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CD4⁺ T-cell subsets in intestinal inflammation

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Summary: Intestinal CD4⁺ T cells are essential mediators of immune homeostasis and inflammation. Multiple subsets of CD4⁺ T cells have been described in the intestine, which represents an important site for the generation and regulation of cells involved in immune responses both within and outside of the gastrointestinal tract. Recent advances have furthered our understanding of the biology of such cells in the intestine. Appreciation of the functional roles for effector and regulatory populations in health and disease has revealed potential translational targets for the treatment of intestinal diseases, including inflammatory bowel disease. Furthermore, the role of dietary and microbiota-derived factors in shaping the intestinal CD4⁺ T-cell compartment is becoming increasingly understood. Here, we review recent advances in understanding the multifaceted roles of CD4⁺ T cells in intestinal immunity.

Keywords: T-cell differentiation, Treg, Th17, Th1, intestinal inflammation

Introduction

The gastrointestinal tract is a major interface with the external environment and a site of high immune challenge. In addition to harboring a resident microbiota estimated to contain 10¹⁴ bacteria, as well as viral and fungal species, the intestine is continuously exposed to dietary and other ingested foreign antigens. On top of this, the gastrointestinal tract is also a key portal of entry for pathogens. To manage these diverse functions, it is populated by a complex and highly specialized network of innate and adaptive immune cells, including the largest population of T cells in the body.

The requirement to tolerate luminal contents while remaining poised to generate appropriate immune responses toward pathogens has led to the development of multiple layers of regulation (1, 2). Anatomical adaptations serve to physically limit the interaction of immune cells with luminal-derived antigens, whereas gut-specific conditioning factors regulate epithelial, stromal, and myeloid cells, generally favoring homeostasis. Multiple specialized lymphocyte populations are found within the intestinal tract, often demonstrating highly compartmentalized distribution and

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functional attributes. Among intestinal lymphocytes, CD4⁺ T cells represent a major population implicated in mediating diverse host protective and homeostatic responses. However, on the dark side, accumulation of CD4⁺ T cells in the intestine is also a key feature of inflammatory bowel disease (IBD) (3, 4). Through elaboration of cytokines that amplify and perpetuate inflammation, CD4⁺ T cells may be drivers of disease in IBD and as such represent an important therapeutic target.

In this review, we discuss recent advances in our understanding of the development, regulation, and function of intestinal CD4⁺ T cells, in both homeostatic settings and in disease. We particularly focus upon murine models of intestinal inflammation, and compare and contrast findings in these model systems with advances in understanding human disease. Finally, we discuss the translational and therapeutic implications of our growing knowledge of this important subset of cells.

Intestinal T-cell subsets and their anatomical distribution

Anatomically, CD4⁺ T cells are distributed within both immunological inductive and effector sites of the small and large intestine (Fig. 1). Inductive sites comprise the mesen-

teric lymph nodes (MLNs) and the gut-associated lymphoid tissue (GALT), including the Peyer's patches (PPs) of the small intestine, smaller isolated lymphoid follicles (ILFs) found throughout the intestines, as well as the cecal patch in mice, and the appendix in humans (5). The anatomy and function of organized lymphoid tissue in intestinal immunity has been reviewed in detail elsewhere (5–7).

In contrast, intestinal effector sites are characterized by the diffuse distribution of lymphocytes among non-immune cells and matrix, and include the intraepithelial (IEL) compartment and the lamina propria (LP). The composition of these effector sites demonstrates significant bias toward specific subsets of lymphocytes. Within the IEL compartment, the majority of T cells express CD8, either as the conventional CD8 $\alpha\beta$ heterodimer or as a CD8 $\alpha\alpha$ homodimer (8). Furthermore, the majority of such cells, at least within the small intestine, use a $\gamma\delta$ T-cell receptor (TCR) rather than the conventional $\alpha\beta$ TCR. While CD4⁺ T cells, the majority of which express an $\alpha\beta$ TCR, are present within the IEL throughout the intestine, they comprise a greater proportion of T cells within more distal segments, including the colon (8, 9). Interestingly, IEL CD4⁺ T-cell populations show significant interstrain variation in mice that may reflect genetic or environmental control (9). Notably, infiltration of the IEL

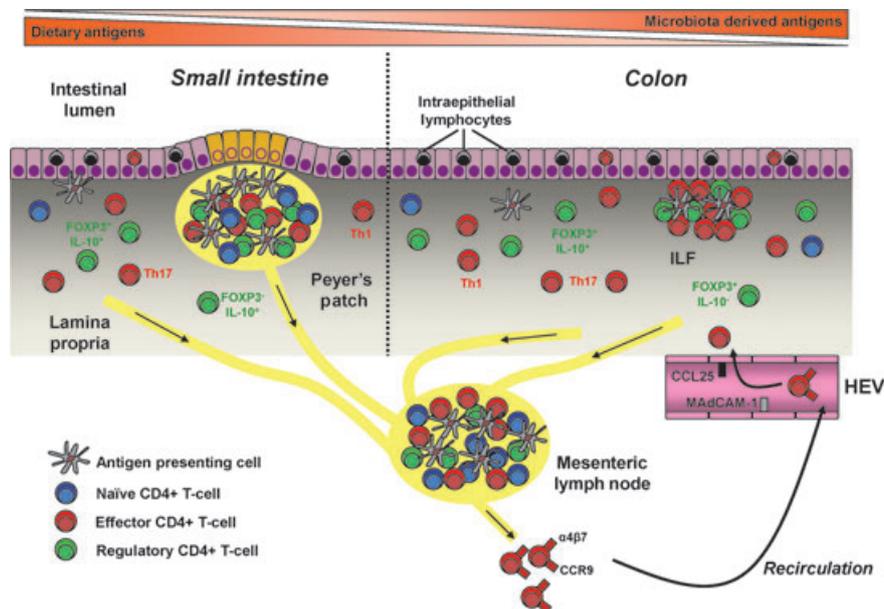


Fig. 1. Anatomical distribution of intestinal CD4⁺ T-cell subsets. At steady state, various populations of CD4⁺ T cells are distributed with the lamina propria of the intestine and associated inductive sites. The intestinal lumen contains a plethora of dietary and microbiota-derived antigens, which are separated from the intestinal immune system by a single layer of epithelial cells. A specialized population of antigen-presenting cells within the intestine contributes to the generation of IL-10-producing regulatory T cells but also effector T cells expressing IL-17A or IFN- γ . Naive CD4⁺ T cells are abundant at inductive sites, but a small proportion of lamina propria CD4⁺ T cells also display surface markers associated with naive T cells. Trafficking of activated CD4⁺ T cells to the intestine is regulated by intestine-specific homing molecules. IL-10, interleukin-10; IFN- γ , interferon- γ ; HEV, High endothelial venule.

by CD4⁺ T cells is a feature of inflammation in experimental models of IBD.

Within the LP of both the small and large intestines, the majority of T cells are CD4⁺, with a smaller population of CD8 $\alpha\beta$ ⁺ cells, although notably the human LP contains a greater proportion of CD8⁺ T cells compared with the murine gut (10, 11). Similar to their distribution within the IEL, CD4⁺ T cells may be more highly represented within the colonic LP. In addition to these conventional T-cell subsets, small populations of various unconventional cells, such as CD4⁻CD8⁻ T cells [including natural killer T (NKT) and mucosal-associated invariant T (MAIT) cells] are present in the healthy LP. The potential role of such cells in intestinal immunity and inflammation has been reviewed elsewhere (12, 13).

Within the steady-state LP of both the small intestine and colon, the majority of CD4⁺ T cells express a CD44^{hi}CD62L⁻ effector memory phenotype of antigen-experienced cells (14, 15). Notable differences exist in the prevailing effector T-cell populations between anatomical niches within the intestine. Acquisition of distinct T-cell effector functions in intestinal niches is discussed in detail below. A small proportion of LP CD4⁺ T cells (up to 10% within the colonic LP) display surface markers associated with naive T cells (16). Whether these cells are tissue-resident or are undergoing normal trafficking through the LP is not fully defined, nor is whether they are able to undergo initial priming and differentiation within the LP. Indeed, the contribution of naive T cells in the LP to immunity in the intestine is an area worthy of further study.

