligand CD155.[27-30] Conversely, treatment-naive pediatric IBD patients with active intestinal inflammation had increased frequencies of activated CD25$^+$CD45RA^{neg} and reduced frequencies of TIGIT$^+$ cells when compared to age-matched healthy controls, indicating that in IBD patients the balance between activated inflammatory and regulatory CD4$^+$ T-cell responses is shifted towards inflammation. Importantly, no differences in the frequencies of Foxp3$^+$ cells in CD38$^+$ effector T cells were detected between IBD patients and controls. Recently, similar observations have been

made in another study that demonstrated that both UC and CD patients had a selective paucity of anti-inflammatory inhibitory receptor-rich CD4$^+$ CD25^{low}Foxp3^{neg} T cells in their peripheral blood.[31]

The analyses of **Chapter 3** were performed on peripheral blood derived from new-onset, treatment-naive pediatric IBD patients with biopsy-proven active intestinal disease and IBD patients in clinical remission who visited the outpatient clinic. In the latter, remission was defined using physician global assessment and clinical disease scores. To obtain insight in disease subtype-specific alterations, we differentiated between UC and CD patients. This yielded differences in specific cell populations between UC and CD, as the increased frequency of CD45RA^{neg} cells were most prominent in CD. In contrast, in patients with UC, the increase in frequency of CD45RA^{neg} cells was more variable between patients and was not significant compared to UC in remission. In addition, the majority of patients with reduced TIGIT$^+$ cell frequencies to treatment were diagnosed with CD. Unfortunately, the number of patients included in the peripheral blood TIGIT analyses in **Chapter 3** were limited in number (n=18) and therefore not powered sufficiently to draw strong conclusions on differences in the frequency of TIGIT$^+$ cells between UC and CD. In a small number patients (n=10), patient charts were reviewed longitudinally for data on response to initiated therapy and duration of clinical remission. Linking clinical follow up data to the observed peripheral blood CD4$^+$ T-cell phenotypes prior to treatment demonstrated that heterogeneity in the percentage of TIGIT$^+$ cells in CD38$^+$ effector T-cell population at disease diagnosis differentiated patients with reduced duration of clinical remission during follow-up. Specifically, a TIGIT$^+$ cell percentage below 25% prior to treatment associated with a reduced duration of clinical remission. These results show that phenotypic characterization of circulating CD4$^+$ T-cell populations can stratify treatment-naive IBD patients into clinically relevant subgroups. In particular, analysis of multiple inhibitory receptors, including TIGIT, on CD38$^+$ effector T cells may further facilitate IBD patient stratification prior to treatment.

Data from ongoing analyses in a large pediatric IBD cohort (PIBD Set Quality, Horizon 2020) have so far validated that frequencies of TIGIT in CD38$^+$ effector T cells are decreased in a subgroup of therapy-naive IBD patients, particularly in CD patients (n=6; inclusion ongoing). In addition, therapy-naive CD patients display increased frequencies of CD38$^+$ effector T cells expressing the proliferation marker Ki67 when compared to age-matched healthy controls. The increased frequencies of Ki67$^+$ cells are mainly observed in TIGIT^{neg}CD38$^+$ effector T cells. Similar analyses will be performed in an ongoing study in which treatment-naive pediatric IBD patients are randomized to step-up (standard induction treatment by oral prednisolone or exclusive enteral nutrition) and top-down (induction by anti-TNFα infusions) treatment arms. In the near future, by selecting multiple CD38$^+$ effector T-cell related parameters such as CD45RA, CD25, TIGIT and Ki67, we aim

to find specific CD4$^+$ T-cell signatures that further enable IBD patient stratification prior to treatment. It will be interesting to determine whether specific CD4$^+$ T-cell signatures present at disease diagnosis correlate to response to the wide range of currently available immune therapies (i.e. anti-TNFα monoclonal antibodies, anti-IL-12 and anti-IL-23 monoclonal antibodies, integrin blockers, and JAK inhibitors).

High intracellular Ca^{2+} following TCR ligation preferentially induces TIGIT on antigen-experienced CD4$^+$ T cells

Why do frequencies of circulating TIGIT$^+$CD38$^+$ effector T cells associate with the disease course of IBD? TIGIT is a receptor of the Ig superfamily that has been shown to have a dampening effect on the immune response through multiple mechanisms that include direct suppression of effector T cells and indirect suppression via modulation of antigen presenting cells (APCs).[27, 32-34] As TIGIT$^+$ cell frequencies were reduced in a subgroup of IBD patients (**Chapter 3**), we aimed to identify factors involved in the induction of TIGIT expression on CD4$^+$ T cells (**Chapter 4**). Others have shown that anti-CD3/anti-CD28 stimulation induces TIGIT expression on total CD4$^+$ T cells, Tregs, naive and memory CD4$^+$ T cells.[27, 32, 35, 36] We show that T-cell receptor (TCR) ligation through anti-CD3 induced TIGIT expression preferably on CD4$^+$ T cells with an antigen-experienced phenotype. In contrast, anti-CD3 nor anti-CD3/CD28 stimulation induced TIGIT expression on naive CD4$^+$ T cells. TCR ligation is known to increase the intracellular Ca^{2+} concentration and result in nuclear import of the transcription factor nuclear factor of activated T cells (NFAT).[37-39] As previously documented, NFAT activation on its own is sufficient to induce a two-fold change in *TIGIT* gene expression in human Jurkat T cells.[40] In agreement, by using the selective Ca^{2+} ionophore ionomycin, we show that Ca^{2+} dependent signaling after TCR ligation was required to induce TIGIT expression in memory CD4$^+$ T cells. Taken together, this suggests that NFAT is a critical component of the signaling pathway that regulates TIGIT expression in CD4$^+$ T cells. These data relates to observations in CD8$^+$ T cells, where NFAT promotes expression of exhaustion-associated genes.[41] Similarly, in both mice and humans, we show that anti-CD28 costimulation decreased the frequency of TIGIT$^+$ cells, which is in line with the established CD28-dependent decrease in the induction of exhaustion associated genes in CD8$^+$ T cells.[41, 42] During homeostasis, resident intestinal APCs express low levels of costimulatory molecules CD80 and CD86.[43, 44] This may allow for reduced CD80/CD86-mediated costimulation through CD28 during CD4$^+$ T-cell priming in mucosa-draining lymph nodes supporting an overall tolerogenic milieu when compared to non-mucosal lymphoid sites. Consequently, TIGIT upregulation may preferentially occur in mucosa-draining lymphoid tissue during intestinal homeostasis, when antigen-experienced intestinal CD4$^+$ T cells receive low levels of anti-CD28 costimulation through encounter of antigens presented by these tissue adapted APCs. During intestinal inflammation,

blood-derived unconditioned APCs accumulate in the intestine, which display enhanced pro-inflammatory characteristics and provide anti-CD28 costimulation during antigen presentation through CD80 and CD86.[45] The percentage of TIGIT on $CD38^+$ effector T cells in the peripheral blood of IBD patients may thus indirectly reflect the inflammatory state of the APCs that $CD4^+$ T cells have encountered in mucosa-draining lymphoid tissue. Hence, it will be interesting to investigate whether patients with reduced $TIGIT^+$ cell frequencies respond favorably to molecules that inhibit APC activation. Alternatively, TIGIT expression may reflect the strength of interaction between the TCR on the $CD4^+$ T cell and the peptide-MHC-complex on the APC. Detailed analyses of $TIGIT^+$ cell frequencies in combination with HLA typing, $CD4^+$ T-cell specificity, phosphoproteomics and Ca^{2+} flux may be required to further investigate this.

The strong association between TIGIT and CD38 is a recurring observation in all our *in vitro* and *in vivo* data reported in **Chapter 3** and **Chapter 4**. CD38 is an ectoenzyme that catalyzes the synthesis of two structurally distinct messengers for intracellular Ca^{2+} mobilization, cyclic ADP-ribose (cADPR) and nicotinic acid adenine dinucleotide phosphate (NAADP), from cytosolic substrates, nicotinamide adenine dinucleotide (NAD) and nicotinamide adenine dinucleotide phosphate (NADP), respectively.[46, 47] As a consequence, this raises the question whether CD38 contributes to TIGIT induction on $CD4^+$ T cells through increasing the intracellular Ca^{2+} concentration. In both mice and humans, CD38 is highly expressed by antigen-experienced $CD62L^{neg}$ lymphocytes in the intestine.[15, 48-50] We have obtained preliminary data indicating that colonic lamina propria $CD4^+$ lymphocytes of CD38-deficient mice (*Cd38*$^{-/-}$) have lower frequencies of $TIGIT^+$ cells compared to wild type mice (collaboration with Dr. Frances E. Lund, University of Alabama at Birmingham). In wild type mice, a mean of 33.5 ± 5.5% of lamina propria lymphocytes express TIGIT versus only 12.9 ± 2.2% in *Cd38*$^{-/-}$ mice ($p < 0.0001$; n=5 in both experimental groups). Although these data demonstrate that CD38 and TIGIT expression are positively correlated, it remains to be investigated whether CD38-mediated Ca^{2+} signaling is involved in inducing or maintaining TIGIT expression on intestinal $CD4^+$ T cells. Using small interfering RNA (siRNA) to interfere with the expression of the *CD38* gene could be used to test this hypothesis in human $CD4^+$ T cells. In addition to CD38, local mucosal factors such as retinoic acid (RA) may be required to induce TIGIT expression on $CD4^+$ T cells. In support of this idea, the addition of RA to anti-CD3 stimulated $CD4^+$ T cells further increased frequencies and mean fluorescence intensity of $TIGIT^+$ cells in both mice and humans (**Chapter 4**). Thus, CD38-inducing factors from the intestinal microenvironment such as RA may further increase TIGIT expression on antigen-experienced $CD4^+$ T cells. Future studies are needed to firmly establish a role for CD38 and CD38-associated environmental factors in TCR-induced TIGIT expression.

In-depth functional characterization of $TIGIT^+CD38^+$ and $TIGIT^{neg}CD38^+$ effector T cells

To gain insight into the functional role of TIGIT on circulating $CD38^+$ effector T cells specifically, we determined whether TIGIT expression on $CD38^+$ effector T cells correlated with immune inhibitory properties (**Chapter 3**). In peripheral blood of adult healthy individuals, $TIGIT^+CD38^+$ cells more often co-expressed a second inhibitory receptor, such as programmed death 1 (PD-1) and cytotoxic T lymphocyte antigen 4 (CTLA-4), and contained lower frequencies of interferon gamma $(IFN\gamma)^+$ cells and higher frequencies of interleukin 10 $(IL\text{-}10)^+$ cells when compared to $TIGIT^{neg}CD38^+$ effector T cells. The association between TIGIT and IL-10 was specific for the $CD38^+$ effector T-cell population, as $TIGIT^+CD38^{neg}$ cells did not have higher frequencies of $IL\text{-}10^+$ cells compared to $TIGIT^{neg}CD38^{neg}$ cells. Importantly, transcriptional profiling as well as flowcytometric analyses showed that $CD38^+$ and $CD38^{neg}$ effector T cells had similar expression of Foxp3 and CD25, indicating no preferential enrichment for Treg-associated molecules in circulating $CD38^+$ effector T cells. As TIGIT was expressed by high frequencies of intestinal $CD4^+$ T cells and its expression increased during bacterial colonization of the gastrointestinal tract (**Chapter 4**), we determined whether TIGIT expression on flagellin-reactive $CD4^+CD154^+$ T cells from healthy adult individuals correlated with a regulatory cytokine profile. TIGIT-expressing $CD4^+CD154^+$ T cells with reactivity to flagellin had increased frequencies of $IL\text{-}10^+$ cells compared to flagellin-reactive $TIGIT^{neg}CD4^+CD154^+$ T cells. Thus, both within circulating $CD38^+$ effector T cells as well as flagellin-reactive $CD4^+CD154^+$ T cells, TIGIT expression associates with a preferential regulatory $CD4^+$ T-cell phenotype. These observations could imply that intestinal disease in IBD patients with a low $TIGIT^+$ cell frequencies (i.e. 20%) is more T-cell driven compared to IBD patients with a high $TIGIT^+$ cell frequencies (i.e. 40%). In order to test this hypothesis, T-cell suppression assays to establish whether $TIGIT^+CD38^+$ effector T cells have the ability to directly suppress effector T-cell proliferation need to be performed. Interestingly, our data in **Chapter 6** indicates that a high frequency of $TIGIT^+CD38^+$ cells can coincide with a strong inflammatory T-cell phenotype and T-cell hyperproliferation. Specifically, we show that a very early onset IBD patient with a *IL2RA* locus duplication has a $TIGIT^+CD38^+$ cell frequency of 45 ± 3.5% which is in a similar range as healthy adult and pediatric individuals. Nevertheless, TCR stimulation of $CD4^+$ T cells in the presence of IL-2 resulted in enhanced proliferation and $IFN\gamma$ secretion compared to controls. Instead of directly affecting T-cell function, $TIGIT^+CD38^+$ cells may exert inhibitory function indirectly via controlling APC function. In this respect, in **Chapter 3** we show that $TIGIT^+CD38^+$ effector T cells have the capacity to modulate inflammatory cytokine expression by monocyte-derived DCs, suggesting that $TIGIT^+$ cells may have a local immunomodulatory effect on surrounding cells when reaching the intestinal tissue. In this respect, it is possible that the significantly lower $IFN\gamma$ expression that we observed in $TIGIT^+CD38^+$ cells compared to $TIGIT^{neg}CD38^+$ cells is a consequence of lower IL-12 production by DCs.

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Importantly, in order to compare phenotype and function of TIGIT$^+$ and TIGIT^{neg} cells within the circulating CD38$^+$ effector T-cell population, it is pivotal to know whether both TIGIT$^+$ and TIGIT^{neg} cells have equal capacity to migrate to the intestinal lamina propria. In order to answer this question, we have biotinylated the clinically used anti-human $\alpha4\beta7$ monoclonal antibody Vedolizumab and optimized $\alpha4\beta7^+$ T-cell detection using flow cytometry on PBMCs from peripheral blood of healthy adults. Through characterization of $\alpha4\beta7$ and CCR9 expression, we have recently observed that TIGIT$^+$CD38$^+$ effector T cells and TIGIT^{neg}CD38$^+$ effector T cells have similar frequencies CCR9$^+$ and $\alpha4\beta7^+$ cells. This indicates that both populations have equal capacities to home to the intestine, which solidifies TIGIT as a marker to discriminate subgroups of IBD patients. In order to investigate whether we could detect patient subgroups with decreased intestinal TIGIT expression, we have analyzed *TIGIT* mRNA expression on intestinal biopsies of treatment-naive IBD patients (not included in this thesis). Due to a large variation, *TIGIT* mRNA expression was not significantly decreased in a particular subgroup of IBD. *TIGIT* mRNA expression strongly correlated with the degree of *CD3* mRNA expression suggesting that *TIGIT* expression was directly related to the amount of T-cell infiltration in biopsies. This emphasizes that, in contrast to analysis of peripheral blood CD38$^+$ effector T cells, a population that reflects ongoing intestinal T-cell priming and differentiation, using intestinal biopsies is more difficult to quantify the balance between regulatory and inflammatory CD4$^+$ T-cell populations. In order to investigate the functional role of TIGIT in the intestine, future analysis of immune responses in TIGIT deficient mice at steady state and after induction of experimental colitis are needed to prove whether TIGIT contributes to regulatory CD4$^+$ T-cell function in the intestine and whether reduced functioning of the TIGIT pathway accelerates or exacerbates intestinal inflammation.

