

# **Role of Th1/Th2/Th3 Regulatory T Cells in Mucosal Immune Development**

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The gastrointestinal tract is in constant contact with food proteins, commensals, and potentially pathogenic microorganisms. To maintain immune homeostasis in this environment, the intestinal immune system has developed redundant regulatory strategies. Soluble factors such as transforming growth factor beta (TGF- $\beta$ ), interleukin-10 (IL-10), thymic stromal lymphopoietin (TSLP), and cells such as tolerogenic dendritic cells (DCs), and a subset of T-helper cells (regulatory T cells or Tregs) play a role in the control of the immune response to avoid overreaction and excessive tissue damage.<sup>1</sup>

Oral administration of protein antigens induces systemic immunological tolerance, a phenomenon largely known as oral tolerance. Failure in the regulatory mechanism may lead to dietary-antigen-derived pathology such as food allergy or celiac disease. Similarly, aberrant immune response against components of commensal flora may trigger inflammatory bowel diseases such as Crohn's disease or ulcerative colitis.

The microenvironment determines the functions of cells recruited to the tissue, with DCs serving as the master coordinator of the mucosal immune response. These cells have a critical influence in changing the balance between active regulatory mechanisms or driving an inflammatory response. Under steady-state conditions, DCs are central to the induction of tolerance to both self and foreign (dietary and commensal) antigens by inducing Tregs. In the presence of inflammation or pathogenic organisms, DCs are activated to express a full range of co-stimulatory molecules and cytokines, ensuring the efficient stimulation and differentiation of effector T cells.

After oral administration of a model antigen (ovalbumin), lamina propria DCs (LPDCs) exhibit regulatory properties, because in vivo antigen-loaded LPDCs transferred into normal recipients induced a state of immune hyporesponsiveness.<sup>2</sup> These LPDCs produce more mRNA for IL-10 and type 1 interferon than spleen DCs and less interleukin (IL)-12, suggesting that there may be a population of DCs in the lamina propria that can preferentially induce T-cell tolerance in the absence of inflammation.

## **Intestinal DC Subsets**

As in peripheral lymph nodes or other tissues, distinct DC (CD11c<sup>+</sup>) subsets are described in intestinal mucosa in mice. With the use of more and more markers, the initial description CD11b<sup>+</sup>, CD8α<sup>+</sup>, CD11b<sup>-</sup>CD8α<sup>-</sup> has become much more complex. Now, additional markers are shown to discriminate different functional populations, and the discrimination between DC subsets CD103 positive or negative and CX3CR1 positive or negative are shown to be important in the understanding of different capabilities of intestinal DC subsets. To increase the complexity of this scenario, distinction between DCs and macrophages in the intestine is not completely elucidated. Finally, because there are no clear markers for human DC subsets, description of human mucosal DCs is more difficult than that of murine counterparts.

In addition to the antigen uptake mechanism by M cells (mainly in Peyer's patches) or antigen passage throughout the tight junctions, intestinal DCs may extend through the enterocyte layer, as nicely described by real-time imaging recording. These extensions are capable not only of sensing the microorganisms in the lumen but also of taking them up and transporting them to the LP. Although the biological relevance of each mechanism is still uncertain, it is clear that DCs can directly take up antigens present in the lumen, process and present orally delivered

antigen, and finally stimulate naïve T cells in mesenteric lymph nodes (MLNs). The outcome of this interaction depends on the characteristics of the DC and signals from the environment.

Consequently, Tregs or effector T cells (Th1, Th2, or Th17) can be induced. Moreover, as part of their differentiation program, these cells upregulate the homing receptors that direct them to the intestinal mucosa.

### **Induction of Tregs in the Intestinal Mucosa**

It is now clear that extrathymic generation of Tregs occurs in the small intestine. Intestinal DC can induce antigen-specific Tregs, which are mainly involved in the control of immune response against dietary antigen and commensal flora. As we will discuss, retinoic acid (RA) is one main player in the induction of Tregs, but also in determining the homing tropism of the activated cells in the mucosa.

By adoptive transference experiments, Sun et al<sup>3</sup> demonstrated that FoxP3<sup>+</sup> cells can be induced in lamina propria of the small intestine and MLNs, and this population may be traced as long as 8 weeks after transference, implying a continual conversion or accumulation. These cells are antigen-specific and selectively express CD103.

LPDCs are especially suited to convert CD4<sup>+</sup> T cells to Tregs, and CD103<sup>+</sup> LPDCs, in particular, are much better than CD103<sup>-</sup> LPDCs. However, TGF- $\beta$  supplementation renders CD103<sup>-</sup> LPDCs equally able to convert FoxP3<sup>+</sup> T cells. TGF- $\beta$  also may induce CD103 expression. LPDCs also are able to expand FoxP3<sup>+</sup> T cells.

Retinoic acid (RA) does not induce FoxP3<sup>+</sup> expression alone, but upregulates its expression when combined with TGF- $\beta$ . Furthermore, RA is a potent inducer of CCR9 and  $\alpha_4\beta_7$  expression, which mediates the gut tropism of Tregs.