Intestinal T-cell homing

Myeloid antigen-presenting cells (APCs) of the intestine are a heterogeneous population consisting of dendritic cells (DCs) and macrophages. These populations are strategically positioned with the LP and in organized lymphoid structures and exhibit a number of adaptations associated with their dual role in tolerance and immunity in the intestine (17). DCs can act as a bridge with the adaptive immune system through their ability to acquire antigen in the intestine and migrate to the MLN where they prime the activation of naive CD4⁺ T cells (18). In addition to presenting antigen, intestine-derived DCs are specialized in their ability to prime T-cell responses that are focused on the intestine through the upregulation of gut-homing molecules on the responding T-cell surface (7). Expression of the ligands for these receptors on endothelial cells of postcapillary venules within

the gut mediates intestinal homing. Specific receptors and their ligands demonstrated to be crucial for physiological T-cell homing in the intestine include the integrin $\alpha_4\beta_7$ and mucosal addressin cell adhesion molecule-1 (MAdCAM-1), lymphocyte function-associated antigen-1, and intercellular adhesion molecule-1, very late antigen-4 ($\alpha_4\beta_1$), and vascular cell adhesion protein 1, CCR9 and CCL25, and P-selectin glycoprotein ligand-1 and P-selectin (7, 19, 20). Notably, however, molecular aspects of T-cell homing have been largely studied in relation to the small intestine, and regulation of lymphocyte homing to the colon is less clear, particularly in the steady state. In the setting of inflammation, expression of $\alpha_4\beta_7$ or $\alpha_4\beta_1$ may contribute to the accumulation of T cells in the colon (20, 21). Similarly, the contribution of the CCR9/CCL25 axis in large intestinal homing and in accumulation within the small intestine in disease states requires further study.

Intestinal homeostasis and consequences of its breakdown

Intestinal CD4⁺ T-cell populations can be broadly functionally divided into effector and regulatory populations. The lack of inflammation in the majority of individuals despite the enormous microbial and antigenic load within the intestine clearly demonstrates the dominance of regulatory mechanisms in the steady state (2). Similarly, the development of colitis in mice transferred with CD4⁺ T cells depleted of regulatory T cells confirms the functional importance of this subset in restraining cells which are otherwise capable of driving inflammatory disease (22–24).

When this balance is perturbed, inflammation results, as typified in humans by the IBDs, ulcerative colitis (UC), and Crohn's disease (CD) (25). These diseases, which affect around 1 in 1000 of the population in developed countries, are characterized by chronic inflammation of the intestinal tract, resulting in bloody diarrhea, abdominal pain, and weight loss. Although understanding of the etiopathogenesis of IBD has advanced rapidly in recent years, particularly through advances in genetics, treatment options remain limited and the majority of patients eventually require surgery. Furthermore, despite the development of increasingly efficacious therapies, such as monoclonal antibodies targeting tumor necrosis factor- α (TNF- α), such drugs have significant adverse event profiles, and are not curative (4).

Understanding of the underlying immunological basis of inflammation in IBD has benefited greatly from complimentary approaches using both animal model systems and studies of

human tissue (4, 25). These studies demonstrate that regardless of the specific initiating factor, infiltration of the intestinal mucosa by CD4⁺ T cells is a critical step in the amplification and chronicity of disease.

Intestinal effector CD4⁺ T cells

Early studies of the functional characteristics of murine LP CD4⁺ T cells described the presence of both interferon- γ (IFN- γ)⁺ T-helper 1 (Th1) and interleukin-4 (IL-4)⁺ Th2 cells, in the healthy and inflamed intestine. However, further studies in mice demonstrated that Th2 cells were largely absent from colonies free of parasitic infection (26). Rodent models of IBD dependent on either Th1 or Th2 responses have been described and characterized, and features aligning CD and UC to each subset have been highlighted (27). However, whereas IFN- γ ⁺ Th1 cells could be clearly demonstrated in CD, IL-4⁺ cells were reported to be reduced in UC, although IL-5 and IL-13 were elevated in the inflamed intestine (28, 29). Later studies provided some explanation of these observations, with the finding of an increased population of IL-5 and IL-13 expressing non-classical CD1d-restricted NKT cells in active UC (29). More recently, description of the Th17 subset of CD4⁺ T cells has led to a reevaluation of the nature of the T-cell populations driving both experimental models of disease and human IBD. Characterized by expression of the transcription factor retinoic acid-related orphan receptor γ t (ROR γ t) and the production of cytokines including IL-17A, IL-17F, IL-21 IL-22, granulocyte-macrophage colony-stimulating factor (GM-CSF), and in humans IL-26, Th17 cells have subsequently been shown to represent a major cellular population within both steady-state and inflamed intestinal mucosae (30).

Intestinal Th1 cells

Critically required for adequate responses to intracellular bacteria and viruses, Th1 cells develop via a molecular program involving activation of signal transducer and activator of transcription 1 (STAT1) and STAT4 by IFN- γ and IL-12, respectively, which leads to the induction of T-bet, an essential transcription factor for the development of Th1 cells. T-bet drives the upregulation of the IL-12R β 2 thereby increasing sensitivity to IL-12 and acts synergistically with STAT4 to drive optimal expression of IFN- γ (31, 32). The type I interferons IFN- α and IFN- β as well as the IL-12 family cytokine IL-27 can also contribute to Th1 differentiation through effects on T-bet induction (31, 33).

Murine models of chronic IBD, including colitis induced by the transfer of naive CD4⁺CD45RB^{hi} T cells into recombinase activating gene deficient (Rag^{-/-}) hosts and bacterially driven intestinal inflammation in the context of IL-10 deficiency, can be effectively prevented by treatment with IFN- γ neutralizing antibody (34, 35). However, T-cell-derived IFN- γ appears to be dispensable for disease pathogenesis (36). Additional studies suggested a highly temporal dependency for IFN- γ in intestinal inflammation, with early but not late administration of an anti-IFN- γ antibody providing protection (34). A role for Th1 cells in intestinal immune pathology is further supported by findings that transfer of naive T cells deficient in key Th1-associated transcription factors T-bet or STAT4 fails to induce disease in the T-cell transfer model of colitis (36, 37). Together these data suggest either redundancy in the development and function of Th1 cells (i.e. STAT4 or T-bet-driven IFN- γ -independent responses) or that other subsets or mediators drive disease in their absence.

Accumulation of T-bet⁺ and IFN- γ ⁺ CD4⁺ T cells is a feature of CD and nuclear extracts from mucosal T cells from patients with CD contain high levels of phosphorylated STAT4 and T-bet and increased expression of IL-12R β 2 (28, 37, 38). Interestingly, an anti-IFN- γ monoclonal antibody was shown to be ineffective in the treatment of established active CD, mirroring the observations from murine systems (39).

The role of the myeloid-derived cytokine IL-27 in regulating intestinal T-cell responses recently has received significant attention. In the steady state, IL-27-receptor deficiency is associated with reduced Th1 populations in the murine colon and MLNs (40). *In vitro*, IL-27 drives Th1 differentiation through STAT-1 and T-bet-dependent effects, and upregulation of IL-12R β 2, implying that the IL-27/T-bet axis might be an important mediator of disease (41). Furthermore, single-nucleotide polymorphisms (SNPs) near the IL27 gene are associated with early onset IBD (42).