Taken together, tracing intestinal disease in peripheral blood is feasible. Analysis of the cellular composition of the circulating CD38$^+$ effector T-cell population allows detection of transient changes in regulatory and inflammatory mucosally-imprinted CD4$^+$ T-cell responses that correlate with the course of IBD. These data have immediate relevance for clinical trials in IBD, as regulatory and inflammatory cell populations within the circulating CD38$^+$ effector T-cell population enable IBD patient stratification prior to treatment. In addition, our findings may be further exploited in other diseases such as graft versus host disease and checkpoint inhibitor-induced intestinal inflammation in the setting of cancer.

Flagellin-reactivity of memory CD4$^+$ T cells in IBD.

The infiltration of the lamina propria by memory CD4$^+$ T cells is a critical step in the chronicity of IBD. What antigens are recognized by these lamina propria CD4$^+$ T cells? Insights from a number of experimental animal models have provided evidence that antigens from commensal microbiota drive intestinal inflammation. First of all, adoptive transfer of

CD4$^+$ T cells activated *in vitro* with bacterial antigens induced colitis in immunodeficient recipient mice, whereas no colitis was induced upon transfer of polyclonally activated CD4$^+$ T cells (using anti-CD3).[51] In addition, in many experimental colitis models, including T-cell transfer colitis and IL-10 deficient colitis, colitis does not develop in germ-free conditions.[52, 53] In some animal models, single bacterial strains can induce colitis.[54-56] For example, germ-free IL-10 deficient mice develop intestinal inflammation after colonization with a pure culture of *Enterococcus faecalis*.[54] In addition, colonization with the intestinal pathogen *Helicobater hepaticus* induces severe colitis in specific pathogen free (SPF) reared IL-10 deficient mice, demonstrating that specific microbial antigens are pivotal for disease development.[55] Altogether, these experimental studies provide support that CD4$^+$ T cells with a specificity for intestinal microbial antigens cause intestinal disease under specific experimental conditions, and demonstrate that not all bacterial antigens are equal in their capacity to induce a pathogenic CD4$^+$ T-cell response. Specifically, these data indicate that loss of immunological tolerance to intestinal microbiota in IBD patients is not global, but that specific bacterial antigens are recognized.

Flagellin: a highly antigenic protein present expressed by commensal as well as pathogenic bacteria

In order to provide insight into the mechanisms that disrupt immunological tolerance to intestinal microbiota in IBD, we studied the frequency and phenotype of flagellin-reactive CD4$^+$ T cells in CD patients with elevated anti-flagellin IgG (**Chapter 5**). The protein flagellin is a structural component of the flagellum, a surface filament present on motile bacteria in the gastrointestinal tract.[57] Flagellin is exclusively expressed by bacteria and is one of the most abundant proteins present on flagellated bacteria. Both commensal as well as pathogenic bacteria can express flagellin, although it is thought that species indigenous to the intestinal microbiome, i.e. bacteria from the phyla *Firmicutes* and *Bacteroides*, may have been selected for the absence or the low *in vivo* expression of flagellin.[58] The flagellin protein contains two to four domains, depending on the bacterial species.[59-62] The D0 and D1 domains are well conserved among bacterial species due to their functional importance in filament formation[60], indicating that there are possible common TCR epitopes among different flagellins. In contrast to D0 and D1, the surface-exposed D2 and D3 domains are highly variable in sequence and structure.[62]

Toll-like receptor 5 (TLR5) recognizes flagellin as a pathogen-associated molecular pattern (PAMP) using the extracellular domain and activates the myeloid differentiation primary response 88 (MyD88)-dependent signaling pathway and nuclear factor κB (NFκB)-mediated production of pro-inflammatory cytokines.[63] Consequently, flagellin is highly antigenic and has successfully been used as a carrier protein and adjuvant to augment immune responses to poorly immunogenic vaccines.[64, 65] The potential binding region

for TLR5 is proposed to be the conserved N- and C-terminal regions in the D1 domain and an adjacent β-hairpin structure.[66, 67] In order to discriminate between commensal and pathogenic flagellated bacteria, intestinal epithelial cells in the intestine display compartmentalized expression of TLR5. For example, *in vitro* analysis of polarized human intestinal epithelial cells demonstrated that TLR5 is expressed exclusively on the basolateral surface of epithelial cells.[68, 69] In line with this, in adult mice, luminal flagellin activates TLR5 only after injury to the epithelial barrier induced by dextran sodium sulphate (DSS).[70]

Adaptive immune responses to microbial flagellin

Longitudinal analyses of commensal antimicrobial antibody responses in the serum of healthy individuals have shown activation of the adaptive immune system to microbial antigens, including flagellins is a healthy feature of our immune system. The initial antibody response to commensal antigens is vigorous in infants and maintained throughout adult life at lower levels.[71] Heightened antibody responses to *Bacteroides* antigens and *Firmicutes* flagellins are observed in infants from 12 to 24 months of age, which is followed by the reduction to more adult-like, lower levels at 7 years of age.[71] These elevated levels of anti-flagellin antibodies could reflect increased microbial antigen translocation in the infant gastrointestinal tract during successive stages of microbial colonization. In adulthood, healthy individuals display low antibody responses to commensal intestinal bacteria[72], including low but detectable levels of anti-flagellin antibodies.[71, 73] In contrast, patients with IBD have variable serological responses to microbial antigens, amongst which bacterial flagellins.[74-77] Elevated anti-flagellin IgG has been detected in sera from subgroups of patients with CD, but not in sera from patients with UC or from healthy individuals.[73, 78, 79] Studying the immune response to flagellin thus provides an opportunity to obtain increased understanding of mechanisms contributing to disease pathogenesis in this particular subgroup of CD patients.

Mouse models have demonstrated that innate immune signaling through TLR5 is essential for the generation of a flagellin-specific antibody response, as MyD88-deficient mice are impaired in their ability to make flagellin-specific antibody responses after intraperitoneal injection of flagellin.[80] Similarly, flagellins from *Helicobacter pylori* and *Campylobacter jejuni*, which are known not to activate TLR5 signaling, do not elicit a flagellin-specific antibody responses.[66, 80] Moreover, studies with αβ T-cell deficient mice have shown that the generation of flagellin-specific antibodies is T-cell dependent.[80] Thus, both TLR5-mediated innate immune activation as well as flagellin-specific CD4$^+$ T cells are likely required for the *in vivo* generation of an increased anti-flagellin antibody response.

Immune mechanisms in CD patients with anti-flagellin adaptive immune responses

In humans, it is unknown whether CD patients with elevated anti-flagellin antibodies have increased anti-flagellin CD4$^+$ T-cell reactivity. In addition, it is unknown whether the increased adaptive immune response to flagellin as observed in subgroups of CD patients are involved in a protective, beneficial immune response or are part of an pathogenic immune response.[81] In **Chapter 5**, we hypothesized that CD patients with elevated anti-flagellin IgG antibodies have increased frequencies of flagellin-reactive CD4$^+$ T cells with a more inflammatory phenotype compared to healthy individuals and CD patients with low anti-flagellin antibody titers. To investigate anti-flagellin antibody responses we used a protein microarray containing 8 recombinant flagellin antigens from the *Firmicutes* phylum, derived from different microbial isolates from the murine intestine belonging to the *Lachnospiraceae* family, including *Roseburia intestinalis*, *Roseburia inulinivorans*, *Butyrivibrio fibrisolvens*.[71, 82] For *in vitro* detection of flagellin-reactive CD4$^+$ T cells we selected a mix of 4 of these recombinant flagellin proteins: 14-2, A4 Fla2, A4 Fla3 and FlaX. As the frequency of flagellin-specific CD4$^+$ T cells in peripheral blood is very low and the precise epitope that is being recognized is unknown, detection of flagellin-specific CD4$^+$ T cells by using MHC-peptide tetramers is not feasible. Instead, we used a novel CD154-based detection method, which detects virtually all functional activated CD4$^+$ T cells irrespective of their differentiation status.[83] Importantly, in order to study flagellin-reactive CD4$^+$ T-cell frequency and phenotype without interference of immunosuppressive drugs, we performed all CD154 stimulation assays on peripheral blood mononuclear cells (PBMCs) of treatment-naive pediatric CD and UC patients.

The preliminary results in **Chapter 5** demonstrate that elevated levels of IgG antibodies to flagellin are associated with increased frequencies of flagellin-reactive CD4$^+$ T cells in treatment-naive CD patients. This observation is in line with the notion that flagellin-specific IgG may be generated upon intestinal damage (i.e. infections or inflammation) when translocation of bacteria to systemic lymphoid tissue drives systemic CD4$^+$ T-cell responses.[84] Why is the compartmentalization of mucosal immune responses lost in this subgroup of CD patients? There is evidence implying that decreased NFκB activation in response to bacteria could result in an upregulated adaptive immune response to bacterial antigens. For example, flagellin-induced IL-6 production by monocytes is lower in CD patients with quantitatively higher anti-CBir1 antibody levels.[75] Moreover, CD patients with NOD2 variants, resulting in a diminished innate response to bacterial muramyl dipeptide (MDP), have increased antibody responses to microbial antigens.[85] Possibly, a relative defect in the innate immune response could lead to increased microbial translocation or decreased bacterial clearance after translocation. In this way, the primary defect in innate immune responses could consequently result in a secondary upregulated adaptive immune response. Conversely, in **Chapter 6**, we present an example of a very early onset IBD patient with enhanced proliferative CD4$^+$ T-cell responses to TCR stimulation, in whom one would expect little microbial translocation. Indeed, analysis of this patient's

plasma did not show elevated levels of anti-flagellin IgG compared to age-matched controls. In addition to defects in innate immunity, increased intestinal permeability due to a defective mucosal barrier, the extent or the location of affected intestinal surface, or increased bacterial pressure are all possible mechanisms that could underlie anti-flagellin IgG responses. In order to differentiate between these various mechanisms, combining immunological disease profiling (including anti-flagellin IgG levels) with extensive clinical characterization of each patient is required. For example, the observation that high levels of anti-CBir1 flagellin antibodies are associated with ileal localization of CD and not with extensive colitis[76], decreases the feasibility that the extent of affected intestinal surface is an important contributor to the generation of anti-flagellin adaptive immune responses.

Importantly, the anti-flagellin antibody response is not a generalized response. In our patient cohort of **Chapter 5**, CD patients with elevated anti-flagellin antibodies did not respond to random recombinant intestinal bacterial proteins (rIBs cloned from the C3H/HeJ mouse cecum).[86] Thus, although previously thought that CD results from a global loss of tolerance, our data suggests that loss of immunological tolerance to microbial flagellin in CD patients is not global. This is in agreement with previous data that could classify CD patients into distinct groups based on seroreactivity to microbial antigens, including anti-*Saccharomyces cerevisiae* antibody (ASCA), I2 protein derived from *Pseudomonas fluorescens* and *Escherichia coli* outer membrane protein C (OmpC).[74] High reactivity to the flagellin CBir1 was seen across all these antibody-defined subgroups, which is consistent with the anti-flagellin IgG response being independent from other, previously-defined antibody responses.[76, 87] In agreement, in our patient cohort, there was no relationship between the IgA and IgG response to OmpC and any of the tested recombinant flagellin antigens. This suggests that an overall decreased innate immunity is likely not the only mechanism required for the generation of an increased anti-flagellin antibody response. Possibly, CD patients with elevated anti-flagellin IgG possess particular HLA subtypes with enhanced affinity for flagellin peptides. Interestingly, CD patients with elevated anti-flagellin IgG often responded to multiple flagellins and strong positive correlations between IgG responses to these multiple flagellin antigens were observed. Hence, it is conceivable that conserved epitopes from the D0 and D1 domains of the flagellin protein are recognized by the adaptive immune system. Unfortunately, our current flagellin-reactive CD4$^+$ T-cell analyses do not clarify whether the increased frequency of flagellin-reactive CD4$^+$ T cells in CD patients with elevated anti-flagellin IgG reflects expansion of cells with defined limited TCR-specificity. Future analyses should address these questions by typing HLA of CD patients with and without elevated anti-flagellin IgG and analyzing the TCR diversity of isolated flagellin-reactive CD4$^+$ T cells.

Phenotype of flagellin-reactive CD4$^+$ T cells in CD patients with elevated antibody response to flagellin

Another key finding of **Chapter 5** is that flagellin-reactive CD4$^+$ T cells of healthy individuals harbor mixed regulatory and inflammatory responses, as evidenced by detection of IL-10$^+$, IFNγ^+ and IL-17$^+$ flagellin-reactive CD4$^+$ T cells. This finding is in line with the general paradigm that harmless exogenous antigens elicit a balanced CD4$^+$ T-cell response in which regulatory cell populations counteract inflammatory cells. CD patients with elevated flagellin-specific IgG had reduced frequencies of IL-10$^+$ flagellin-reactive CD4$^+$ T cells when compared to those of healthy individuals and patients with low levels of flagellin-reactive IgG. Moreover, frequencies of flagellin-reactive CD4$^+$ T-cells expressing the inhibitory receptor TIGIT were reduced in this patient group. Therefore, on the basis of these results, we favor the hypothesis that flagellin-reactive CD4$^+$ T cells from CD patients with elevated IgG plasma antibodies may have altered functional characteristics with reduced regulatory features. It is possible that this decreased regulatory phenotype is a consequence of an altered innate immune response. Alternatively, the altered functional characteristics of flagellin-reactive CD4$^+$ T cells could be a result of signals from the inflammatory intestinal environment. In mice, CD4$^+$ T cells with a transgenic flagellin (CBir1)-specific TCR have a Th1 profile when generated during *Toxoplama gondii* infection, whereas chemical disruption of the intestine by DSS induces a Th17 profile.[88] These data suggest that loss of intestinal barrier integrity in combination with an inflammatory environment determines the phenotype of the ensuing microbiota-specific CD4$^+$ T-cell response. It would be of interest to ascertain whether CD4$^+$ T cells with the same TCR specificity can adopt both regulatory and inflammatory features in response to flagellin.