However, additional contrasting effects observed including suppression of effector T-cell proliferation, antagonism of Th2 and Th17 development, and augmentation of IL-10 production have been noted in other systems (43). In IL-10R knockout mice, deficiency of IL-27R significantly delayed but did not prevent the development of colitis and was associated with reduced expression of Th1-associated genes such as IFN- γ and IL-12p35 (44). By contrast, in a T-cell transfer model of colitis, IL-27R^{-/-} T cells were impaired in their ability to induce intestinal inflammation, associated with reduced accumulation of IFN- γ ⁺ Th1 cells

within lymphoid tissues, including the MLNs (45). Intriguingly, Th1 populations in the colonic LP were largely unaffected. However, the most striking observation was the marked shift toward forkhead box protein 3 (Foxp3)⁺ T-regulatory cells (Tregs), in line with *in vitro* evidence that IL-27 can suppress TGF- β 1-mediated Foxp3 induction in a cell-intrinsic STAT-3-dependent manner (46). The recent finding that IL-27 can also promote protective Treg responses in the intestine during infection with *Toxoplasma gondii* adds further complexity to the biology of this cytokine (47). Further understanding of the pleiotropic effects of IL-27 in intestinal immunity and how this pathway may be manipulated therapeutically remains of significant interest.

Intestinal Th17 cells

Studies of the role of the Th1-polarizing cytokine IL-12 in models of IBD demonstrated significant protection from disease to be afforded by antibody-mediated blockade of the IL-12p40 subunit (36, 48, 49). However, it was also noted that whereas IFN- γ blockade was only effective when administered at the onset of disease, treatment with anti-IL-12p40 could effectively treat established disease (48). Although initially interpreted as showing additional effects for IL-12 beyond driving IFN- γ production from Th1 cells, the description of IL-23, a novel heterodimeric cytokine comprising the IL-12p40 subunit combined with a novel IL-23p19 subunit, led to a re-evaluation of these findings (50). Studies using a variety of approaches to target the p19 subunit have now revealed IL-23 to instead be the critical cytokine required for the development of intestinal inflammation in a variety of both acute and chronic murine models (51–54). The functional receptor for IL-23 is composed of IL-12R β 1 and the IL-23R subunit, and signals predominantly through Jak2-STAT3 but can also weakly activate STAT1, STAT4, and STAT5 (55). It should be noted that limited information exists with regard to signaling events downstream of IL-23R and a detailed understanding of the IL-23 signaling pathway is currently lacking. Genome-wide association studies in human IBD have provided strong evidence of a functional role for the IL-23 axis in intestinal inflammation. SNPs linked to IBD have been identified in key components of this axis including IL23R, IL12B (encoding IL-12p40), JAK2, STAT3, and CCR6 (56, 57).

Because Th17 cells have been implicated in the pathogenesis of IBD, it was originally thought that IL-23 is necessary for the generation of Th17 cells. However, it is now clear that IL-23 is dispensable for Th17 differentiation, and

emerging data suggest that rather than driving Th17 development, IL-23 modulates Th17 effector function and pathogenicity (58–60). The development of Th17 cells in mice requires the combination of signals via the TCR and from TGF- β 1, IL-1 β , and a STAT-3-activating cytokine, such as IL-6 or IL-21 (61). Downstream of the TCR, the transcription factors BATF and IRF4 mediate chromatin accessibility, and together with STAT3, these factors initiate a transcriptional program that is modulated further through activation of the lineage-specific transcription factor ROR γ t, which drives expression of key genes in Th17 cell differentiation (62). Importantly, sustained expression of ROR γ t is required for the maintenance of the Th17 program as small molecule inhibitors of ROR γ t downregulate Th17 signature genes, such as IL-17A and IL-23R, in established Th17 cells and inhibit Th17-driven immunopathology *in vivo*, making this pathway especially amenable to therapeutic intervention (63, 64).

In the steady state, Th17 cells preferentially locate to the LP of the small intestine and to a lesser extent the colon, where they produce IL-17A but not IFN- γ (65). In contrast to other mucosal sites, CD4⁺ T cells are the dominant IL-17A-expressing cells in homeostatic settings within the small intestine of adult mice (66). Interestingly, expansion of Th17 cell populations in the small intestine may occur in the setting of extraintestinal infectious or autoimmune disease, without detectable mucosal inflammation (67). Loss of Th17 cells into the intestinal lumen has recently been proposed as a mechanism controlling this population, although the general relevance of this remains to be established (67). The murine small intestine therefore appears to be an important site for the generation and regulation of Th17 cells. The basis for this observation might reflect the presence of specific antigens, distinct population of APCs, or the prevailing cytokine milieu, and is an intense area of study. The absence of Th17 cells in the intestines of germ-free mice provided an early indication of the critical role of components of the microbiota in the generation of such cells (66, 68), although conflicting reports as to the status of Th17 populations in the absence of intestinal bacteria have been published (69). The role of the microbiota in the development of Th17 cells is discussed in detail below.

The importance of the intestine as a site of Th17 cell development may reflect the prevailing cytokine environment, which is rich in TGF- β 1, IL-1 β , and IL-6. However, the persistent presence of large numbers of Th17 cells in the healthy intestine also argues against a simple, primarily pathogenic, function for Th17 cells. In the steady state, mice

deficient in ROR γ t or STAT3 have markedly reduced Th17 cell numbers, although a small population persists, thought to develop via an alternative ROR α -dependent pathway (65, 70–72). Studies of mice deficient in cytokines involved in Th17 differentiation indicate that important differences in developmental requirements may exist between intestinal and systemic populations. Based on studies in mice deficient in myeloid differentiation factor 88 (MyD88), which is downstream of Toll-like receptor (TLR) and IL-1R signaling pathways, initial reports concluded that Th17 cells were unaffected by the absence of IL-1 β signaling (66, 68). In contrast, significantly attenuated numbers of intestinal Th17 cells were seen in mice deficient in IL-6, despite normal peripheral populations (65, 73). By contrast, it was recently reported that IL-6 may be dispensable for the development of steady-state intestinal Th17 cells, but that IL-1 β is both necessary and sufficient to drive mucosal Th17 accumulation in a cell-intrinsic manner, but has less consistent effects on systemic populations (74). These seemingly contrasting findings may reflect differences in the definition of Th17 cells. The latter study used a reporter-mouse approach and defined Th17 cells based on expression of Ror γ t^{gfp}, whereas earlier studies used IL-17A production following *ex vivo* restimulation to quantify Th17 cells, an approach that may overestimate the prevalence of such cells (75). Intestinal Th17 cell numbers are maintained or even expanded in IL-21R-deficient mice, confirming results in inflammatory settings where the contribution of IL-21 to Th17 differentiation is only revealed in the absence of IL-6 (61, 66). The intestines of mice deficient in TGF- β 1, or having impaired ability to activate the latent form of TGF- β 1 have been reported to be effectively devoid of Th17 cells (66, 76). However, more recently it has been reported that murine Th17 cells may not require TGF- β 1 for their development (59).

The factors controlling intestinal Th17 accumulation in states of inflammation may differ from those operating in the steady state. For example, IL-1R-deficient CD4⁺ T cells are unaffected in their ability to differentiate to Th17 cells, but are impaired in their accumulation in the colonic LP in T-cell transfer colitis (77). In addition, in a model of chronic inflammation of the central nervous system-specific deletion of TGF- β 1 in T-effector cells was shown to prevent the development of disease, suggesting that Th17 cells may amplify their own differentiation in an autocrine manner (78). Thus, the *in vivo* cytokine requirements for Th17 development in the intestine and beyond require further clarification.

The lack of clarity around this issue may reflect the functional heterogeneity of Th17 cells studied in different ana-

tomical locations and in homeostasis and inflammation. Historically, Th17 cells have been defined by production of IL-17A; however, it is increasingly apparent that the functional characteristics of these cells are determined by the combination of a number of cytokines. Studies of *in vitro*-differentiated cells, and those generated in mice in the absence of specific cytokines involved in Th17 development, suggest significant differences may exist in the pathogenic potential of the resulting T cells (58, 79). Examination of the transcriptional profile of such cells has revealed signatures associated with pathogenicity in models of extraintestinal inflammatory disease (59). A recent study by Kuchroo et al. (80) identified TGF- β 3 as a novel target of IL-23 in CD4⁺ T cells. The authors found that TGF- β 3, mainly produced by Th17 cells themselves in response to IL-23, drives a distinct transcriptional signature in Th17 cells that is associated with heightened pathogenic potential. Although the role of TGF- β 3 in intestinal inflammation is currently unclear, the important question of the relationship of intestinal steady-state Th17 cells to those implicated in driving intestinal inflammation clearly requires further study, including definition of their corresponding transcriptional and functional attributes.

In both mice and humans, impaired Th17 responses through deficiency in critical molecular components of this axis are associated with disease. Mutations in STAT3 result in hyper-immunoglobulin E (IgE) syndrome (Job's syndrome), characterized by a marked reduction in the number of IL-17-secreting cells in the periphery and susceptibility to recurrent *Staphylococcal* and fungal infection (81, 82). Impaired Th17 development due to gain-of-function STAT1 mutations is associated with chronic mucocutaneous candidiasis (83), a disease also seen in patients with circulating neutralizing antibodies against key effector cytokines of the Th17 axis, including IL-17A, IL-17F, and IL-22 (84). Together, these data indicate that Th17 cells are an important component of host defense at mucosal surfaces including the intestine.

However, accumulation of IL-17A-expressing cells in the mucosa is an important feature of numerous models of intestinal inflammation. In human CD, Th17-associated molecules such as IL-17A, IL-17F, IL-21, IL-22, CCR6, and IL-23R are increased compared with controls or UC patients; however, similar elevations in UC have been noted by other groups (85).

Mediators of Th17 effector T-cell function

Although a role for the IL-23/Th17 pathway has been convincingly demonstrated in multiple murine models of

intestinal inflammation, the roles of its downstream mediators are less well understood. Whereas the transfer of wild-type (WT) naive CD4⁺ T cells into *Rag*^{-/-} hosts results in intestinal inflammation and wasting, the transfer of *RORγ*^{-/-} or *IL-23R*^{-/-} T cells, or the use of *IL-23p19*^{-/-} recipient mice, results in highly attenuated intestinal disease (60, 86, 87). In mice receiving WT T cells, significant elevation in multiple Th17 cytokines is observed in the intestinal mucosa. By allowing the transfer of T cells deficient in individual cytokines, this model is a particularly elegant system in which the specific role of CD4⁺ T-cell-derived cytokines in intestinal inflammation can be examined.

IL-17A and IL-17F

Using this approach, multiple groups have now demonstrated that T cells deficient in IL-17A are unimpaired in their ability to drive both the intestinal and systemic features of this model (86–88). Indeed, transfer of IL-17A-deficient T cells may result in an exacerbated wasting phenotype, proposed to occur through a T-cell-intrinsic requirement for IL-17A signals to suppress IFN- γ production (89). In contrast, disease induced by Treg-specific STAT3 deficiency was significantly attenuated by anti-IL-17A treatment indicating a pathogenic role in some settings (90). Similar mixed findings have been observed using chemically induced models of colitis where epithelial injury is a prominent feature of the pathogenesis. Thus, in dextran-sodium sulfate (DSS) colitis, both protective (91, 92) and pathogenic roles (93) for IL-17A have been documented. However, chemically driven models of disease are T-cell independent, somewhat complicating the interpretation of these findings.

A number of the functions of IL-17A, such as prominent effects on neutrophil recruitment, are shared by the related cytokine IL-17F, as both cytokines utilize a common heterodimeric signaling receptor composed of IL-17RA and IL-17RC subunits (94). Studies in T-cell transfer colitis suggest redundant effects of these cytokines in driving colitis as only concomitant ablation of IL-17A and IL-17F resulted in attenuated colitis (86). In *Citrobacter rodentium* infection, a murine model of enteropathogenic *Escherichia coli* infection, both IL-17A and IL-17F exert protective effects (95). However, in other systems, such as DSS colitis, the function of IL-17F appears to oppose that of IL-17A, exacerbating disease (92). Together the data paint a complicated picture of the roles of IL-17A and IL-17F in intestinal inflammation that encompasses both redundancy and a contextual nature that

may be dictated by specifics of the model of inflammation or environmental conditions.

Interestingly, recent experience in a clinical trial of an anti-IL-17A monoclonal antibody in active CD appears to confirm a similar redundancy or possibly even protective effect for IL-17A in established human disease (96).

IL-22

IL-22 is produced by Th17 cells as well as other T cells and appears to have an IL-23 dependency (97). In *IL-23p19*^{-/-} mice, whereas overall IL-17A⁺ CD4⁺ T cells are relatively unaffected, a specific reduction in IL-17⁺IL-22⁺ cells has been reported (66). In human IBD, serum IL-22 levels are increased in active disease, and may correlate with disease-associated IL23R polymorphisms (98, 99). Interestingly, it has been reported that remission in UC is associated with the presence of IL-17A⁺IL-22⁺CD4⁺ T cells, in contrast to the absence of IL-22-producing cells in active disease (100).

IL-22R is highly expressed on non-hematopoietic cells of the intestine, including stromal and epithelial cells, and activates STAT3 to promote the elaboration of antimicrobial peptides, including RegIII β , RegIII γ , and mucins (97). IL-22 has been reported to play a protective role in experimental models of IBD, including CD45RB^{hi} T-cell transfer, DSS colitis, and in the spontaneous intestinal inflammation developing in *TCR α* ^{-/-} mice (101, 102). Importantly, whereas IL-22 is protective in the *Citrobacter rodentium* model of acute colitis (101), non-Th17 cell sources may be critical, including CD4⁺ lymphoid tissue-inducer (LTi) cells (103). In contrast with these protective properties, IL-22 has been shown to promote colitis in some settings. Colitis induced in *Rag*^{-/-} mice transferred with Treg-depleted CD45RB^{lo} memory T cells is in part driven by IL-22 (104). Similarly, colitis induced by *Helicobacter hepaticus* infection is promoted by IL-22 in both innate and lymphocyte replete models (authors' unpublished observations). In reconciling these apparently contradictory results, it is interesting to note that highly context-specific effects of IL-22 are seen in experimental lung inflammation, dependent upon the presence of IL-17A (105). Such context-specific effects await exploration in the setting of intestinal disease.

IL-21

The role of IL-21 in intestinal inflammation is less well characterized. IL-21 is both an important differentiation factor and a product of Th17 cells, and it may prevent Treg development (61). In both acute and chronic DSS-induced

disease and in colitis induced by treatment with trinitrobenzene sulfonic acid (TNBS), IL-21 has been shown to be pathogenic (106, 107). Mechanisms for this may include effects on the generation of Th17 cell responses and upon intestinal chemokine secretion and metalloproteinase production. However, while IL-21 is an important Th17 cell mediator, it is produced in large quantities by other cells, including NK cells (61). In the intestine in active human IBD, the majority of IL-21-producing cells are IFN- γ ⁺ Th1 cells and are not IL-17A⁺ (108). Therefore, any specific requirement for Th17 cell-derived IL-21 in disease pathogenesis remains to be further defined.

GM-CSF

GM-CSF has recently been proposed to be a critical pathogenic effector of Th17 responses in experimental autoimmune encephalomyelitis (EAE), a murine model of multiple sclerosis (109, 110). In this model, GM-CSF is produced by both Th1 and Th17 cells, with production downstream of IL-1 β and IL-23. In the context of intestinal inflammation, antibody-mediated neutralization of GM-CSF ameliorates disease (111), whereas GM-CSF-deficient T cells were unimpaired in their ability to induce colitis (77), suggesting that T cells may not be an essential source of GM-CSF in the intestine. The colitogenic potential of GM-CSF involves the accumulation of granulocyte-macrophage progenitors in the intestine linking an inflammatory Th17 response to dysregulated hematopoiesis (111).

Plasticity of intestinal effector T cells

It is increasingly clear that Th17 cells present in the intestine during inflammation may differ from steady-state populations in terms of their effector functions. Whereas steady-state Th17 cells are largely IL-17A single positive, significant proportions of the cells accumulating in inflammatory settings produce additional mediators such as IFN- γ or GM-CSF (60, 109, 110). Human Th17 cells display differential cytokine secretion patterns depending on the eliciting pathogen. Whereas Th17 cells generated in response to *Candida albicans* produce IL-17A and IFN- γ , *Staphylococcus aureus*-specific Th17 cells produce IL-17A and the immunoregulatory cytokine IL-10 (112). In mice, colitis is associated with the accumulation of IL-17A⁺IFN- γ ⁺ CD4⁺ T cells in the mucosa in an IL-23-dependent manner (60). In this scenario, IL-23 functions through effects on proliferation within the intestine, as well as directly promoting the emergence of IL-17A⁺IFN- γ ⁺ cells in a cell-intrinsic manner. Interestingly, abrogation of IL-23

signaling does not affect the presence of IL-17A⁺IFN- γ ⁻ or IL-17A⁻IFN- γ ⁺ cells in this model.