Our finding of reduced regulatory features of flagellin-reactive CD4$^+$ T cells in CD patients with elevated flagellin IgG may seem in contrast with recent literature showing increased IL-17A production in IBD patients after stimulation with heat-inactivated bacteria[89] or flagellin proteins (FlaX, A4 Fla-2).[90] However, in our cohort, CD patients with low anti-flagellin IgG levels also displayed increased relative frequencies of IL-17A$^+$ and TNFα^+ flagellin-reactive T cells. Recent evidence suggests that effector CD4$^+$ T-cell responses to commensals can also support intestinal homeostasis by producing barrier-protective cytokines.[89, 91] Specifically, Th17 cells can cooperate with Treg to promote the repair of damaged epithelial barrier during colitis.[91, 92] Based on these data, is interesting to speculate that, instead of contributing to tissue pathology, these flagellin-reactive IL-17 secreting CD4$^+$ T cells promote intestinal barrier integrity through IL-17A and TNFα induced tissue repair in CD patients with low levels of anti-flagellin IgG. This argues that inflammatory cytokines other than IL-17, such as IFNγ, possibly drive the generation of increased anti-flagellin antibody responses in a subgroup of CD patients. Increasing the duration of *in vitro* flagellin stimulation may be required to detect flagellin-reactive CD4$^+$ T

cells with increased expression of pro-inflammatory IFNγ.

Our observation of reduced regulatory features of flagellin-reactive CD4$^+$ T cells in CD patients with elevated anti-flagellin IgG are of particular interest in the light of previous results obtained with TCR transgenic mice specific for CBir1 flagellin (CBir-Tg mice).[93] In this model, under steady state conditions, intestinal IgA production was regulated by Tregs in an antigen-specific manner. Depletion of Tregs substantially reduced intestinal IgA levels within days, in part due to interruption of survival signals to lamina propria IgA$^+$ B cells provided by Tregs.[93] These data imply that Tregs are the major helper T cell for induction and maintenance of intestinal IgA$^+$ B-cell responses directed to flagellin. Taken together with our data, one hypothesis is that, in healthy individuals, low frequencies of flagellin-specific CD4$^+$ T cells are present locally in the lamina propria that support intestinal IgA$^+$ B-cell responses directed to flagellin. This idea is in line with observations that IgA is the main immunoglobulin in the intestine of healthy individuals.[94-99] Conversely, in specific subgroups of CD patients, where anti-flagellin CD4$^+$ T-cell specific regulatory features are lost (i.e. due to decreased innate immune responses or inflammatory environmental factors), the preferential anti-flagellin IgA response might disappear. As intestinal IgA was found to block mucosal flagellin uptake and systemic T-cell activation in CBir-Tg mice[93], the decreased levels of anti-flagellin IgA in the intestine could result in elevated levels of anti-flagellin IgG in the serum and increased frequencies of circulating flagellin-specific CD4$^+$ T cells. Hence, it will be of interest to determine the amount of secretory IgA in the intestinal lumen as well as identify IgA-coated bacteria in feces of CD patients with and without elevated anti-flagellin IgG. In addition, in order to characterize the regulatory phenotype of flagellin-reactive CD4$^+$ T-cell responses in detail, determining expression of Foxp3 expression in future experiments is desired. Additional activation-dependent proteins such as CD137, which has recently been associated with Treg phenotype[100], should also be taken into account. Combined analysis of CD154 and CD137 following short-term stimulation with flagellin would enable in parallel detection of conventional CD4$^+$ effector T cells, Foxp3$^+$ Tregs and CD4$^+$Foxp3^{neg} T cells responding to the same antigen.

Anti-flagellin adaptive immune responses: future experiments

It is important to emphasize that the CD154-assay described in **Chapter 5** was performed on only a limited number of patients. Currently, we are performing CD154 analyses in a larger IBD patient cohort with detailed information on clinical disease and therapy response. In addition, longitudinal analyses of anti-flagellin antibody responses are being performed to assess whether anti-flagellin IgG levels in treatment-naive CD patients decrease after initiation of treatment. Previous studies have suggested that anti-microbial antibody levels, including anti-flagellin IgG levels[76], are relatively stable over time, and do not correspond with active or remission disease states.[74] However, these analyses were performed on infliximab-treated CD patients who experienced a change in their clinical disease score[74] and surgical CD patients in whom anti-flagellin IgG levels were analyzed at time of small-bowel surgery and at least 6 months or more after the surgery.[76] In contrast, we aim to analyze levels of anti-flagellin IgG in plasma of treatment-naive pediatric CD and UC patients before and after initiation of therapy. Preliminary analyses (not included in this thesis) indicate that anti-flagellin IgG levels significantly decrease after 10 weeks of induction treatment. This is reminiscent of observations in patients with celiac disease, an immunological intolerance to wheat gluten (consisting of gliadin and glutenin components), in which anti-gliadin and anti-transglutaminase IgA and IgG antibodies disappear after commencement of a gluten-free diet[101, 102], and rise again when gluten is reintroduced into the diet.[103] Thus, in celiac disease, antibody levels mirror the immune reaction triggered by gluten in the intestine, and can therefore be used to diagnose disease and monitor disease remission. In CD patients, one possible mechanism for the decreased levels of anti-flagellin antibodies after 10 weeks of immunosuppressive treatment is reduced CD4$^+$ T-cell help to flagellin-specific IgG$^+$ B cells. Combining data on anti-flagellin IgG levels together with data on the activation state of CD38$^+$ effector T cells and flagellin-reactive CD4$^+$ T cells in peripheral blood will help to test this hypothesis. If anti-flagellin IgG levels reflect the efficacy of the initiated treatment to suppress CD4$^+$ T-cell responses, determining antibody levels throughout the course of disease would aid in monitoring response to treatment.

Looking beyond CD: evidence for anti-commensal reactivity in UC and very early onset IBD

IgG production is dramatically increased in the intestines of patients with IBD, including both CD and UC patients.[94-99] Thus, why do elevated levels of anti-flagellin antibodies not develop in UC patients even though this disease is believed to be a loss of tolerance to microbiota and an intestinal imbalance in IgG and IgA levels can be observed? Defective epithelial barrier function, which has been proposed as a mechanism of UC[104, 105], could allow excessive translocation of microbial antigens into the lamina propria. However, UC patients may not have a concomitant innate defect or HLA subtype required for the generation of an increased anti-flagellin antibody response. Alternatively, different microbial species may colonize the colon of UC patients, resulting in a different localization of mucosal inflammation and generation of a different type anti-commensal IgG response. In line with this hypothesis, UC patients do have altered adaptive immune responses to the intestinal microbiota when compared to healthy controls. For example, in our analyses **Chapter 5**, a subgroup of UC patients did show antibody reactivity to the recombinant flagellin 3-1-57 (also derived from *Lachnospiraceae*). In addition, recent evidence suggests that anti-commensal IgG in UC patients contributes to UC tissue pathology via increasing Fc gamma receptor (FcγR)-dependent expression of IL-1β, a Th17-polarizing cytokine, by intestinal APCs.[106, 107] Importantly, these new insights in different disease-causing mechanisms in

specific subgroups of IBD patients could have therapeutic implications. For example, CD patients with elevated anti-flagellin IgG levels might benefit from agents used to boost innate immunity, whereas in UC patients in whom intestinal APCs have a lower cellular activation threshold might benefit from anakinra, a recombinant IL-1 receptor antagonist.

Our data in **Chapter 6** further illustrate that microbiota-immune system interactions can be crucial in driving intestinal disease in IBD patients. In this chapter, we characterized the immune function of a patient with a *de novo* duplication of the 10p15.1 chromosomal region, including the *IL2RA* gene. The patient developed an UC-like acute severe colitis at 2 years of age, with endoscopy showing severe pancolitis with edematous mucosa and multiple erosions. The intestinal disease was refractory to standard immunosuppressive, immunomodulatory and biological therapy, but strikingly, subtotal colectomy effectively induced clinical remission in the patient without the need for maintenance therapy. There have been reports of patients with similar duplications for whom no intestinal disease was reported. Together, this argues that the hyperproliferative $CD4^+$ T-cell response that we observed in the context of *IL2RA* duplication is not enough to cause intestinal disease. Instead, considering the observation that colectomy, resulting in a reduced bacterial load, effectuated disease cure, an environmental factor such as a specific microbial antigen may have initiated colonic disease in this particular patient. Although the plasma of the patient did not show elevated levels of anti-flagellin IgG compared to age-matched controls, anti-microbial antibodies with a wide range of specificity were detectable in the patient's plasma, most particularly to whole cell extracts from bacteria belonging to the *Prevotalla* genus. Moreover, the affected colonic tissue contained many T-bet^+, IFNγ^+ and IL-21^+ cells, but only very few IL-17^+ cells, which is in agreement with the increased levels of TCR-induced IFNγ in the presence of IL-2 observed *in vitro* (**Chapter 6**). Thus, these data demonstrate that, although defects causing overall T-cell hyperproliferation and inflammation contribute to disease susceptibility, an additional trigger was needed to license the expansion of inflammatory $CD4^+$ T cells activated in the antigen-rich intestinal environment.

Conclusion and future challenges: Targeting treatment to IBD immune subclasses.

Traditionally, medical treatment for IBD was limited to non-biological therapies, including aminosalicylates, corticosteroids, immunosuppressive agents (azathioprine, mercaptopurine and methotrexate), and in the pediatric population, exclusive enteral nutrition. However, the increased understanding of the immunopathogenesis of IBD has led to the development of new treatment options, including anti-TNFα antibodies, which have dramatically influenced treatment outcomes in IBD.[108, 109] Unfortunately, these agents are not effective in all patients, and patients who initially respond to anti-TNFα antibodies can lose responsiveness over time. Recently, antibodies that target

inflammatory cytokines (i.e. ustekiumab[110]) and that impede immune cell homing (i.e. vedolizumab[111]) have been approved for IBD. In addition, other promising targets for new therapeutic strategies are currently under investigation, including small molecule JAK/STAT signaling pathway inhibitors[112] and a sphingosine-1-phosphate receptor-1 selective agonist[113].The question now arises, what drug should be used in which patient? As these novel treatment options have different mechanisms of action, it is crucial to select patients subgroups who are most likely to respond to these targeted therapies. As outlined above, in order to achieve this, in-depth patient characterization can identify patients with similar immune pathways involved in their disease pathogenesis. In line with this idea, current clinical trials are becoming increasingly selective in their patient inclusion with respect to disease characteristics. In addition, prior to initiating treatment monitoring patient's cellular response to a panel of small molecules and therapeutic antibodies may reveal which targeted therapies should be used preferentially.

Interestingly, IBD is strongly associated with other immune-mediated inflammatory diseases (IMIDs).[114-116] Recently, a large population-based study demonstrated that nearly one-in-four patients with IBD had a concomitant IMID.[114] Why do certain patients develop more than one IMID? Generally speaking, IMIDs are characterized by inappropriately regulated immune responses and ongoing tissue damage, which promotes a vicious cycle leading to chronic tissue-localized disease. A key feature shared by IMIDs is an inflammatory T-cell response drives disease chronicity. As such, similar disease-causing mechanisms and common therapeutic targets could be present in different IMIDs. In line with this idea, a large number of IBD-associated gene loci have been implicated in other IMIDs.[117] Uncovering the shared disease-driving mechanisms between IMIDs could create a new taxonomy of IMIDs that moves away from a traditional organ-based approach towards a mechanism-based pathway-driven classification. Taking into account that subgroups of patients with various IMIDs can benefit from the same drug, experimental therapeutics could be screened across multiple patient populations in an early-phase of development.

Recent data indicates that there is a distinct and relatively rare type of $CD4^+$ T lymphocytes that is common to multiple IMIDs.[118] When compared to healthy individuals, patients with celiac disease, systemic lupus erythematosus and systemic sclerosis had elevated numbers of $CD4^+$ T cells with a $CD45RA^{neg}CD62L^{neg}CD25^{neg}CD38^+$ phenotype[118], which is reminiscent of the circulating $CD38^+$ effector T-cell population that we characterize in-detail in this thesis. Previously, we have shown that almost all of the gluten-specific T cells in peripheral blood of patients with celiac disease a $CD38^+$ effector T-cell phenotype.[15] This thesis adds to this data by demonstrating that changes in regulatory versus inflammatory cell composition of $CD38^+$ effector T cells can be detected in peripheral blood of IBD patients. Hence, it is of interest to investigate whether these cells might also be the key disease-driving T cells in other IMIDs. Detailed analyses (including flow cytometry,

mass cytometry, TCR sequencing, transcriptome analyses and functional analyses) of circulating CD38^{+} effector T cells across multiple patient populations with different IMIDs could aid in dissecting the complex pathogenesis of these diseases, with the ultimate aim to achieve better treatment strategies to induce long lasting disease remission. Data for these analyses are currently being collected in the national, multicenter TIMID project (new taxonomy and treatment strategies for T cell driven Immune Mediated Inflammatory Diseases).