IL-23 signals into Th17 cells were shown to drive IFN- γ production in a STAT4 and T-bet-dependent manner *in vitro* (113). Therefore, an important question to ask is what relationship mucosal IFN- γ single-positive cells have to Th17 cells in terms of *in vivo* plasticity, as the developmental pathways of Th1 cells present in the steady-state or inflamed intestine are incompletely defined. Recently, fate mapping experiments of IL-17A-producing T cells during the course of EAE demonstrated that the majority of IL-17A⁺IFN- γ ⁺ and IFN- γ single-producing cells arise from Th17 cell progeny (75). Importantly, this transition of Th17 cells into IFN- γ -producing T cells required IL-23 and correlated with increased expression of T-bet. A similar experimental approach will be required to delineate the origins of IFN- γ ⁺ CD4⁺ T cells in homeostatic and inflammatory settings in the intestine and could provide important mechanistic insights into the role of plasticity in intestinal immunity. Significantly, the emergence of a population of T cells capable of producing both IL-17A and IFN- γ has also been described in intestinal biopsies of IBD patients (114, 115).

Human T-cell clones derived from the mucosa of patients with CD were able to convert from Th17 or Th1/Th17 phenotypes to IL-17A⁻IFN- γ ⁺ Th1 cells following *in vitro* stimulation with IL-12, indicating similar plasticity or progression may occur in human T cells, although the specific signals required may differ (114). In this regard, murine studies have highlighted the importance of context and prevailing cytokine environment in regulating Th17 to Th1 transition. Stimulation of Th17 cells with IL-12 could drive the acquisition of a Th1 phenotype *in vitro* even in the presence of TGF- β 1; however, IL-23 could exert a similar effect only in the absence of TGF- β 1 (113). Furthermore, continued antigenic stimulation via the TCR also favored progression from a Th17 to Th1 phenotype *in vitro*. Such studies highlight the important role of TGF- β 1 in controlling T-cell fate.

The emergence of mixed Th1/Th17 cells in the inflamed mucosa, in contrast to their absence in the steady state suggests such cells may contribute to the pathogenesis of disease. Interestingly, in disease induced by the transfer of C_Bir-specific T cells, recognizing bacterial flagellin, severity correlated with the proportion of Th1/Th17 cells present in the LP. Furthermore, using *in vitro*-differentiated cells polarized to Th1 or Th17 phenotypes, it was shown that whereas transfer of Th1 cells resulted in mild disease, transfer of similar numbers of Th17 cells induced severe colitis (116, 117). Analysis of the colons of animals receiving Th17 cells

revealed populations of Th17, Th1/Th17, and Th1 cells, demonstrating the potential for plasticity of Th17 cells in the intestine *in vivo*. Although T-cell plasticity has been studied extensively *in vitro*, the anatomical sites relevant to intestinal T-cell plasticity remain unknown.

In addition to the ability to adopt a Th1/Th17 phenotype, Th17 cells may also acquire IL-10 production under appropriate circumstances, a phenomenon reported for all known lineages of effector T cells and thought to be a feature of continued antigenic stimulation (118). Acquisition of IL-10 production by Th17 cells may be an important regulatory mechanism for such cells, as suggested by the appearance in the intestine of IL-17A⁺IL-10⁺ T cells following treatment with anti-CD3 antibody, which is known to result in an immunosuppressive environment within the intestinal mucosa (67). While further definition of the role of such cells in intestinal immunity is awaited, in experimental neurological inflammation exposure of Th17 cells to IL-23 limits IL-10 production and promotes pathogenicity of Th17 cells, suggesting that inhibition of IL-10 might abrogate the regulatory capacity of Th17 cells (79). Beyond an ability to express IL-10, Th17 cells exhibit a specific developmental relationship with Foxp3⁺ inducible Tregs (iTregs), discussed in detail below.

Intestinal regulatory CD4⁺ T cells

Despite the presence of a large population of potentially pathogenic effector T cells in close proximity to the largest antigen load in the body, the majority of individuals do not develop intestinal inflammation. This clearly points to the presence of dominant suppressive and regulatory mechanisms, restraining innate and T-cell responses in the steady state (2). In mice, early adoptive transfer experiments demonstrated such regulatory activity to be a function of CD4⁺ T cells contained within the CD45RB^{lo} population of antigen-experienced cells, in particular those expressing CD25, the α -chain of the IL-2 receptor (119). Cotransfer of CD45RB^{lo} or CD25⁺ cells prevented the development of colitis normally seen with the transfer of naive T cells alone and could cure disease in some settings. These observations established the concept of an important functional role for CD4⁺ Treg cells in maintaining intestinal homeostasis and led to efforts to identify further phenotypic markers and effector mechanisms utilized by regulatory populations. A number of subsets of T cells possessing regulatory or suppressive activity have now been characterized, but those expressing the transcription factor Foxp3 and IL-10-producing

cells appear to be of particular functional importance in intestinal homeostasis and the control of inflammation (2).

In comparison with systemic immune compartments, the intestine is enriched for the presence of Treg cells. In the colon of specific pathogen-free (SPF) mice, approximately 30–40% of CD4⁺ T cells produce IL-10, with the overwhelming majority coexpressing Foxp3 (120). Although IL-10⁺Foxp3⁺ Treg cells are also found in abundance in the small intestinal LP, a sizable fraction of IL-10⁺ CD4⁺ T cells in this location do not express Foxp3, exhibiting a Tr1 phenotype (121). Notably, such cells are also the major IL-10-producing population within the small intestinal IELs, but are rare at any site within the colon. These data support the concept of highly compartmentalized regulatory cell populations and suggest overlapping, but non-redundant functions for these cells.

The anatomical basis of the interaction between regulatory and effector T cells is incompletely understood. In the prevention model of T-cell transfer disease, in which Treg cells are cotransferred with naive CD45RB^{hi} CD4⁺ cells, both cell types preferentially accumulate in lymphoid tissues outside of the intestine (122). By contrast, where Treg cells are transferred into mice with established disease, regulation appears to occur within the intestinal LP (120, 123). Precisely how specific anatomical interactions between effector and regulatory populations influence regulatory T-cell adaptation and function is an important area for further study.

Foxp3⁺ Treg cells

An essential functional role for Foxp3 in restraining inflammatory responses in the normal intestine is demonstrated by the development of intestinal inflammation as a key feature of disease in the immune dysregulation, polyendocrinopathy, enteropathy, X-linked syndrome, which develops in humans with germ line mutations in Foxp3 (124). Natural Treg cells (nTregs) develop in the thymus under the influence of appropriate TCR stimulation, which activates NF- κ B-dependent pathways and suppresses the phosphoinositol 3-kinase-Akt-mammalian target of rapamycin pathway. Further development requires signals from the costimulatory molecule CD28 and common γ chain cytokines, including IL-2 and IL-15, to activate STAT5 (125). Expression of Foxp3 is considered essential to the regulatory properties of these cells.

In addition to thymus-dependent nTreg cells, the intestinal Foxp3⁺ cell population contains a large population of non-thymically derived Foxp3⁺ iTreg cells. Although nTreg

and iTreg cells have been shown to have different transcriptional signatures (126, 127), they are hard to distinguish once generated. Recently, markers including Helios, a member of the Ikaros family of transcription factors, and the transmembrane protein neuropilin-1 have been reported to permit segregation of iTreg from nTreg cells (128–130). Although the validity of such markers continues to be debated, it is notable that up to 45% of total colonic Foxp3⁺ cells in the intestine can be defined as iTregs by such criteria. Such cells are conspicuously reduced in the colons but not MLNs of germ-free mice, and reconstitution with components of the normal microbiota drives the accumulation of Foxp3⁺ cells with a Helios⁺Neuropilin-1⁺ iTreg phenotype in the colon (129).

Recent studies have given some insight into the relative contribution of nTreg and iTreg cells to homeostasis. Specific deletion of the conserved non-coding sequence CNS1 in the Foxp3 locus demonstrated that this element was specifically required for iTreg induction, whereas its deletion did not affect nTreg development (131). Surprisingly, loss of Foxp3 CNS1 led to exacerbated Th2-type immunopathology in the lung and intestine, demonstrating that iTreg cells are particularly important for restraining Th2-type responses at mucosal sites (132). In addition, cooperation between iTreg and nTreg cells appears to be required for protection from the autoimmune disease that develops in Foxp3-deficient mice (133), and protection from colitis induced by transfer of naive CD4⁺ T cells (127). However, the molecular basis of this cooperation is currently unknown.

The large iTreg population in the intestine might reflect the presence of specialized populations of APCs favoring iTreg development (18). Alternatively, this might also reflect the presence of their cognate antigen at that site, whether host or exogenous in nature. Studies of TCR specificities among CD4⁺ T cells recovered from steady-state mice reveal that little overlap exists between regulatory and effector populations (134). Furthermore, TCR repertoires expressed by intestinal Treg cells may differ significantly from cells isolated from secondary lymphoid tissues, with a significant proportion of the most highly represented Treg-TCRs in the colon reported to show marked reactivity against components of the microbiota (135). Interestingly, in the setting of colitis, some CD4⁺ effector T cells express TCRs normally associated with steady-state Treg cells, suggesting that in the appropriate inflammatory environment, such cells might be skewed toward a highly pathogenic effector phenotype.

IL-10⁺ Treg cells

Among multiple regulatory mechanisms described for Foxp3⁺ Treg cells, production of the immunosuppressive cytokine IL-10 is a key pathway. Whereas in the colon Foxp3⁺ cells are the almost exclusive T-cell source of IL-10, in the small intestine Foxp3-IL-10⁺ cells (Tr1 cells) may play an important role (136). Furthermore, IL-10 production by cells primarily demonstrating an effector phenotype is well recognized. IL-10-producing Th1, Th2, and Th17 cells have all been described, with their generation apparently related to chronic immune stimulation (118). However, many IL-10-producing cells, particularly within the small intestine, do not produce additional effector cytokines, and express a more Tr1-like phenotype. Whether this is related to a particular state of differentiation or a *bona fide* functionally specialized T-cell subset remains to be established.

The contrasting phenotypes seen in Foxp3 or IL-10 deficiency point to non-redundant functions for each molecule and cell type in aspects of intestinal immune regulation. Whereas Foxp3-deficient mice develop fatal autoimmunity, ablation of the IL-10 gene renders mice susceptible to intestinal inflammation driven by otherwise non-pathogenic components of the intestinal microbiota, such as *Helicobacter hepaticus* (136). Notably, intestinal disease in IL-10^{-/-} mice is dependent on the presence of CD4⁺ T cells and does not occur in Rag^{-/-} or CD4-depleted animals (137).

The cellular targets of IL-10 in suppressing intestinal inflammation are increasingly understood. Suppression of Th1 CD4⁺ T cells appears not to occur in a cell-intrinsic fashion, which corresponds with the description of low levels of IL-10R expression on such cells (138, 139). Instead effects on APCs, including downregulation of activation, major histocompatibility complex class II antigen presentation, and the expression of costimulatory molecules have been proposed to underlie suppression of Th1-dependent responses (140). However, recent studies suggest this mechanism may be of less importance in Th17-driven disease. High levels of expression of IL-10R on this subset compared with other effector T cells points to a potential direct role for IL-10 signaling in controlling Th17 responses (139).

Using mice expressing a dominant negative-IL-10Ra subunit under control of the CD4 promoter (resulting in T-cell-specific ablation of IL-10 signaling), it was shown that the emergence of intestinal IL-17A⁺ and IL-17A⁺IFN- γ ⁺ cells following anti-CD3 treatment was directly inhibited by cell-intrinsic IL-10 signals. In contrast, IFN- γ ⁺ Th1 cells were

similar in number to those seen in WT mice (139). In models of colitis driven by IL-17A⁺ or IL-17A⁺IFN- γ ⁺ T cells, suppression of disease by Foxp3⁺ Treg or Tr1 cells required direct IL-10 signaling into the effector T cells. Interestingly, Foxp3⁺ cells lacking a functional IL-10R are unable to suppress Th17 cell-driven colitis (141). Similar observations have been made using Treg cells lacking STAT3, a mediator of both IL-10 signaling and IL-10 production in Tregs (90). These results suggest that in addition to direct activity on Th17 effector cells, Treg cells are themselves important targets of IL-10 signaling in the *in vivo* control of inflammatory responses.

The key role of IL-10 in intestinal homeostasis revealed in mouse models has also been validated in human IBD. Loss of function mutations in *IL10*, *IL10RA*, or *IL10RB* is responsible for severe early onset IBD in some infants (142). Furthermore, SNPs in *IL10* have been associated with adult IBD in GWAS studies (25).

TGF- β 1

TGF- β 1 plays a critical role in the development and function of Treg cells, including Foxp3⁺ and Tr1 cells (2). Despite the abundance of other cellular sources within the intestine, T cells may represent a non-redundant population of TGF- β 1-producing cells, as CD4⁺ T-cell-specific TGF- β 1 deficiency can result in spontaneous colitis (143, 144). However, in T-cell transfer models, TGF- β 1^{-/-} Treg cells may retain the ability to suppress disease, although conflicting reports exist in this regard (136). Naive T cells that are unable to respond to TGF- β due to expression of a dominant negative (DN) TGF- β II receptor are refractory to control by Treg cells and induce severe intestinal inflammation (145). Similarly, overexpression of *Smad7*, which inhibits TGF- β 1 signaling, results in colitogenic T cells resistance to suppression by Treg cells (146). Notably, T cells from patients with IBD exhibit upregulation of *SMAD7*. Together the data highlight the role of sensitivity of target T cells to TGF- β for appropriate Treg-mediated control raising the possibility that interference with the *SMAD7* pathway may be a therapeutic target in IBD (147).

IL-35

IL-35 is a recently described heterodimer cytokine expressed at high levels by CD4⁺ Foxp3⁺ Treg cells (148, 149). Comprised of the IL-12p35 subunit of IL-12 and Epstein–Barr virus-induced gene-3 (*Ebi-3*), itself a component of IL-27, IL-35 is produced at high levels by activated Treg cells dur-

ing suppression of effector T cells *in vitro*. Treg cells rendered deficient in IL-35 activity by genetic deletion of *Il12a* (encoding IL-12p35) or *Ebi3* are impaired in their ability to cure intestinal and systemic features of disease in the CD45RB^{hi} T-cell transfer model (149). Furthermore, IL-35 signaling into conventional T cells may induce a suppressive Foxp3⁻ phenotype characterized by production of IL-35 but not IL-10 or TGF- β , which also demonstrates suppressive activity in the cure model of T-cell transfer disease (150). The role of IL-35 in human Treg function is less well documented, although production of biologically active IL-35 from human T cells has been reported (150, 151).

OX40

OX40 is a member of the TNF superfamily of costimulatory molecules, whose expression was originally described on activated conventional T cells, where it may have a functional role in T-cell memory responses (152). However, OX40 expression has also been noted on Treg cells, particularly among colonic Foxp3⁺ populations (153). Whereas OX40 deficiency is associated with reduced steady-state colonic Treg cell populations, it appears to be redundant for their accumulation in inflammatory settings. However, OX40^{-/-} Tregs are unable to suppress disease when co-transferred with CD45RB^{hi} naive T cells, implying a functional requirement for OX40 to control inflammatory effector pathways. Notably, OX40 also promotes colitogenic Th1 responses, highlighting the overlapping costimulatory requirements of effector and regulatory responses (153).