REFERENCES

1. Louis E, Van Kemseke C, Reenaers C. Necessity of phenotypic classification of inflammatory bowel disease. Best Pract Res Clin Gastroenterol 2011;25 Suppl 1:S2-7.
2. Satsangi J, Silverberg MS, Vermeire S, et al. The Montreal classification of inflammatory bowel disease: controversies, consensus, and implications. Gut 2006;55:749-753.
3. Levine A, Griffiths A, Markowitz J, et al. Pediatric Modification of the Montreal Classification for Inflammatory Bowel Disease: The Paris Classification. Inflammatory Bowel Diseases 2011;17:1314-1321.
4. Magro F, Langner C, Driessen A, et al. European consensus on the histopathology of inflammatory bowel disease. J Crohns Colitis 2013;7:827-51.
5. Masclee A, Jonkers D, Wintjens D, et al. OP002 Assessment of disease activity patterns during the first 10 years after diagnosis in a population-based Crohn's disease cohort shows a quiescent disease course for a substantial proportion of the population. Journal of Crohn's and Colitis 2018;12:S001-S003.
6. Peyrin-Biroulet L, Panes J, Sandborn WJ, et al. Defining Disease Severity in Inflammatory Bowel Diseases: Current and Future Directions. Clin Gastroenterol Hepatol 2016;14:348-354 e17.
7. Yarur AJ, Strobel SG, Deshpande AR, et al. Predictors of aggressive inflammatory bowel disease. Gastroenterol Hepatol (N Y) 2011;7:652-9.
8. Jostins L, Ripke S, Weersma RK, et al. Host-microbe interactions have shaped the genetic architecture of inflammatory bowel disease. Nature 2012;491:119-24.
9. Cho JH. The genetics and immunopathogenesis of inflammatory bowel disease. Nat Rev Immunol 2008;8:458-66.
10. Uhlig HH. Monogenic diseases associated with intestinal inflammation: implications for the understanding of inflammatory bowel disease. Gut 2013;62:1795-805.
11. Bennett CL, Christie J, Ramsdell F, et al. The immune dysregulation, polyendocrinopathy, enteropathy, X-linked syndrome (IPEX) is caused by mutations of Foxp3. Nat Genet 2001;27:20-1.
12. Glocker EO, Kotlarz D, Boztug K, et al. Inflammatory bowel disease and mutations affecting the interleukin-10 receptor. N Engl J Med 2009;361:2033-45.
13. Zenlea T, Peppercorn MA. Immunosuppressive therapies for inflammatory bowel disease. World J Gastroenterol 2014;20:3146-52.
14. Maynard CL, Weaver CT. Intestinal effector T cells in health and disease. Immunity 2009;31:389-400.
15. du Pre MF, van Berkel LA, Raki M, et al. 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;106:1147-59.
16. van Leeuwen MA, du Pre MF, van Wanrooij RL, et al. Changes in natural Foxp3(+)Treg but not mucosally-imprinted CD62L(neg)CD38(+)Foxp3(+)Treg in the circulation of celiac disease patients. PLoS One 2013;8:e68432.
17. Maul J, Loddenkemper C, Mundt P, et al. Peripheral and intestinal regulatory CD4+ CD25(high) T cells in inflammatory bowel disease. Gastroenterology 2005;128:1868-78.
18. Ban H, Andoh A, Shioya M, et al. Increased number of Foxp3+CD4+ regulatory T cells in inflammatory bowel disease. Mol Med Rep 2008;1:647-50.
19. Li Z, Vermeire S, Bullens D, et al. Restoration of Foxp3+ Regulatory T-cell Subsets and Foxp3- Type 1 Regulatory-like T Cells in Inflammatory Bowel Diseases During Anti-tumor Necrosis Factor Therapy. Inflamm Bowel Dis 2015;21:2418-28.
20. Veltkamp C, Anstaett M, Wahl K, et al. Apoptosis of regulatory T lymphocytes is increased in chronic inflammatory bowel disease and reversed by anti-TNFalpha treatment. Gut 2011;60:1345-53.
21. Guidi L, Felice C, Procoli A, et al. Foxp3(+) T regulatory cell modifications in inflammatory bowel disease patients treated with anti-TNFalpha agents. Biomed Res Int 2013;2013:286368.
22. La Scaleia R, Morrone S, Stoppacciaro A, et al. Peripheral and intestinal CD4+ T cells with a regulatory phenotype in pediatric patients with inflammatory bowel disease. J Pediatr Gastroenterol Nutr 2010;51:563-72.
23. Li Z, Arijs I, De Hertogh G, et al. Reciprocal changes of Foxp3 expression in blood and intestinal mucosa in IBD patients responding to infliximab. Inflamm Bowel Dis 2010;16:1299-310.
24. Di Sabatino A, Biancheri P, Piconese S, et al. Peripheral regulatory T cells and serum transforming growth factor-beta: relationship with clinical response to infliximab in Crohn's disease. Inflamm Bowel Dis 2010;16:1891-7.

25. Boschetti G, Nancey S, Sardi F, et al. Therapy with anti-TNFalpha antibody enhances number and function of Foxp3(+) regulatory T cells in inflammatory bowel diseases. Inflamm Bowel Dis 2011;17:160-70.
26. Grundstrom J, Linton L, Thunberg S, et al. Altered immunoregulatory profile during anti-tumour necrosis factor treatment of patients with inflammatory bowel disease. Clin Exp Immunol 2012;169:137-47.
27. Yu X, Harden K, Gonzalez LC, et al. The surface protein TIGIT suppresses T cell activation by promoting the generation of mature immunoregulatory dendritic cells. Nat Immunol 2009;10:48-57.
28. Joosse ME, Menckeberg CL, de Ruiter LF, et al. Frequencies of circulating regulatory TIGIT(+)CD38(+) effector T cells correlate with the course of inflammatory bowel disease. Mucosal Immunol 2019;12:154-163.
29. Burton BR, Britton GJ, Fang H, et al. Sequential transcriptional changes dictate safe and effective antigen-specific immunotherapy. Nat Commun 2014;5:4741.
30. Lozano E, Dominguez-Villar M, Kuchroo V, et al. The TIGIT/CD226 axis regulates human T cell function. J Immunol 2012;188:3869-75.
31. Brockmann L, Soukou S, Steglich B, et al. Molecular and functional heterogeneity of IL-10-producing CD4(+) T cells. Nat Commun 2018;9:5457.
32. Joller N, Hafler JP, Brynedal B, et al. Cutting edge: TIGIT has T cell-intrinsic inhibitory functions. J Immunol 2011;186:1338-42.
33. Anderson AC, Joller N, Kuchroo VK. Lag-3, Tim-3, and TIGIT: Co-inhibitory Receptors with Specialized Functions in Immune Regulation. Immunity 2016;44:989-1004.
34. Manieri NA, Chiang EY, Grogan JL. TIGIT: A Key Inhibitor of the Cancer Immunity Cycle. Trends Immunol 2017;38:20-28.
35. Chihara N, Madi A, Kondo T, et al. Induction and transcriptional regulation of the co-inhibitory gene module in T cells. Nature 2018;558:454-459.
36. Yan J, Zhao Q, Gabrusiewicz K, et al. FGL2 promotes tumor progression in the CNS by suppressing CD103(+) dendritic cell differentiation. Nat Commun 2019;10:448.
37. Kapturczak MH, Meier-Kriesche HU, Kaplan B. Pharmacology of calcineurin antagonists. Transplant Proc 2004;36:25S-32S.
38. Smith-Garvin JE, Koretzky GA, Jordan MS. T cell activation. Annu Rev Immunol 2009;27:591-619.
39. Gaud G, Lesourne R, Love PE. Regulatory mechanisms in T cell receptor signalling. Nat Rev Immunol 2018.
40. Brignall R, Cauchy P, Bevington SL, et al. Integration of Kinase and Calcium Signaling at the Level of Chromatin Underlies Inducible Gene Activation in T Cells. J Immunol 2017;199:2652-2667.
41. Martinez GJ, Pereira RM, Aijo T, et al. The transcription factor NFAT promotes exhaustion of activated CD8(+) T cells. Immunity 2015;42:265-278.
42. Kamphorst AO, Wieland A, Nasti T, et al. Rescue of exhausted CD8 T cells by PD-1-targeted therapies is CD28-dependent. Science 2017;355:1423-1427.
43. Iwasaki A, Kelsall BL. Freshly isolated Peyer's patch, but not spleen, dendritic cells produce interleukin 10 and induce the differentiation of T helper type 2 cells. J Exp Med 1999;190:229-39.
44. Lutz MB, Schuler G. Immature, semi-mature and fully mature dendritic cells: which signals induce tolerance or immunity? Trends Immunol 2002;23:445-9.
45. Bain CC, Mowat AM. Macrophages in intestinal homeostasis and inflammation. Immunol Rev 2014;260:102-17.
46. Zhao YJ, Zhu WJ, Wang XW, et al. Determinants of the membrane orientation of a calcium signaling enzyme CD38. Biochim Biophys Acta 2015;1853:2095-103.
47. Rah SY, Kim UH. CD38-mediated Ca(2+) signaling contributes to glucagon-induced hepatic gluconeogenesis. Sci Rep 2015;5:10741.
48. Schneider M, Schumacher V, Lischke T, et al. CD38 is expressed on inflammatory cells of the intestine and promotes intestinal inflammation. PLoS One 2015;10:e0126007.
49. Deaglio S, Mallone R, Baj G, et al. Human CD38 and its ligand CD31 define a unique lamina propria T lymphocyte signaling pathway. Faseb J 2001;15:580-2.
50. Lord JD, Shows DM, Chen J, et al. Human Blood and Mucosal Regulatory T Cells Express Activation Markers and Inhibitory Receptors in Inflammatory Bowel Disease. PLoS One 2015;10:e0136485.
51. Cong Y, Brandwein SL, McCabe RP, et al. CD4+ T cells reactive to enteric bacterial antigens in spontaneously colitic C3H/HeJBir mice: increased T helper cell type 1 response and ability to transfer disease. J Exp Med 1998;187:855-64.
52. Aranda R, Sydora BC, McAllister PL, et al. Analysis of intestinal lymphocytes in mouse colitis mediated by transfer of CD4+, CD45RBhigh T cells to SCID recipients. J Immunol 1997;158:3464-73.

53. Sellon RK, Tonkonogy S, Schultz M, et al. Resident enteric bacteria are necessary for development of spontaneous colitis and immune system activation in interleukin-10-deficient mice. Infect Immun 1998;66:5224-31.
54. Rath HC, Herfarth HH, Ikeda JS, et al. Normal luminal bacteria, especially Bacteroides species, mediate chronic colitis, gastritis, and arthritis in HLA-B27/human beta2 microglobulin transgenic rats. J Clin Invest 1996;98:945-53.
55. Kullberg MC, Ward JM, Gorelick PL, et al. Helicobacter hepaticus triggers colitis in specific-pathogen-free interleukin-10 (IL-10)-deficient mice through an IL-12- and gamma interferon-dependent mechanism. Infect Immun 1998;66:5157-66.
56. Balish E, Warner T. Enterococcus faecalis induces inflammatory bowel disease in interleukin-10 knockout mice. Am J Pathol 2002;160:2253-7.
57. Winstanley C, Morgan JA. The bacterial flagellin gene as a biomarker for detection, population genetics and epidemiological analysis. Microbiology 1997;143 (Pt 10):3071-84.
58. Rumbo M, Nempont C, Kraehenbuhl JP, et al. Mucosal interplay among commensal and pathogenic bacteria: lessons from flagellin and Toll-like receptor 5. FEBS Lett 2006;580:2976-84.
59. Samatey FA, Imada K, Nagashima S, et al. Structure of the bacterial flagellar protofilament and implications for a switch for supercoiling. Nature 2001;410:331-7.
60. Yonekura K, Maki-Yonekura S, Namba K. Complete atomic model of the bacterial flagellar filament by electron cryomicroscopy. Nature 2003;424:643-50.
61. Maki-Yonekura S, Yonekura K, Namba K. Conformational change of flagellin for polymorphic supercoiling of the flagellar filament. Nat Struct Mol Biol 2010;17:417-22.
62. Beatson SA, Minamino T, Pallen MJ. Variation in bacterial flagellins: from sequence to structure. Trends Microbiol 2006;14:151-5.
63. Hayashi F, Smith KD, Ozinsky A, et al. The innate immune response to bacterial flagellin is mediated by Toll-like receptor 5. Nature 2001;410:1099-103.
64. Skountzou I, Martin Mdel P, Wang B, et al. Salmonella flagellins are potent adjuvants for intranasally administered whole inactivated influenza vaccine. Vaccine 2010;28:4103-12.
65. Liu G, Tarbet B, Song L, et al. Immunogenicity and efficacy of flagellin-fused vaccine candidates targeting 2009 pandemic H1N1 influenza in mice. PLoS One 2011;6:e20928.
66. de Zoete MR, Keestra AM, Wagenaar JA, et al. Reconstitution of a functional Toll-like receptor 5 binding site in Campylobacter jejuni flagellin. J Biol Chem 2010;285:12149-58.
67. Alexander KL, Targan SR, Elson CO, 3rd. Microbiota activation and regulation of innate and adaptive immunity. Immunol Rev 2014;260:206-20.
68. Gewirtz AT, Navas TA, Lyons S, et al. Cutting edge: bacterial flagellin activates basolaterally expressed TLR5 to induce epithelial proinflammatory gene expression. J Immunol 2001;167:1882-5.
69. Gewirtz AT, Simon PO, Jr., Schmitt CK, et al. Salmonella typhimurium translocates flagellin across intestinal epithelia, inducing a proinflammatory response. J Clin Invest 2001;107:99-109.
70. Rhee SH, Im E, Riegler M, et al. Pathophysiological role of Toll-like receptor 5 engagement by bacterial flagellin in colonic inflammation. Proc Natl Acad Sci U S A 2005;102:13610-5.
71. Christmann BS, Abrahamsson TR, Bernstein CN, et al. Human seroreactivity to gut microbiota antigens. J Allergy Clin Immunol 2015;136:1378-86 e1-5.
72. Haas A, Zimmermann K, Graw F, et al. Systemic antibody responses to gut commensal bacteria during chronic HIV-1 infection. Gut 2011;60:1506-19.
73. Lodes MJ, Cong Y, Elson CO, et al. Bacterial flagellin is a dominant antigen in Crohn disease. J Clin Invest 2004;113:1296-306.
74. Landers CJ, Cohavy O, Misra R, et al. Selected loss of tolerance evidenced by Crohn's disease-associated immune responses to auto- and microbial antigens. Gastroenterology 2002;123:689-99.
75. Shen C, Landers CJ, Derkowski C, et al. Enhanced CBir1-specific innate and adaptive immune responses in Crohn's disease. Inflamm Bowel Dis 2008;14:1641-51.
76. Targan SR, Landers CJ, Yang H, et al. Antibodies to CBir1 flagellin define a unique response that is associated independently with complicated Crohn's disease. Gastroenterology 2005;128:2020-8.
77. Mitsuyama K, Niwa M, Takedatsu H, et al. Antibody markers in the diagnosis of inflammatory bowel disease. World J Gastroenterol 2016;22:1304-10.
78. Macpherson A, Khoo UY, Forgacs I, et al. Mucosal antibodies in inflammatory bowel disease are directed against intestinal bacteria. Gut 1996;38:365-75.