Intestinal regulatory and effector T-cell relationships

Cells coexpressing ROR γ t and Foxp3 are found in intestinal tissues (154, 155). Notably, both Treg cells and Th17 cells require TGF- β 1 for their development, and the presence or absence of further factors, including the STAT3-activating cytokines IL-6 and IL-23, may determine the balance of these populations in the steady-state or inflamed intestine (156). IL-23 favors the accumulation of Th17 cells in the inflamed intestine at the expense of Foxp3⁺ Treg cells in a cell-intrinsic manner (60). Similarly high levels of IL-6, as seen in inflammation, favor a Th17 response, and anti-IL-6R treatment increases the proportion of CD4⁺ T cells expressing Foxp3 in intestinal and peripheral tissues (61). Foxp3⁺ Tregs have also been reported to express key transcription factors associated with other effector T-cell subsets, including STAT3, T-bet, IRF-4, and GATA3 (90, 157–159). Expression of these transcription factors by Foxp3⁺ Treg

cells appears to be an essential component of their adaptation to particular environmental conditions and important for their ability to effectively control specific types of immune responses.

The acquisition of transcription factors more normally associated with effector T cells raises the question of the stability of the immune-suppressive Foxp3-driven program. This is currently hotly debated, and although Foxp3⁺ cells have been shown to acquire effector functions (160), the physiological importance of this in intestinal inflammation remains to be established.

Regulation of the development of intestinal CD4⁺ T-cell subsets

The intestine is a site of immense immune challenge, home to a dense microbiota, numbering up to 10¹⁴ bacteria, as well as viral and fungal components, and a vast array of dietary-derived antigens, adjuvants, and metabolically active components. Unsurprisingly, it has long been recognized that environmental factors influence intestinal T-cell populations and their responses (Fig. 2). Prime among such environmental factors is commensal bacteria. In addition to other immunological defects, germ-free mice exhibit failure

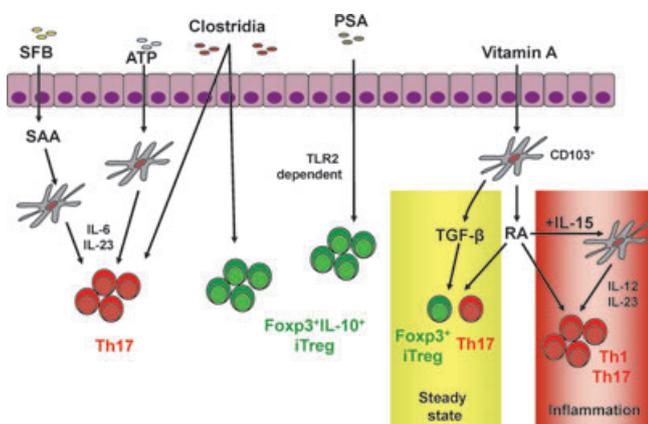


Fig. 2. Regulation of intestinal CD4⁺ T-cell subsets by environmental factors. The microbiota directs the accumulation of both Treg cells and Th17 cells in the intestinal lamina propria. Clostridia species induce IL-10-producing iTregs. Colonization with the human commensal *Bacteroides fragilis* leads to induction of iTregs and IL-10 production through PSA–TLR2 interactions. Segmented-filamentous bacteria promote the induction of Th17 responses, possibly by promoting expression of IL-6 and IL-23 from antigen-presenting cells through serum amyloid A. The vitamin A metabolite retinoic acid can promote both iTreg and effector T-cell differentiation, depending on the prevailing cytokine environment. The relative balance of Treg and Th17 cells can alter the outcome of local as well as systemic immune responses. IL-10, interleukin; PSA, polysaccharide A; TLR, Toll-like receptor.

of development of GALT and reduction in LP T-cell populations (161). Conventionalization of such animals, by co-housing with specific pathogen-free mice, results in development of normal LP T-cell numbers. However, knowledge of the contribution of individual constituents to this process is limited. Recent evidence suggests that species-specific differences exist in the requirement for specific components of the microbiota for immune maturation. However, the mechanisms underlying this are poorly understood.

Th17 cells

Whereas defects in all intestinal T-cell subsets are seen in germ-free mice, a specific deficit in Th17 was noted early in the study of factors involved in their development (66, 68). Using germ-free colonies and mice obtained from multiple commercial sources, it was noted that Th17 cells were absent from the intestines of both germ-free mice and specific pathogen-free animals obtained from Jackson Labs (66). Cohousing Jackson Labs mice with those from other commercial sources restored the lack of Th17 cells, implying an essential role for a transmissible component of the microbiota. Analysis of the composition of the microbiota of several strains of mice revealed the presence of segmented-filamentous bacteria (SFB) to be necessary and sufficient for the generation of Th17 responses in the small intestines of mice (162). SFB belong to a currently uncultivable group of spore-forming Gram-positive bacteria related to Clostridia, and SFB are resident in the flora of the terminal ileum in many mouse colonies, including those maintained under SPF conditions. Mechanistically, it has been shown that SFB may drive Th17 accumulation in the small intestinal LP via a serum amyloid A protein-dependent effect, resulting in IL-6 and IL-23 production from dendritic cells (162). Other groups have reported that SFB may exert more diverse effects on intestinal T-cell populations, including Th1 and Treg cells (163). Interestingly, in mice expressing human α -defensin, reduced Th17 populations were associated with a reduced intestinal burden of SFB, whereas Th1 cells were unaffected (164).

The molecular and functional attributes of SFB inducing the development of Th17 cells remain unclear. The recent sequencing of the SFB genome may result in important insights into functional aspects of microbial determinants of intestinal T-cell development (165, 166). Whether additional species of bacteria are similarly able to induce intestinal Th17 accumulation awaits further study. Importantly, colonization of germ-free mice with a defined

cocktail of innocuous bacteria (excluding SFB) contained in altered-Schaedlers flora was able to promote Th17 accumulation when IL-10 signaling was blocked, demonstrating that Th17 responses can be generated in the absence of SFB when regulatory pathways are defective (167).

The presence of Th17 cells in mice lacking key molecules involved in the sensing of microbes makes it quite unclear how the necessary host–microbiota interactions are mediated. MyD88^{-/-}Trif^{-/-} double knockout mice, which lack functional signaling via the Toll-like receptor pathway, generate normal Th17 responses in the steady state (66, 68). Similarly, deficiency of receptor interacting protein 2 (RIP2), an adapter of the nucleotide oligomerization domain (NOD) pathway, does not impair Th17 development (162). Intriguingly, Tlr9^{-/-} mice are reported to have altered mucosal Th17/Treg ratios, but this is difficult to reconcile with the reported lack of effect of MyD88 deficiency, through which TLR9 signals (168). Similarly, interaction with microbes via other mechanisms, including sensing of bacteria-derived ATP, has been proposed, but is unlikely to explain the specific effect of SFB as it does not activate this pathway (68). Furthermore, the requirement for specific pathways to drive Th17 cell development in conditions of infection or inflammation may differ. TLR1 has been reported to be required for the generation of protective Th17 responses in oral *Yersinia enterocolitica* infection, via an IL-6- and IL-23-dependent mechanism (169). An important unresolved issue is the antigen specificity of Th17 cells generated in response to microbial colonization. It has been suggested that intestinal Th17 cells arise in the absence of cognate antigen, but whether this is a general phenomenon remains to be determined (170).

Treg cells

Similar to the observations made in studies of Th17 accumulation during ontogeny (66), intestinal Foxp3⁺ or IL-10⁺ cell numbers increase rapidly in the first month of life in mice, with a rapid accumulation seen at the time of weaning (171). In contrast to observations for Th17 cells, Treg populations are maintained in the small intestines of germ-free animals (66, 171, 172). By contrast, Treg cells within the colon are highly dependent on the presence of the flora, being significantly reduced in germ-free conditions or by antibiotic treatment, and accumulating when germ-free mice are ‘conventionalized’ by feeding fecal pellets from SPF animals. In such studies, analysis of specific components of the flora has implicated a key role for members of the clostrid-

ium family, particularly clusters IV and XIVa. Colonization with a defined flora composed of 46 *Clostridium* species was sufficient to induce accumulation of CTLA-4^{hi} IL-10⁺Helios⁻ iTreg cells in the colon (171). Notably, achieving greater levels of colonization resulted in more pronounced Treg activity and protection in DSS and oxazolone-induced colitis. Although species-specific differences in the composition of the microbiota might limit the translational significance of these findings, it is notable that patients with IBD have a lower representation of clostridia within their flora, including reduced clostridium clusters IV and XIVa (173, 174).