79. Brandwein SL, McCabe RP, Cong Y, et al. Spontaneously colitic C3H/HeJBir mice demonstrate selective antibody reactivity to antigens of the enteric bacterial flora. J Immunol 1997;159:44-52.
80. Sanders CJ, Yu Y, Moore DA, 3rd, et al. Humoral immune response to flagellin requires T cells and activation of innate immunity. J Immunol 2006;177:2810-8.
81. Gewirtz AT. TLRs in the Gut. III. Immune responses to flagellin in Crohn's disease: good, bad, or irrelevant? Am J Physiol Gastrointest Liver Physiol 2007;292:G706-10.
82. Duck LW, Walter MR, Novak J, et al. Isolation of flagellated bacteria implicated in Crohn's disease. Inflamm Bowel Dis 2007;13:1191-201.
83. Bacher P, Scheffold A. Flow-cytometric analysis of rare antigen-specific T cells. Cytometry A 2013;83:692-701.
84. Macpherson AJ, Gatto D, Sainsbury E, et al. A primitive T cell-independent mechanism of intestinal mucosal IgA responses to commensal bacteria. Science 2000;288:2222-6.
85. Devlin SM, Yang H, Ippoliti A, et al. NOD2 variants and antibody response to microbial antigens in Crohn's disease patients and their unaffected relatives. Gastroenterology 2007;132:576-86.
86. Konrad A, Cong Y, Duck W, et al. Tight mucosal compartmentation of the murine immune response to antigens of the enteric microbiota. Gastroenterology 2006;130:2050-9.
87. Papadakis KA, Prehn J, Moreno ST, et al. CCR9-positive lymphocytes and thymus-expressed chemokine distinguish small bowel from colonic Crohn's disease. Gastroenterology 2001;121:246-54.
88. Hand TW, Dos Santos LM, Bouladoux N, et al. Acute gastrointestinal infection induces long-lived microbiota-specific T cell responses. Science 2012;337:1553-6.
89. Hegazy AN, West NR, Stubbington MJT, et al. Circulating and Tissue-Resident CD4(+) T Cells With Reactivity to Intestinal Microbiota Are Abundant in Healthy Individuals and Function Is Altered During Inflammation. Gastroenterology 2017;153:1320-1337 e16.
90. Calderon-Gomez E, Bassolas-Molina H, Mora-Buch R, et al. Commensal-Specific CD4(+) Cells From Patients With Crohn's Disease Have a T-Helper 17 Inflammatory Profile. Gastroenterology 2016;151:489-500.e3.
91. Song X, Dai D, He X, et al. Growth Factor FGF2 Cooperates with Interleukin-17 to Repair Intestinal Epithelial Damage. Immunity 2015;43:488-501.
92. Kinugasa T, Sakaguchi T, Gu X, et al. Claudins regulate the intestinal barrier in response to immune mediators. Gastroenterology 2000;118:1001-11.
93. Cong Y, Feng T, Fujihashi K, et al. A dominant, coordinated T regulatory cell-IgA response to the intestinal microbiota. Proc Natl Acad Sci U S A 2009;106:19256-61.
94. Scott BB, Goodall A, Stephenson P, et al. Rectal mucosal plasma cells in inflammatory bowel disease. Gut 1983;24:519-24.
95. Scott MG, Nahm MH, Macke K, et al. Spontaneous secretion of IgG subclasses by intestinal mononuclear cells: differences between ulcerative colitis, Crohn's disease, and controls. Clin Exp Immunol 1986;66:209-15.
96. Ruthlein J, Ibe M, Burghardt W, et al. Immunoglobulin G (IgG), IgG1, and IgG2 determinations from endoscopic biopsy specimens in control, Crohn's disease, and ulcerative colitis subjects. Gut 1992;33:507-12.
97. Rosekrans PC, Meijer CJ, van der Wal AM, et al. Immunoglobulin containing cells in inflammatory bowel disease of the colon: a morphometric and immunohistochemical study. Gut 1980;21:941-7.
98. Baklien K, Brandtzaeg P. Comparative mapping of the local distribution of immunoglobulin-containing cells in ulcerative colitis and Crohn's disease of the colon. Clin Exp Immunol 1975;22:197-209.
99. Brandtzaeg P, Baklien K, Fausa O, et al. Immunohistochemical characterization of local immunoglobulin formation in ulcerative colitis. Gastroenterology 1974;66:1123-36.
100. Nowak A, Lock D, Bacher P, et al. CD137+CD154- Expression As a Regulatory T Cell (Treg)-Specific Activation Signature for Identification and Sorting of Stable Human Tregs from In Vitro Expansion Cultures. Front Immunol 2018;9:199.
101. Savilahti E, Viander M, Perkkio M, et al. IgA antigliadin antibodies: a marker of mucosal damage in childhood coeliac disease. Lancet 1983;1:320-2.
102. Kilander AF, Dotevall G, Fallstrom SP, et al. Evaluation of gliadin antibodies for detection of coeliac disease. Scand J Gastroenterol 1983;18:377-83.
103. Koninckx CR, Giliams JP, Polanco I, et al. IgA antigliadin antibodies in celiac and inflammatory bowel disease. J Pediatr Gastroenterol Nutr 1984;3:676-82.
104. Lee JY, Wasinger VC, Yau YY, et al. Molecular Pathophysiology of Epithelial Barrier Dysfunction in Inflammatory Bowel Diseases. Proteomes 2018;6.
105. Odenwald MA, Turner JR. The intestinal epithelial barrier: a therapeutic target? Nat Rev Gastroenterol Hepatol 2017;14:9-21.

106. Chung Y, Chang SH, Martinez GJ, et al. Critical regulation of early Th17 cell differentiation by interleukin-1 signaling. Immunity 2009;30:576-87.
107. Castro-Dopico T, Dennison TW, Ferdinand JR, et al. Anti-commensal IgG Drives Intestinal Inflammation and Type 17 Immunity in Ulcerative Colitis. Immunity 2019;50:1099-1114.e10.
108. Hyams J, Crandall W, Kugathasan S, et al. Induction and maintenance infliximab therapy for the treatment of moderate-to-severe Crohn's disease in children. Gastroenterology 2007;132:863-73; quiz 1165-6.
109. Crandall W, Hyams J, Kugathasan S, et al. Infliximab therapy in children with concurrent perianal Crohn disease: observations from REACH. J Pediatr Gastroenterol Nutr 2009;49:183-90.
110. Feagan BG, Sandborn WJ, Gasink C, et al. Ustekinumab as Induction and Maintenance Therapy for Crohn's Disease. N Engl J Med 2016;375:1946-1960.
111. Feagan BG, Greenberg GR, Wild G, et al. Treatment of ulcerative colitis with a humanized antibody to the alpha4beta7 integrin. N Engl J Med 2005;352:2499-507.
112. Panes J, Sandborn WJ, Schreiber S, et al. Tofacitinib for induction and maintenance therapy of Crohn's disease: results of two phase IIb randomised placebo-controlled trials. Gut 2017;66:1049-1059.
113. Danese S, Furfaro F, Vetrano S. Targeting S1P in Inflammatory Bowel Disease: New Avenues for Modulating Intestinal Leukocyte Migration. J Crohns Colitis 2018;12:S678-s686.
114. Burisch J, Jess T, Egeberg A. Incidence of Immune-mediated Inflammatory Diseases Among Patients With Inflammatory Bowel Diseases in Denmark. Clin Gastroenterol Hepatol 2019.
115. Halling ML, Kjeldsen J, Knudsen T, et al. Patients with inflammatory bowel disease have increased risk of autoimmune and inflammatory diseases. World J Gastroenterol 2017;23:6137-6146.
116. Bar Yehuda S, Axlerod R, Toker O, et al. The Association of Inflammatory Bowel Diseases with Autoimmune Disorders: A Report from the epi-IIRN. J Crohns Colitis 2019;13:324-329.
117. Van Limbergen J, Radford-Smith G, Satsangi J. Advances in IBD genetics. Nat Rev Gastroenterol Hepatol 2014;11:372-85.
118. Christophersen A, Lund EG, Snir O, et al. Distinct phenotype of CD4(+) T cells driving celiac disease identified in multiple autoimmune conditions. Nat Med 2019.

Appendices

English Summary

The intestine is continuously exposed to harmless antigens from the diet and commensal bacteria, but also provides harmful pathogens access to the body. Consequently, the intestinal immune system continuously tailors regulatory and inflammatory responses to maximize host defense without unnecessary tissue damage. The balance between regulatory and inflammatory intestinal immune responses is known as intestinal homeostasis.

The immune system in the intestine.

Up to 60% of lymphocytes residing in the intestinal tissue are antigen-experienced $CD4^+$ T cells that have the unique ability to exert memory to previously encountered antigens. Anatomically, $CD4^+$ T cells are located within both inductive and effector sites of the intestine. The gut-draining lymph nodes and gut-associated lymphoid tissue (GALT) are "inductive sites", the main location for priming naive T- and B-cell responses (cells that have not yet encountered cognate antigen within the periphery). The mucosal epithelium and underlying lamina propria are the "effector sites" of the intestinal immune system, which harbor large populations of antigen-experienced $CD4^+$ T cells and antibody-secreting plasma cells.

Antigen presenting cells (APCs) migrate from the lamina propria to inductive sites in order to present intestinal antigen-derived peptides to naive $CD4^+$ T cells. Naive $CD4^+$ T cells migrate from the peripheral blood into the gut-draining lymph nodes and GALT using the lymphoid tissue homing receptors CD62L and chemokine receptor C-C motif receptor 7 (CCR7). T-cell responses are initiated when a naive $CD4^+$ T cells encounter APCs expressing the appropriate peptide-MHC-complex. Activation, proliferation and differentiation of naive $CD4^+$ T cells is dependent on three APC-derived signals: the interaction of the T-cell receptor (TCR) with a specific peptide-MHC complex (signal 1), costimulation (signal 2) and soluble cytokines (signal 3). In addition, signals from the APC and microenvironment in the lymph nodes and GALT drive the acquisition of T-cell specific integrins and chemokine receptors required for preferential T-cell homing towards the intestine. Consequently, after recognition of cognate antigen, antigen-experienced $CD4^+$ T cells exit the lymph node, enter the blood and migrate to the intestinal lamina propria or enter the epithelial layer where they reside as long-lived antigen-experienced $CD4^+$ T cells.

Intestinal $CD4^+$ T cells.

Both regulatory and inflammatory $CD4^+$ T cells differentiate from naive $CD4^+$ T cells upon antigen encounter by intestinal APCs in the GALT and intestinal draining lymph nodes. During intestinal homeostasis, environmental factors in the intestine alter the functional properties of intestinal APCs, maintaining a tolerogenic state in the intestine by skewing $CD4^+$ T-cell differentiation in favor of a regulatory phenotype. In consequence, harmless exogenous antigens elicit a balanced intestinal $CD4^+$ T-cell response in which regulatory cell populations counteract inflammatory cells.

Regulatory T cells

Regulatory $CD4^+$ T cells are defined by their functional capacity to suppress an inflammatory T-cell response. Regulatory $CD4^+$ T cells in the intestine can be subdivided into $CD4^+Foxp3^+$ regulatory T cells (Tregs) and IL-10-producing $CD4^+Foxp3^{neg}$ T cells. Tregs and IL-10-producing $CD4^+Foxp3^{neg}$ T cells exert regulatory function in different intestinal compartments, with a prominent role for $Foxp3^+$ Treg cells in the colon and $Foxp3^{neg}CD4^+$ T cells in the small intestine. Both populations use multiple mechanisms to suppress inflammatory immune responses, the best characterized of which involves IL-10 production, which is essential to restrain local inflammation in the intestine.

Inflammatory T cells

In addition to regulatory $CD4^+$ T-cell subsets, the lamina propria harbors diverse populations of pro-inflammatory effector $CD4^+$ T cells. There are different subgroups of inflammatory effector $CD4^+$ T cells, denoted as $CD4^+$ T helper (Th) cells, including Th1, Th2 and Th17. Each Th cell subset is associated with predominance of specific effector cytokines. Although inflammatory effector $CD4^+$ T cells are critically required for elimination of invasive mucosal pathogens and controlling resident commensal microbiota, infiltration of the lamina propria by inflammatory $CD4^+$ T-cell populations is a key characteristic of chronic intestinal inflammation, as seen in inflammatory bowel disease (IBD).

Inflammatory bowel disease.

Uncontrolled inflammatory $CD4^+$ T-cell responses to commensal bacteria, as seen in patients with inflammatory bowel diseases (IBD), can result in tissue damage and ensuing chronic intestinal inflammation. As a group, IBD is a heterogeneous disease and varies in terms of clinical symptoms, location of intestinal inflammation, disease extent and severity, presence of extra-intestinal manifestations and response to therapy. The two most prevalent clinical forms of IBD are Crohn's disease (CD) and ulcerative colitis (UC). Although the precise etiology may differ per patient, the current theory is that IBD is caused by a dysregulated immune response to antigens of the intestinal bacteria in a genetically susceptible host. Evidence that dysfunction of the immune system is involved for the development of IBD has become stronger over the past decade. First of all, genome-wide association studies (GWAS) have identified IBD-associated susceptibility loci containing genes involved in immune pathways. In addition, several genetic deficiencies in immune genes have been shown to cause IBD-like intestinal inflammation. These monogenic disorders have illustrated that both insufficient host defense (i.e. chronic

granulomatous disease) as well as insufficient immune regulation (i.e. IPEX syndrome) can result in dysregulated immune responses to bacterial antigens. Lastly, the success of immunosuppressive therapies to decrease intestinal disease further emphasizes the important role of dysfunctional immune responses in IBD. Together, this argues that classification on the basis of immune dysfunction could identify subgroups of IBD patients with similar immune pathways involved in their disease pathogenesis.

This thesis.

The aim of this thesis was to identify immune regulatory processes that are pivotal for intestinal homeostasis in order to yield parameters that classify immunological disease in patients with IBD. In addition, by combining immunological disease profiling with extensive clinical characterization of each patient, the research presented in this thesis aimed to identify which clinical and immunological factors can be used to predict disease course and response to therapy.

As outlined in **Chapter 1**, $CD4^+$ T cells play a key role in the pathogenesis of IBD. The intestinal pathology is characterized by infiltration of $CD4^+$ T cells that secrete large amounts of pro-inflammatory cytokines. Inhibitory receptors serve as checkpoints to these cells controlling their T-cell receptor (TCR)- and CD28-mediated activation and modulating the phenotype of neighboring APCs. In **Chapter 2**, we highlight the emerging insights in the role of inhibitory receptors in intestinal homeostasis. In the intestine, inhibitory receptors are expressed by both $CD4^+Foxp3^+$ Tregs, which express high levels of cytotoxic T lymphocyte antigen 4 (CTLA-4), as well as IL-10-producing $CD4^+Foxp3^{neg}$ T cells, which are characterized by high expression of programmed death 1 (PD-1) and lymphocyte activation gene-3 (Lag-3). Our review describes the diverse range of inhibitory receptors, expressed by both $CD4^+Foxp3^+$ Tregs and $CD4^+Foxp3^{neg}$ T cells, and their role in intestinal homeostasis.