No difference in the small intestinal Treg population was seen in either germ-free mice or those colonized with the cocktail of clostridia, suggesting important differences in the biology of regulatory cells exists within discrete compartments of the intestinal tract. Furthermore, as is the situation for Th17 cells the relevant pathways driving host–microbial interaction in this scenario are uncertain, as Treg populations were unaffected by deficiency of MyD88, Rip2, or Card9 (171).

Colonization with the human symbiont *Bacteroides fragilis* has been reported to protect mice from *Helicobacter hepaticus*-driven pathology in a variation in the T-cell transfer model, via the induction of IL-10 production from CD4⁺ T cells (175). This protection was dependent on microbial expression of polysaccharide A (PSA). Monocolonization with *B. fragilis* was reported to induce functional IL-10 producing iTregs in a PSA and TLR2-dependent manner (176, 177). Interestingly, such cells did not produce TGF- β 1 or the Ebi-3 component of IL-35, but were able to afford protection in the TNBS model of colitis. Notably, the effect of PSA on mucosal T cells, favoring regulatory responses, contrasts with its Th1-promoting effects on cells within secondary lymphoid tissues (178). The intestinal bacteria *Faecalibacterium prausnitzii*, isolated from the flora of patients with CD, has also been shown to promote IL-10 production, suggesting this characteristic may be conserved across multiple species of bacteria (174).

Retinoic acid

An important role for the vitamin A derivative retinoic acid (RA) in intestinal immune responses has been demonstrated in numerous experimental systems. Such studies highlight multiple mechanisms by which RA might exert its effects.

Mice with impaired RA signaling or those fed diets deficient in vitamin A have reduced populations of activated T cells in the intestinal LP (179). This may reflect a requirement for RA for the upregulation of gut-homing molecules

including $\alpha_4\beta_7$ and CCR9 on T cells. This imprinting is mediated by populations of specialized macrophages and DCs within the LP and GALT, characterized by the expression of CD103 (α_E -integrin), and capable of metabolizing vitamin A to RA via retinal dehydrogenases (RALDH) (18). Mucosal and MLN CD103⁺ DCs are enriched for basal expression of *Aldh1a2*, encoding RALDH2, whereas CD103⁺ DCs present in other immune compartments express much lower levels of RALDH2. Interestingly, RALDH expression may be linked to the presence of the intestinal flora, with TLR2 activation shown to be a particularly potent inducing signal (180). Furthermore, intestinal epithelial and MLN stromal cells are also capable of metabolizing RA and may support local conditioning of DCs (18, 181). Therefore, multiple specialized cell populations within the intestine may be involved in the regulation of RA metabolism and hence influence downstream effects on T cells.

In addition to effects on T-cell homing, RA may also directly influence the differentiation and development of T-cell subsets in the intestine. Along with TGF- β 1 and IL-2, RA acts as a cofactor in the generation of iTregs, also via a CD103⁺ APC-dependent mechanism (18). Vitamin A-deficient mice demonstrate impaired iTreg generation in response to oral antigen feeding in an oral tolerance model (182). However, the effect of vitamin A deficiency on induction of oral tolerance may relate to combined effects on both homing and iTreg development (183). Vitamin A-deficient mice have maintained overall Treg cell populations, and IL-10⁺ Tr1 cells are unaffected, implying the requirement for RA signals is specific to Foxp3⁺ iTreg cells (182, 184). However, RA alone is insufficient to induce upregulation of Foxp3, and RA requires the additional presence of TGF- β 1 (179). The mechanism underlying this synergy may reflect activity of both RA and TGF- β 1 on SMAD3 pathways.

Conversely, RA may inhibit the Th17 axis, via inhibition of IL-6 and IL-23R expression and consequent repression of Th17 development (179). However, it is notable that steady-state intestinal Th17 populations are ablated in vitamin A-deficient mice, associated with reduced levels of the cytokine IL-6. These data therefore suggest that RA may exert paradoxical effects on Th17 cells related to the nature of the exposure and additional factors, such as activation status and prevailing cytokine environment. In support of this concept is the interesting observation that in the presence of high levels of IL-15, a scenario observed in the intestine in human celiac disease, RA acts as an adjuvant to suppress iTreg generation while favoring effector T-cell responses (185). RA acted at the level of the DC, increasing IL-15-dri-

ven IL-12 and IL-23 production, and directly on T cells, where it synergized with these downstream cytokines, favoring Th1 and Th17 responses.

Manipulation of intestinal T cells in the treatment of IBD

In view of the central role of CD4⁺ T cells in intestinal homeostatic or inflammatory responses, manipulation of constituent subsets may be a highly effective therapeutic approach. Intestinal CD4⁺ T cells isolated from the inflamed intestine in IBD demonstrate relative resistance to apoptosis (186). Therefore, approaches to reinstate sensitivity to induction of apoptosis represent an important potential mechanism of action for novel therapeutics.

The efficacy of two major classes of drugs currently used in the treatment of IBD has been linked to induction of apoptosis in CD4⁺ T cells. Anti-TNF monoclonal antibodies induce rapid apoptosis in colonic mucosal T cells *in vivo*, and this has been linked to therapeutic efficacy (187). Furthermore, whereas anti-TNF drugs which do not induce T-cell apoptosis are effective in treating other systemic inflammatory diseases, their lack of benefit in IBD has been suggested to reflect a specific and critical requirement for this mechanism of action for therapeutic efficacy in IBD (188).

Among multiple other roles, IL-6 is proposed to be an important mediator of antiapoptotic pathways in IBD in a STAT3-Bcl-dependent manner (189). Antibody blockade of IL-6R signaling renders T cells susceptible to apoptosis, and treatment with an anti-IL-6R antibody suppressed colitis in the T-cell transfer model (88). Investigation of the efficacy of anti-IL-6 therapies in IBD is limited.

Another major class of drugs currently used in the treatment of IBD, the thiopurines (azathioprine and 6-mercaptopurine), may also exert their effects via induction of CD4⁺ T-cell apoptosis *in vivo* through a mechanism involving the CD28-Rac-1-Vav1 pathway (190).

The complementary approach of preventing accumulation of CD4⁺ T cells in the gut through targeting of molecules involved in the homing of cells to the intestine is currently the subject of intense interest. Trials of anti- α_4 integrin treatment have demonstrated efficacy in active CD, but their widespread use in IBD is prevented by concerns over their safety profile (191, 192). In particular, a number of patients treated with anti- α_4 integrin therapy have developed the fatal neurological disease progressive multifocal leukoencephalopathy, associated with JC-polyomavirus reactivation, presumably reflecting the role of α_4 in T-cell homing to the central nervous system (192). Trials of agents targeting

more intestine-specific homing molecules including anti- $\beta 7$, anti-MadCAM1, and anti-CCR9 are ongoing in human IBD, with promising initial observations (193).

Among anticytokine therapies, anti-TNF remains the most efficacious, although many patients are non-responsive (194). As mentioned earlier, anti-IL-17A was not effective in CD (96), and it may be that stratification of patients with distinct 'pathotypes' of disease is required to see beneficial effects in patient subgroups with blockade of particular effector cytokines.

In addition to inhibiting pathological T-cell responses, complementary therapies may be necessary to restore and maintain intestinal homeostasis. These may include strategies to enhance intestinal barrier function and the number and function of intestinal Treg cells. As these effects should be focused on the intestine, manipulation of the microbiota may offer a tool to deliver these goals and evidence from model systems is encouraging (25).

Conclusions

Recent advances have contributed significantly to our understanding of the biology of CD4⁺ T cells in the intestine.

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