Monitoring loss of balance between inflammatory and regulatory intestinal $CD4^+$ T-cell responses in IBD patients is highly desired to classify patients and predict their disease course. In **Chapter 3**, in order to investigate whether changes in regulatory and inflammatory $CD4^+$ T-cell responses would enable IBD patient stratification prior to treatment, we focused our analyses on circulating $CD38^+$ effector T cells, which are known to be strongly enriched in gut-homing $CD4^+$ T cells. We show that, in healthy individuals as well as IBD patients, the circulating $CD38^+$ effector T-cell population contained both regulatory and inflammatory cell populations. In healthy individuals, who have a balanced mucosal tolerance to commensal microbiota, $CD38^+$ effector T cells had a bias towards regulation with low frequencies of activated $CD25^+CD45RA^{neg}$ and high frequencies of inhibitory receptor T cell Ig and ITIM domain $(TIGIT)^+$ cells. In IBD patients, the balance between activated inflammatory and regulatory $CD4^+$ T-cell populations was shifted towards

inflammation, as treatment-naive pediatric IBD patients with active intestinal inflammation had increased frequencies of activated $CD25^+CD45RA^{neg}$ and reduced frequencies of $TIGIT^+$ cells when compared to age-matched healthy controls. No differences in the frequencies of $Foxp3^+$ cells in $CD38^+$ effector T cells were detected between IBD patients and controls. Importantly, the percentage of inhibitory receptor $TIGIT^+$ cells in the $CD38^+$ effector T-cell population at disease diagnosis differentiated patients with reduced duration of clinical remission during follow-up. Specifically, TIGIT percentages below 25% before treatment associated with shorter duration of clinical remission. These results show that phenotypic characterization of circulating $CD4^+$ T-cell populations can stratify treatment-naive IBD patients into clinically relevant subgroups. In particular, analysis of multiple inhibitory receptors, including TIGIT, on $CD38^+$ effector T cells may further facilitate IBD patient stratification prior to treatment.

As $TIGIT^+$ cell frequencies were reduced in a subgroup of IBD patients, we aimed to identify factors involved in the induction of TIGIT expression on $CD4^+$ T cells. In **Chapter 4**, using human and murine experimental approaches, we studied TIGIT expression in various $CD4^+$ T-cell subpopulations and determined the stimulatory signals required for TIGIT expression. We show that TCR ligation through anti-CD3 induced TIGIT expression preferably on $CD4^+$ T cells with an antigen-experienced phenotype. Given the reduced $TIGIT^+$ cell frequencies in a subgroup of patients with IBD, we hypothesized that TIGIT expression on antigen-experienced $CD4^+$ T cells may be involved in regulating immune responses to intestinal bacteria. Conventionalization of germ-free mice with specific pathogen free microbiota resulted in a two- to three-fold increase in intestinal TIGIT expression. As a large proportion of intestinal T cells is expected to have specificity to microbial antigen, we investigated whether TIGIT was expressed by microbiota-reactive $CD4^+$ T cells. Although the majority bacterial antigen-reactive $CD4^+$ T cells did not express TIGIT, a clear population of approximately 7%-10% $TIGIT^+$ cells could be identified among bacterial antigen-reactive $CD4^+$ T cells. Bacterial antigen-reactive $TIGIT^+CD4^+$ T cells contained higher frequencies of $IL\text{-}10^+$ cells compared to bacterial antigen-reactive $TIGIT^{neg}CD4^+$ cells, suggesting that TIGIT-expressing $CD4^+$ T-cell populations may be involved in regulating immune responses to intestinal bacteria.

Interestingly, the strong association between TIGIT and CD38 is a recurring observation in all our *in vitro* and *in vivo* data reported in **Chapter 3** and **Chapter 4**. CD38 is an ectoenzyme that catalyzes the synthesis of two structurally distinct messengers for intracellular Ca^{2+} mobilization. Using the calcineurin inhibitor tacrolimus and calcium ionophore ionomycin, we show that calcium dependent signaling after TCR ligation was required to induce TIGIT expression on antigen-experienced $CD4^+$ T cells. Future experiments are needed to establish whether CD38 contributes to TIGIT induction on $CD4^+$ T cells through increasing the intracellular Ca^{2+} concentration.

Elevated humoral responses to bacterial flagellin, a bacterial protein expressed by both commensal and pathogenic bacteria, are present in a subgroup of CD patients at risk for aggressive and complicated disease. As antibody responses rely on the activation of $CD4^+$ T cells, we hypothesized in that CD patients with elevated anti-flagellin antibody titers have increased frequencies of flagellin-reactive $CD4^+$ T cells (**Chapter 5**). In addition, we tested whether these cells had a more inflammatory phenotype compared to flagellin-reactive cells from healthy individuals and CD patients with low anti-flagellin antibody titers. Our preliminary data showed that treatment-naive pediatric CD patients with high anti-flagellin IgG titers had increased frequencies of flagellin-reactive $CD4^+$ T cells in peripheral blood when compared to UC patients and healthy controls. In both healthy and diseased individuals, flagellin-reactive T cells had phenotypic regulatory and inflammatory features and could express $\alpha4\beta7$ and CD38. However, CD patients with elevated flagellin-specific IgG and reactivity to 8/8 flagellins had reduced frequencies of $IL\text{-}10^+$ and $TIGIT^+$ flagellin-reactive $CD4^+$ T cells when compared to those of healthy controls and patients with low levels of flagellin-reactive IgG. In line with previous findings, TIGIT and IL-10 expression appeared related as $TIGIT^+$ flagellin-reactive $CD4^+$ T cells were enriched in IL-10-producing cells when compared to $TIGIT^{neg}CD4^+$ T cells, irrespective of health or disease.

In **Chapter 6**, the analysis of peripheral blood lymphocytes and intestinal tissue derived from a very early onset IBD patient with a genetic duplication of the *IL2RA* locus, allowed us to investigate the requirements for intestinal inflammation in the context of a hyperinflammatory $CD4^+$ T-cell defect. The patient developed an UC-like acute severe colitis at 2 years of age, with endoscopy showing severe pancolitis with edematous mucosa and multiple erosions. Histologically, the intestinal disease was characterized by hyperproliferative $CD4^+$ T cells in the lamina propria and epithelial layer, and many interferon gamma $(IFN\gamma)^+$ and $pSTAT5^+$ lesions. The intestinal disease was refractory to standard immunosuppressive, immunomodulatory and biological therapy, but strikingly, subtotal colectomy effectively induced clinical remission in the patient without the need for maintenance therapy. There have been reports of patients with similar duplications for whom no intestinal disease was reported. Together, this argues that the hyperproliferative $CD4^+$ T-cell response that we observed in the context of *IL2RA* duplication is not enough to cause intestinal disease. Instead, considering the observation that colectomy, resulting in a reduced bacterial load, effectuated disease cure, an environmental factor such as a specific microbial antigen may have initiated colonic disease in this particular patient. Thus, these data demonstrate that, although defects causing overall T-cell hyperproliferation and inflammation contribute to disease susceptibility, an additional trigger was needed to license the expansion of inflammatory $CD4^+$ T cells activated in the antigen-rich intestinal environment.

The disease course of IBD can be complicated by local (i.e. cancer, **Chapter 7** and **Chapter 8**) and extra-intestinal complications (i.e. sclerosing cholangitis, a severe liver disease leading to destruction of bile ducts, **Chapter 9**) that further influence the disease course. Given the rarity of these complications, more detailed information on pediatric-onset IBD patients who develop cancer or sclerosing cholangitis (SC) is needed to obtain more insight in predictive factors of these severe outcomes. In **chapter 7**, we provide a literature overview of patients with pediatric-onset IBD patients who developed cancer or suffered a fatal outcome at any point later in life. In **chapter 8**, we report the largest number of pediatric-onset IBD patients with cancer and/or fatal outcomes to date. Malignancies followed by infections were the major causes of mortality. Although it is believed that pediatric-onset SC runs a milder course in children than in adults, we identified SC as a significant risk factor for cancer-associated mortality. In **Chapter 9**, in order to compare long-term disease outcomes between pediatric- and adulthood-onset SC patients, we evaluated time-to-complication curves in two independent cohorts from the same geographical area. Our data demonstrate that pediatric and adult-onset SC run a similar disease course regarding time-to-transplantation and SC-related death. Our results emphasize the importance of follow-up of young patients with rare diseases, whose disease course is blurred by transition to adult-oriented care.

The results of this thesis are put into perspective in **Chapter 10**. When combining the original data presented in the various chapters of this thesis, a recurrent observation is that, when compared to healthy individuals, IBD patients have an altered balance between regulatory and inflammatory $CD4^+$ T-cell responses. We provide evidence for both reduced regulatory (**Chapter 3 and 5**) as well as increased inflammatory features (**Chapter 3 and 6**). We demonstrate that characterization of inhibitory receptor expression on $CD4^+$ T cells allows IBD patient stratification prior to treatment. Thus, the data in this thesis substantiate the idea that microbial-host mutualism is lost in IBD due to a predominating inflammatory $CD4^+$ T-cell responses to commensal microbial antigens, promoting tissue destruction and chronic inflammation in the intestine. The main challenge for future research lies within classifying IBD patients on the basis of immune dysfunction, in order to select patient subgroups who are most likely to respond to targeted therapies. In addition, uncovering shared disease-driving mechanisms between IBD and other immune-mediated inflammatory diseases (IMIDs) could identify subgroups of patients across various IMIDs that can benefit from the same targeted treatment.

Nederlandse Samenvatting

Het slijmvlies (mucosa) van ons maag-darm kanaal wordt continu blootgesteld aan grote hoeveelheden lichaamsvreemde eiwitten uit voedsel en van onschadelijke darmbacteriën. Om infecties met schadelijke indringers tegen te gaan, bevat de darm een groot aantal afweercellen (immuuncellen) die ziekteverwekkers signaleren en vernietigen. Het afweersysteem (immuunsysteem) van de darm moet er voor zorgen dat het schadelijke darmbacteriën aanvalt, maar de onschadelijke darmbacteriën (commensalen) met rust laat. Om dit te bewerkstelligen, bestaat het immuunsysteem van de darm uit zowel ontstekingsbevorderende (inflammatoire) als ontstekingsremmende (regulerende) immuuncellen. In een gezonde darm is er een balans tussen regulerende en inflammatoire immuuncellen. Deze balans wordt homeostase genoemd.

Het immuunsysteem in de darm.
Het immuunsysteem in de darm bestaat uit cellen van het aangeboren en het verworven immuunsysteem. Het darmweefsel onder het darmepitheel (laag van dicht aaneengesloten cellen) bevat antigeen-presenterende cellen (APC), waaronder dendritische cellen (DC) en macrofagen. Deze cellen van het aangeboren afweersysteem nemen lichaamsvreemde eiwitten (antigenen) op en verzorgen daarmee de eerstelijns afweer. De darm bevat ook een groot aantal immuuncellen van het verworven immuunsysteem, waaronder T-cellen. Deze cellen dragen een receptor die 'op maat' gemaakt kan worden voor het herkennen van antigenen en hebben een geheugen waardoor ze bij een tweede ontmoeting met hetzelfde antigeen snel kunnen reageren. Anatomisch gezien wordt het immuunsysteem in de darm onderverdeeld in inductie en effector plaatsen. De darm-geassocieerde lymfoïde weefsels en de lokale darm drainerende lymfeklieren zijn de inductie plaatsen, waar naïeve (niet eerder aan antigeen blootgestelde) T-cellen worden geactiveerde door APC. Het darmweefsel en darmepitheel zijn de effector plaatsen waar antigeen-specifieke effector T-cellen hun acties uitvoeren.

APC nemen antigenen op en presenteren deze op hun MHC moleculen op het celmembraan. Vervolgens reizen ze naar de lymfeklieren om daar naïeve T-cellen te activeren. Naïeve T-cellen uit perifeer bloed reizen ook naar de lymfeklieren met behulp van specifieke migratie-receptoren. Activatie van T-cellen wordt in gang gezet wanneer een naïeve T-cel een APC tegenkomt die het juiste antigeen-MHC-complex tot expressie brengt. Als een sleutel in een slot zorgt de binding van antigeen-MHC-complex aan de T-cel receptor dat de naïeve T cel actief wordt, deelt en differentieert waardoor er een groot aantal antigeen-specifieke effector en geheugen T-cellen ontstaan. Dit proces is afhankelijk van drie signalen: de verbinding van het antigeen-MHC complex van de APC met de T-cel receptor (signaal 1), co-stimulatoire moleculen aanwezig op de APC (signaal 2) en cytokines (kleine, oplosbare eiwitten) die door de APC worden uitgescheiden (signaal 3). Deze signalen zorgen ook voor het tot expressie komen van migratie-receptoren die T-cellen specifiek naar de effector plaatsen van de darm leiden. Dus, na herkenning van antigeen in darm-geassocieerde lymfoïde weefsels en lokale darm drainerende lymfeklieren, verlaten T-cellen de lymfoïde weefsels om zich naar de darm te begeven, waar ze verblijven als lang levende antigeen-specifieke effector en geheugen T-cellen.

$CD4^+$ T-cellen in de darm.
Wanneer een naïeve T-cel een APC tegenkomt met het juiste antigeen-MHC-complex kan dit leiden tot differentiatie van zowel inflammatoire (ontstekingsbevorderende) als regulerende (ontstekingsremmende) T-cellen. In de darm zijn veel factoren aanwezig die APC leren tolerant te zijn tegen onschuldige antigenen, en dus preferentieel de ontwikkeling van regulerende T-cellen te stimuleren. Als gevolg hiervan leidt herkenning van een onschadelijk antigen tot een gebalanceerde immuunreactie, waarbij regulerende T-cellen in balans zijn met inflammatoire T-cellen.

Regulerende (ontstekingsremmende) T-cellen
Regulerende T-cellen worden gekenmerkt door eigenschap dat zij in staat zijn ongewenste reacties van andere immuuncellen te onderdrukken. Regulerende T-cellen in de darm worden onderverdeeld in $CD4^+Foxp3^+$ regulerende T-cellen (Tregs) en IL-10-producerende $CD4^+Foxp3^{neg}$ T-cellen. Tregs en IL-10-producerende $CD4^+Foxp3^{neg}$ T-cellen oefenen hun regulerende functies uit in verschillende delen van de darm. Tregs zijn in grote getalen aanwezig in de dikke darm (colon), terwijl IL-10-producerende $CD4^+Foxp3^{neg}$ T-cellen een prominente rol spelen in de dunne darm. Beide regulerende T-cel populaties gebruiken meerdere mechanismen om inflammatoire immuunreacties te onderdrukken, waarvan interleukine 10 (IL-10)-productie het meest onderzocht is. Dit cytokine is van essentieel belang is om ontsteking in de darm te voorkomen.

Inflammatoire (ontstekingsbevorderende) T-cellen
Naast regulerende T-cellen bevat het darmweefsel diverse groepen van inflammatoire T-cellen. Er zijn verschillende groepen van inflammatoire T-cellen, aangeduid als T-helper (Th)-cellen, waaronder Th1, Th2 en Th17 cellen. Deze T-celpopulaties kunnen worden onderscheiden op basis van hun cytokine profiel en hebben elk een andere functie. Hoewel inflammatoire T-cellen van essentieel belang zijn voor de eliminatie van invasieve pathogene bacteriën, wordt chronische darmontsteking, zoals gezien wordt in patiënten met inflammatoire darmziekten (IBD), vaak gekenmerkt door uitgebreide infiltratie van het darmweefsel met inflammatoire T-celpopulaties.

Inflammatoire darmziekten (IBD).
Door fouten in tolerantie voor onschadelijke bacteriën kan chronische darmontsteking

ontstaan, in het Engels “inflammatory bowel disease (IBD)” genoemd. De twee bekendste chronische darmontstekingen zijn colitis ulcerosa en de ziekte van Crohn (ZvC). IBD verschilt van patiënt tot patiënt en zit soms overal en soms alleen op een plek. De precieze oorzaak van IBD is onbekend, maar drie factoren spelen een oorzakelijke rol. Deze factoren zijn genetische belasting (gevoeligheidsgenen), darmbacteriën en het immuunsysteem van de darm. Er wordt gedacht dat IBD wordt veroorzaakt door een ontregelde immuunreactie op antigenen van onschuldige darmbacteriën in een genetisch-gevoelig persoon. Over de afgelopen decennia is er steeds meer bewijs gekomen dat ontregeling van het immuunsysteem betrokken is bij de ontwikkeling van IBD. Ten eerste, uit genetische analyses van grote groepen IBD patiënten zijn ongeveer 160 gen gebieden (waarin zich meerdere genen bevinden) gevonden die een genetische aanleg voor IBD zouden kunnen veroorzaken. Van deze gen gebieden is een groot deel betrokken bij immuunreacties van het aangeboren en verworven immuunsysteem. Ten tweede, genetische deficiëntie van bepaalde immuungenen zijn geassocieerd meet IBD-achtige darmontsteking. Deze monogenetische ziekten (veroorzaakt door één gen) hebben laten zien dat zowel een tekort aan aangeboren immuunreacties als een tekort aan regulerende immuunreacties kan leiden tot chronische ontsteking van de darm. Tot slot, de darmontsteking kan in een groot deel van de IBD patiënten met succes worden behandeld met afweerremmende medicijnen. Dit suggereert dat metingen van ontregelde immuunreacties in de toekomst misschien kunnen helpen om IBD niet meer alleen onder te verdelen in colitis ulcerosa en ZvC maar in ‘afweerfouten’ waarop een therapie is af te stemmen.

Dit proefschrift.

Dit proefschrift richt zich op het classificeren van kinderen met IBD op basis van de onderliggende afweerfout om zo het ziektebeloop en reactie op behandeling beter te voorspellen. Zoals beschreven in **Hoofdstuk 1** spelen T-cellen een belangrijke rol in het ontstaan van chronische darmontsteking. De darmontsteking in IBD wordt gekenmerkt door infiltratie van T-cellen die grote hoeveelheden inflammatoire cytokines uitscheiden. Remmende receptoren op het celmembraan van deze T-cellen dienen als “checkpoints” die activatie via de T-cel receptor (signaal 1) en co-stimulatoire moleculen (signaal 2) dempen. In **Hoofdstuk 2** bespreken we de nieuwe inzichten in de rol van deze remmende receptoren in darm homeostase. Ons review beschrijft de verschillende remmende receptoren die door zowel $CD4^+Foxp3^+$ Tregs als IL-10-producerende $CD4^+Foxp3^{neg}$ T-cellen tot expressie worden gebracht, en hun rol in darm homeostase.

Het meten van de ontregelde balans tussen regulerende en inflammatoire T-cellen in IBD patiënten zou kunnen helpen om patiënten te classificeren en hun ziektebeloop te voorspellen. In **Hoofdstuk 3** onderzochten we of we veranderingen in regulerende en inflammatoire T-cellen in het bloed van IBD patiënten konden detecteren. Om dit te onderzoeken hebben we onze analyses gericht op circulerende $CD38^+$ effector T-cellen, een T-cel populatie waarvan we eerder hebben aangetoond dat deze sterk verrijkt is aan T-cellen die naar de darm toe migreren. We laten zien dat gezonde individuen en IBD patiënten deze circulerende $CD38^+$ effector T-cellen hebben en dat deze cellen zowel regulerende als inflammatoire cel populaties bevatten. Bij gezonde individuen, die gebalanceerde immuunreacties in de darm hebben, hadden $CD38^+$ effector T-cellen bij voorkeur een regulatoir fenotype met lage frequenties van geactiveerde $CD25^+CD45RA^{neg}$ en hoge frequenties van T-cellen met de remmende receptor T-cel Ig en ITIM-domein (TIGIT) op hun oppervlak. Bij een deel van de IBD patiënten was de balans tussen regulerende en inflammatoire T-cellen verschoven richting inflammatie. In vergelijking met gezonde controles, hadden IBD patiënten met actieve darmziekte (voor aanvang van therapie) verhoogde frequenties van $CD25^+CD45RA^{neg}$ T-cellen en verlaagde frequenties van $TIGIT^+$ T-cellen. Er werden geen verschillen in de frequenties van $Foxp3^+$ Tregs in $CD38^+$ effector T-cellen gezien tussen IBD patiënten en gezonde controles. Het percentage van $TIGIT^+$ cellen in $CD38^+$ effector T-cellen kon patiënten met korte duur van klinische ziekteremissie na start van therapie identificeren. Om precies te zijn, TIGIT-percentages lager dan 25% vóór behandeling waren geassocieerd met kortere duur van klinische ziekteremissie. Onze resultaten tonen aan dat fenotypische karakterisering van circulerende $CD4^+$ T-celpopulaties in het bloed van IBD patiënten klinisch-relevante subgroepen kan onderscheiden. In het bijzonder, analyse van meerdere remmende receptoren, waaronder TIGIT, op $CD38^+$ effector T-cellen zou kunnen helpen om IBD patiëntstratificatie voorafgaand aan behandeling verder te optimaliseren.

Omdat een subgroep van IBD patiënten verlaagde $TIGIT^+$-cel frequenties hadden, wilden we factoren identificeren die betrokken zijn bij de inductie van TIGIT-expressie op het celmembraan van $CD4^+$ T-cellen. In **Hoofdstuk 4**, door zowel humaan als muis materiaal te gebruiken, analyseerden we TIGIT expressie op verschillende $CD4^+$ T-cel populaties en bepaalden we de signalen die noodzakelijk zijn voor TIGIT expressie op het celmembraan. We laten zien dat activatie via de T-cel receptor middels anti-CD3 TIGIT expressie induceert op antigeen-specifieke effector T-cellen. Omdat een subgroep van IBD patiënten verlaagde $TIGIT^+$-cel frequenties hadden, veronderstelde we dat TIGIT expressie op T-cellen betrokken kan zijn bij het reguleren van immuunreacties tegen darmbacteriën. Het koloniseren van kiemvrije muizen (muizen zonder darmbacteriën) met specifieke pathogeen-vrije (SPF) darmbacteriën resulteerde in een twee- tot drievoudige toename van TIGIT expressie in darmweefsel. Omdat een groot deel van de T-cellen in de darm verondersteld wordt een specificiteit voor bacteriële antigenen heeft, onderzochten we of TIGIT tot expressie werd gebracht door T-cellen die specifiek bacteriële antigenen herkenden. Hoewel de meerderheid van de bacterieel antigeen-reactieve T-cellen geen TIGIT tot expressie brachten, kwam TIGIT tot expressie op circa 7%-10% van de bacterieel

antigeen-reactieve T-cellen. Bacterieel antigeen-reactieve TIGIT^{+}CD4^{+} T-cellen bevatten hogere frequenties van IL-10^{+} cellen in vergelijking met bacterieel antigeen-reactieve TIGIT^{neg}CD4^{+} cellen. Dit impliceert dat TIGIT^{+}CD4^{+} T-cellen betrokken kunnen zijn bij het reguleren van immuunreacties tegen darmbacteriën.

In zowel **Hoofdstuk 3** als **Hoofdstuk 4** zagen we een sterke associatie tussen TIGIT en CD38. CD38 is een ectoenzym dat betrokken is bij de synthese van twee signaleringsmoleculen die noodzakelijk zijn voor intracellulaire Ca^{2+} mobilisatie. Met behulp van de calcineurine-remmer tacrolimus en calcium ionophore ionomycine (dat de intracellulaire Ca^{2+} concentratie verhoogd) laten we zien dat calcium-afhankelijke signalering na T-cel receptor activatie vereist was om TIGIT expressie op T-cellen te induceren. Toekomstige experimenten zijn nodig om vast te stellen of CD38 bijdraagt aan TIGIT inductie op T-cellen door de intracellulaire Ca^{2+} concentratie te verhogen.

Een subgroep van patiënten met de ZvC heeft verhoogde antistof responsen tegen bacterieel flagelline, een eiwit dat tot expressie wordt gebracht door zowel onschuldige als pathogene bacteriën. Deze antistoffen zijn geassocieerd met een verhoogd risico op agressieve en gecompliceerde ziekte. Antistof responsen zijn vaak afhankelijk van de activatie van T-cellen. Daarom veronderstelden we dat patiënten met de ZvC met verhoogde anti-flagelline antistof titers ook verhoogde frequentie van flagelline-reactieve T-cellen zouden hebben (**Hoofdstuk 5**). Ook hebben we getest of deze flagelline-reactieve T-cellen een meer inflammatoir fenotype hadden vergeleken met flagelline-reactieve cellen van gezonde personen en ZvC-patiënten met lage titer aan anti-flagelline antistoffen. Onze analyses tonen aan dat ZvC patiënten met een hoge anti-flagelline IgG-titers verhoogde frequenties van flagelline-reactieve T-cellen in perifeer bloed hadden vergeleken met gezonde controles. Zowel bij gezonde als zieke personen hadden flagelline-reactieve T-cellen regulatoire en inflammatoire kenmerken en konden $\alpha4\beta7$ en CD38 tot expressie brengen. ZvC-patiënten met een verhoogde anti-flagelline IgG-titers en reactiviteit op 8/8 flagellines hadden verminderde frequenties van IL-10^{+} en TIGIT^{+} flagelline-reactieve T-cellen in vergelijking met gezonde controles en ZvC-patiënten met lage anti-flagelline IgG-titers. In overeenstemming met eerdere bevindingen, leken TIGIT en IL-10 expressie positief gecorreleerd aan elkaar. TIGIT^{+} flagelline-reactieve T-cellen waren namelijk verrijkt in IL-10-producerende cellen in vergelijking met TIGIT^{neg}CD4^{+} T-cellen.

In **Hoofdstuk 6** hebben we T-cellen uit perifeer bloed en darmweefsel bestudeerd afkomstig van een patiënt met een genetische duplicatie van de *IL2RA* locus, die op zeer jonge leeftijd IBD ontwikkelde. Dit stelde ons in staat de kenmerken van darmontsteking in de context van een hyper-inflammatoir T-celdefect te onderzoeken. De patiënt ontwikkelde op 2-jarige leeftijd een colitis ulcerosa-achtige colitis, waarbij een ernstige pancolitis met oedemateus slijmvlies en meerdere erosies werd gezien bij endoscopie. Histologie van aangedane darmbiopten toonde hyper-proliferatieve T-cellen in het lamina propria en darmepitheel en vele interferon-gamma (IFNγ)$^{+}$ en pSTAT5^{+} laesies. De darmontsteking was ongevoelig voor standaard immunosuppressieve en immunomodulerende medicijnen en biologicals. De patiënt moest een subtotale colectomie ondergaan, wat ervoor heeft gezorgd dat de patiënt blijvend in klinische remissie is zonder dat hiervoor nog onderhoudstherapie nodig is. Er zijn meldingen geweest van patiënten met vergelijkbare genduplicaties bij wie geen darm-gerelateerde klachten werden beschreven. Deze observaties suggereren dat het hyper-proliferatieve T-celfenotype dat we observeerden in de context van *IL2RA* duplicatie niet voldoende kan zijn om darmontsteking te veroorzaken. In plaats daarvan kan het zijn dat een omgevingsfactor, zoals een specifiek bacterieel antigeen, chronische ontsteking heeft geïnitieerd in de darm van deze specifieke patiënt. Dus, ondanks dat defecten die algemene T-celhyperproliferatie veroorzaken bijdragen aan de gevoeligheid om IBD te ontwikkelen, is er een extra “trigger” nodig om expansie van inflammatoire T-cellen die in de antigeenrijke darmomgeving zijn geactiveerd te veroorzaken.

Het ziekteverloop van IBD kan worden gecompliceerd door lokale (bijvoorbeeld kanker, **Hoofdstuk 7** en **Hoofdstuk 8**) en extra-intestinale complicaties (bijvoorbeeld scleroserende cholangitis, een ernstige leveraandoening die leidt tot destructie van galgangen, **Hoofdstuk 9**) die het ziekteverloop negatief beïnvloeden. Gezien het feit dat deze complicaties niet frequent voorkomen, is meer gedetailleerde informatie over IBD patiënten die op jonge leeftijd die kanker of scleroserende cholangitis (SC) ontwikkelen nodig om meer inzicht te krijgen in voorspellende factoren van deze ernstige uitkomsten. In **Hoofdstuk 7** geven we een literatuuroverzicht van patiënten die op kinderleeftijd IBD ontwikkelden en later in het leven kanker kregen of een fatale afloop hadden. In **Hoofdstuk 8** rapporteren we het grootste aantal kinder IBD patiënten met kanker en/of een fatale afloop tot nu toe. Kanker en infecties waren de belangrijkste oorzaken van mortaliteit. Ondanks dat vaak wordt verondersteld dat SC bij kinderen een mildere ziektebeloop heeft dan bij volwassenen, lieten onze data zien dat SC een significante risicofactor was voor kanker-geassocieerde mortaliteit. In **Hoofdstuk 9** evalueerden we de tijd-tot-complicatiecurven in twee onafhankelijke cohorten uit hetzelfde geografische gebied om het ziektebeloop op de lange termijn te vergelijken tussen SC-patiënten op de kinderleeftijd en op de volwassen leeftijd. Onze gegevens tonen aan tijd tot transplantatie en SC-gerelateerde sterfte niet verschilt tussen patiënten die SC op de kinderleeftijd ontwikkelden versus patiënten die SC op volwassen leeftijd kregen. Onze resultaten benadrukken het belang van de nauwkeurige follow-up van jonge patiënten met zeldzame ziekten, waarvan het ziektebeloop vaak niet goed in kaart wordt gebracht door de overgang naar volwassen-gerichte zorg.

De resultaten van dit proefschrift worden in **Hoofdstuk 10** in perspectief geplaatst. Wanneer alle data uit dit proefschrift wordt gecombineerd, is een terugkerende observatie dat, in

vergelijking met gezonde individuen, IBD patiënten een ontregelde balans hebben tussen regulerende en inflammatoire T-cellen. We leveren bewijs aan voor zowel verminderde regulerende T-cel kenmerken (**Hoofdstuk 3 en 5**) als voor verhoogde inflammatoire T-cel kenmerken (**Hoofdstuk 3 en 6**). We tonen aan dat expressie van remmende receptoren op T-cellen van IBD patiënten stratificatie mogelijk maakt voorafgaand aan de behandeling. Met deze bevindingen hebben we een basis gelegd voor de detectie van immuun-disfunctie in patiënten met IBD. De belangrijkste uitdaging voor toekomstig onderzoek is om IBD patiënten te classificeren op basis van immuun-disfunctie, met als doel subgroepen van IBD patiënten te identificeren die het meest gebaat zijn bij gerichte therapieën. Bovendien kan het ontdekken van de overeenkomsten in ziekte-veroorzakende mechanismen tussen IBD en andere immuun-gemedieerde inflammatoire ziekten (IMID's) subgroepen van patiënten identificeren in verschillende IMID's die baat kunnen hebben bij dezelfde gerichte behandeling.

Dankwoord

“He who loves, flies, runs, and rejoices; he is free and nothing holds him back.”
– Henri Matisse –

DANKWOORD

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Het Samsom Lab.

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De kinder-MDL.

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Department of Pediatric Gastroenterology, Child Life and Health, University of Edinburgh, Edinburgh, United Kingdom.

List of Publications

LIST OF PUBLICATIONS

Included in this thesis

Joosse ME*, Haisma SM*, Sterk MFM, van Munster KN, Ponsioen CIJ, Houwen RHJ, Koot BHP, de Meij T, van Rheenen PF, de Koning BAE. Disease progression in pediatric- and adult-onset sclerosing cholangitis: results from two independent Dutch registries. Liver Int. 2019 Jun 1 Epub ahead of print.

Joosse ME, Nederlof I, Walker LSK, Samsom JN. Tipping the balance: inhibitory checkpoints in intestinal homeostasis. Mucosal Immunol. 2019 Jan;12(1):21-35.

Joosse ME, Menckeberg CL, de Ruiter LF, Raatgeep HC, van Berkel LA, Simons-Oosterhuis Y, Tindemans I, Muskens AFM, Hendriks RW, Hoogenboezem RM, Cupedo T, de Ridder L, Escher JC, Veenbergen S, Samsom JN. Frequencies of circulating regulatory TIGIT^{+}CD38^{+} effector T cells correlate with the course of inflammatory bowel disease. Mucosal Immunol. 2019 Jan;12(1):154-163.

Aardoom MA*, **Joosse ME***, de Vries ACH, Levine A, de Ridder L. Malignancy and Mortality in Pediatric-onset Inflammatory Bowel Disease: A Systematic Review. Inflamm Bowel Dis. 2018 Mar 19;24(4):732-741.

Joosse ME*, Aardoom MA*, Kemos P, Turner D, Wilson DC, Koletzko S, Martin-de-Carpi J, Fagerberg UL, Spray C, Tzivinikos C, Sladek M, Shaoul R, Roma-Giannikou E, Bronsky J, Serban DE, Ruemmele FM, Garnier-Lengline H, Veres G, Hojsak I, Kolho KL, Davies IH, Aloi M, Lionetti P, Hussey S, Veereman G, Braegger CP, Trindade E, Wewer AV, Hauer AC, de Vries ACH, Sigall Boneh R, Sarbagili Shabat C, Levine A, de Ridder L; Pediatric IBD Porto group of ESPGHAN. Malignancy and mortality in pediatric-onset inflammatory bowel disease: a 3-year prospective, multinational study from the pediatric IBD Porto group of ESPGHAN. Aliment Pharmacol Ther. 2018 Sep;48(5):523-537.

Joosse ME, Charbit-Henrion F, Raatgeep HC, J. Lindenbergh-Kortleve D, Costes LMM, Nugteren S, Veenbergen S, Malan V, Nowak JK, Mearin ML, Escher JC, Cerf-Bensussan N, Samsom JN. Duplication of the *IL2RA* locus causes excessive IL-2 signaling and predisposes to very early onset colitis. Manuscript submitted for publication.

Not included in this thesis

Veenbergen S, Li P, Raatgeep HC, Lindenbergh-Kortleve DJ, SimonsOosterhuis Y, Farrel A, Costes LMM, **Joosse ME**, van Berkel LA, de Ruiter LF, van Leeuwen MA, Winter D, Holland SM, Freeman AF, Wakabayashi Y, Zhu J, de Ridder L, Driessen GJ, Escher JC, Leonard WJ, Samsom JN. IL-10 signaling in dendritic cells controls IL-1β-mediated IFNγ secretion by human CD4^{+} T cells: relevance to inflammatory bowel disease. Accepted for Mucosal Immunology.

Liu L, Dong Y, Ye M, Jin S, Yang J, **Joosse ME**, Sun Y, Zhang J, Lazarev M, Brant SR, Safar B, Marohn M, Mezey E, Li X. The Pathogenic Role of NLRP3 Inflammasome Activation in Inflammatory Bowel Diseases of Both Mice and Humans. J Crohns Colitis. 2017 Jun 1;11(6):737-750.

Joosse ME, Samsom JN, van der Woude CJ, Escher JC, van Gelder T. The Role of Therapeutic Drug Monitoring of Anti-Tumor Necrosis Factor Alpha Agents in Children and Adolescents with Inflammatory Bowel Disease. Inflamm Bowel Dis. 2015 Sep;21(9):2214-21.

Joosse ME, Schipper ME, Libbrecht L, van Buuren HR, de Man RA. Recurrent cholangitis in a 65-year-old man. Biliary papillomatosis. Gut. 2015 Jun;64(6):883, 910.

Haisma SM, Weersma RK, **Joosse ME**, de Koning BAE, de Meij T, Koot BGP, Wolters V, Norbruis O, Daly MJ, Stevens C, Xavier RJ, Rivas MA, Visschedijk MC, Verkade HJ, Barbieri R, Jansen BH, Festen EAM, van Rheenen PF, van Diemen CC. Exome Sequencing in Patient-Parent Trios Reveals New Candidate Genes for Early-onset Primary Sclerosing Cholangitis. Manuscript submitted for publication.

Winter DA, **Joosse ME**, de Wildt SN, Taminiau JAJM, de Ridder L, Escher JC. Pharmacokinetics, pharmacodynamics and immunogenicity of infliximab in pediatric IBD: a systematic review. Manuscript submitted for publication.

* Shared first authors

About the Author

ABOUT THE AUTHOR

Linda Joosse was born on the 20th of January 1989 in Goes, the Netherlands. She completed her bilingual secondary school education (Gymnasium, International Baccalaureate English Higher Level, cum laude) at the Pontes Het Goese Lyceum in 2007. In the same year she moved to Rotterdam to start her medical training at medical faculty of the Erasmus University Rotterdam. In 2008, she successfully participated in the Erasmus Honours Program, an interdisciplinary academic training program to explore the boundaries of science together with students from other disciplines. From 2008-2009 she chaired STOLA, a foundation that organizes internships in developing countries for Erasmus MC medical students. In 2009, she commenced with the Master of Science program in Infection an Immunity (Molecular Medicine Postgraduate School) and received a scholarship to perform research at the Johns Hopkins University, Baltimore, USA. Here, she conducted research on extra-intestinal manifestations of IBD in a murine model under the supervision of Dr. Xuhang Li. She obtained her Dutch medical degree in August 2013 (cum laude), graduated from the research master Infection and Immunity a year later (cum laude) and was awarded the Prof. Bruins prize for best research master student at the Erasmus University Rotterdam. In 2014, she received a research grant provided by the Molecular Medicine Postgraduate School that enabled her to start a PhD project in the Laboratory of Pediatrics, division Gastroenterology and Nutrition, at the Erasmus MC in Rotterdam, under the supervision of Dr. Janneke Samsom and Prof. dr. Johanna Escher. During this time she spent a research period abroad at the University of Alabama at Birmingham, Alabama, under supervision of Prof. dr. Charles O. Elson III. The research was concluded in September 2018, and the results are described in this thesis.

Since 2015, Linda is registered as a ECFMG/USMLE certified physician in the USA and organizes an annual USMLE Step 1 Preparation Course for Dutch medical students and medical graduates at the Erasmus MC. In October 2018, she started working as a resident (ANIOS) in internal medicine at the Ikazia Hospital in Rotterdam. In the near future, she hopes to start her residency training in internal medicine, aiming to combine clinical work with immunological research and her passion for medical teaching. Linda lives in Rotterdam, together with her fiancé Floris Dammeijer.

PhD PORTFOLIO

Summary of PhD training and teaching

Name PhD student: M.E. (Linda) Joosse	PhD period: 2014 - 2018
Erasmus MC Department: Lab. of Pediatrics, Div. Gastroenterology and Nutrition	Promotor: Prof. Dr. J.C. Escher
Research School: Molecular Medicine Postgraduate School	Co-promotor: Dr. J.N. Samsom

1. PhD training	**Year**	**Workload (ECTS)**
General academic skills		
Medical Immunology course	2018	1.0
Good Clinical Practice (BROK)	2015	1.5
Laboratory Animal Science (LAS) course	2016	3.5
Scientific Integrity Course	2015	0.3
Advanced Immunology Course	2014	3.0
Research skills		
Basic introduction course on SPSS	2016	0.8
Workshop on Adobe InDesign CS5	2016	0.15
Biomedical English Writing Course	2015	2.0
Workshop on Access Basic	2014	0.3
Workshop on Excel Basic	2014	0.3
Workshop on Adobe Photoshop and Illustrator	2014	0.3
(Inter)national scientific presentations and conferences		
• *Oral presentations*		
European Conference for Immunology (ECI), Amsterdam	2018	1.0
European Mucosal Immunology Group (EMIG) meeting, Oxford, UK	2018	1.0
European Crohn's and Colitis Organisation (ECCO), Vienna, Austria	2018	1.0
Bright Spark, NVVI Annual Meeting, Noordwijkerhout	2017	1.0
Theme Sophia Research Day, Rotterdam	2017	1.0
Digestive Disease Days, NVGE, Veldhoven	2017	1.0
50th anniversary of Laboratory of Pediatrics, Het Nieuwe Instituut, Rotterdam	2017	1.0
ECCO Conference, Amsterdam	2016	1.0
Sophia Research Day, Rotterdam	2015	1.0
Sophia Research Day, Rotterdam	2014	1.0
• *Poster presentations*		
ICMI, Washington DC, USA	2017	0.3
BSI and NVVI Joint Meeting, Liverpool, UK	2016	0.3
EMIG Meeting, Copenhagen, Denmark	2016	0.3
ECCO Conference, Amsterdam	2016	0.3
20th Molecular Medicine Day, Rotterdam	2016	0.3
NVVI Annual Meeting, Noordwijkerhout	2015	0.3
ECCO Conference, Barcelona, Spain	2015	0.3
NVVI Annual Meeting, Kaatsheuvel	2014	0.3
18th Molecular Medicine Day, Rotterdam	2014	0.3

2. Teaching		
Supervising research master theses		
Anne van Schoonhoven, MSc Infection and Immunity student (MolMed)	2017-2018	5.0
Iris Nederlof, medical student and MSc student Leiden University	2017	5.0
Coaching		
Bachelor Coach for medical students at Erasmus MC	2015-2018	3.0
Student education		
• Teacher Human Physiology, Erasmus University College (EUC), Rotterdam	2018	3.0
• USMLE SOS co-founder: USMLE Info Night, USMLE Prep Night, USMLE Step 1 Preparation Course and web-based USMLE guidance at www.usmlesos.nl	2016-2018	n/a
• Guest teacher for International Baccalaureate students (4 VWO), Scholengroep Pontes het Goese Lyceum	2014-2018	4.0
• Nurse Specialists Refresher Course, Hogeschool Rotterdam Lecture: "Het immuun systeem: hoe zit het ook al weer met die cellen?"	2016	0.3
• Student Information Night, Erasmus School of Medicine, Rotterdam Lecture: "Acing the USMLE Step 1"	2014-2016	0.9
3. Other		
Chairperson Poster Walk at ECI European Conference for Immunology (ECI)	2018	1.0
Participation in TULIPS PhD program	2016-2018	5.0
Organizer of "Lab – Clinic Meetings" of Pediatric Gastroenterology	2014-2018	1.0
Student Information Nights on Research Masters, Woudestein Campus, Rotterdam	2014-2015	0.3
Member of the Sophia Research Committee Educational committee	2015	1.0
Medical Business Masterclass, Medical Business, Amsterdam	2015	0.9
Junior scientist for the Molecular Medicine Postgraduate School Re-evaluation	2015	0.3
4. Awards and grants		
Travel grant of the ECI, Amsterdam	2018	-
Travel grant of the EMIG, Oxford, UK	2018	-
Travel grant of the ECCO, Vienna, Austria	2018	-
Travel grant of the BSI-NVVI Joint Meeting, Liverpool, UK	2016	-
Travel grant of the EMIG, Copenhagen, Denmark	2016	-
Grant for USMLE S.O.S. by Trustfond Rotterdam (€3,175)	2016	-
Prize for best oral presentation at Sophia Research Day	2015	-
Prof. Bruins Prize for best research master student at Erasmus University (€4,500)	2014	-
PhD grant provided by the Molecular Medicine Postgraduate School (€200,000)	2014	-
Total		**55.6**

ECTS = European Credit Transfer and Accumulation System (1 ECTS represents 28 hours)

About the Cover

The cover image of this thesis depicts a Native American boy performing a Powwow dance. Native American communities lived in many different environments ranging from flatlands and forests to deserts and prairies. They were very close to their environment. Any change in nature – including the climate, landscape, plants and animals – required a change in their own habits. By adequately responding to changing environmental factors, they successfully adapted to and survived in dynamic environments that were constantly shifting and changing. The Powwow dance, in which every motion that is performed on one foot has to be repeated on the other, was performed on social gatherings to remind the community of the need for balance between human life and nature.

Like a Native American community, the immune system in the intestine has to monitor and adapt to a constantly changing mucosal landscape. In the intestinal environment, immune cells continuously encounter a variety of external agents, including antigens derived from dietary components, commensal bacteria and infectious pathogens. Adaptation of immune cells to the intestinal environment requires constant discrimination between harmless antigens derived from commensal bacteria and harmful pathogens that need to be cleared. A large proportion of immune cells in the intestinal tissue are $CD4^+$ memory T cells that have the unique ability to exert memory to previously encountered antigens. These $CD4^+$ memory T-cell responses are essential for protective immunity against pathogens, but need to be tightly regulated as inadequately controlled immune responses to commensal bacterial antigens can result in chronic intestinal inflammation. As such, a dynamic balance between regulatory and inflammatory $CD4^+$ T-cell populations determines the outcome between intestinal health and disease. In search for new parameters to classify immunological disease in patients with inflammatory bowel disease (IBD), this thesis investigates the underlying immune mechanisms that establish and maintain this balance.

A DYNAMIC BALANCE

Regulatory and inflammatory T-cell responses in inflammatory bowel disease