It is clear that Tregs conversion occurs in the intestinal mucosa, and FoxP3<sup>+</sup> T cells can be observed in LP, Peyer's patches, and MLN. However, LPDCs, particularly CD103<sup>+</sup> LPDCs, which enter the MLN from LP, are the more potent inducers of Treg conversion.<sup>4</sup>

## **Regulation of Immune Response**

The immune system contains potent mechanisms that eliminate pathogenic microorganisms and infected cells, and parallel regulatory mechanisms that keep effector cells under control during physiological conditions and after the activation elicited by an inflammatory process. In particular, in this inflammatory situation, immune regulation is required to reduce the tissue damage caused by excessive immune activation.

In recent years, the study of suppressor T cells, most commonly Tregs, has been intense. The transcription factor FoxP3<sup>+</sup>, originally thought to be uniquely expressed by Tregs cells, helped to identify the initial descriptions of Treg functions. Humans and mice that do not have functional FoxP3<sup>+</sup> T cells as a consequence of mutations in the FoxP3 gene suffer from inflammatory and autoimmune disease. This highlights the essential role of Treg cells in control of the immune response.

Thymic-borne Tregs migrate as naïve FoxP3<sup>+</sup> cells to secondary lymph nodes for activation and differentiation into memory type FoxP3<sup>+</sup> T cells. Then Tregs can migrate to B-cell areas, nonlymphoid tissues, or sites of Th1 or Th2 inflammation. In addition, naïve FoxP3<sup>-</sup> cells can be converted to FoxP3<sup>+</sup> cells by different factors; RA is one of the modulators.

## **Murine and Human Tregs**

Human and mice Tregs are different in many aspects regarding the impact of or regulation of FoxP3 expression. In humans, FoxP3<sup>+</sup> expression is very low and transient in activated T cells. These FoxP3<sup>+</sup> cells do not suppress T-cell proliferation. Overexpression of FoxP3 induces hyporesponsiveness and suppression of IL-2 production but does not convert T cells in Tregs, suggesting that additional signals are required in humans. TGF-β can induce FoxP3 expression but alone is not sufficient to induce a full Tregs programming in humans. RA is sufficient to convert FoxP3<sup>+</sup> T cells in humans, but in mice TGF-β is required in addition to RA to generate functional FoxP3<sup>+</sup> Tregs cells from FoxP3<sup>-</sup> T cells.

### **Mechanism of Suppression by Tregs**

Evidence exists of the suppressive effects displayed by Tregs, but information about the actual pathways determining regulation or suppression of effector T cells is scarce. One of these mechanisms consists of redirecting the DC function to a regulatory/tolerogenic phenotype as a consequence of IL-10 secretion by Tregs. Regulatory DCs later can induce expansion/differentiation of new Tregs. This is an indirect manner to induce regulation, and mechanisms having a direct effect on effector T cells are less evident.

When molecules like cytokines are the functional mechanism of action, one must keep in mind that most of them have a short half-life. Thus, Tregs must be located in close proximity to the target cells, although this does not necessarily imply cell contact.

At present, data suggest that overlapping and, to some extent, redundant mechanisms may be required to obtain the maximal suppressive effect. Mechanisms for the suppressive effects of Tregs can be grouped into four types, as described next.<sup>5</sup>

**Suppression by inhibitory cytokines.** IL-10, TGF- $\beta$ , and, more recently, IL-35 have been described as mediators of the suppressive effects by Tregs. All three have suppressive activity but the mechanisms and relevance of each still are unclear. However, different studies have reported that the three are involved in distinct pathogenic or homeostatic situations and have non-overlapping functions.<sup>5</sup>

Production of IL-10 is clearly demonstrated in the mouse model of colitis, in which secretion by Tregs is essential to control inflammation. The situation for TGF- $\beta$  is more complex because the biological activity is mostly due to the TGF- $\beta$  tethered to the plasma membrane of Tregs.

One member of the IL-12 family, IL-35, is preferentially expressed by mouse FoxP3<sup>+</sup> Tregs cells but not by activated effector T cells. IL-35 also is significantly upregulated in Tregs that are actively suppressing and is sufficient to confer regulatory function to naïve T cells or to suppress T-cell proliferation in vitro, although the pathways for these effects still are undefined.

**Suppression by cytotoxicity.** Tregs have suppressive effects on target cells by the induction of granzyme A and granzyme B, in human and mouse, respectively, resulting in cytotoxicity.

**Suppression by metabolic disruption.** One of the mechanisms, largely debated, is deprivation of IL-2 by Tregs through the high expression of CD25 (the IL-2R $\alpha$  high affinity chain) that starves dividing effector T cells and induces apoptosis. In addition, it recently was observed that release of adenosines can inhibit effector T-cell function by inhibiting the secretion of IL-6 and inducing the production of TGF- $\beta$ , which in turn favors the generation of Tregs compared with Th17 cells. It also has been proposed that release of cAMP by Tregs through gap junctions can mediate strong suppressive effects on target cells.

**Suppression through dendritic cells.** As mentioned previously, Tregs may influence DCs by altering their maturation and function. CTLA-4, which is constitutively expressed by Tregs,

modulates the priming and co-stimulatory properties of DCs, thereby attenuating the DC activation of effector T cells. The co-stimulatory function of DCs can be impaired by decreasing the expression of CD80 and CD86 mediated by CTLA-4 or via IL-10 and TGF- $\beta$ . More recently, it was proposed that LAG3 (CD223) may block DC maturation because it is able to bind with high affinity to MHC class II molecules and deliver suppressive signals.<sup>5</sup> This correlates with higher suppressive effects of human MHC class II<sup>+</sup> Tregs cells compared to MHC class II<sup>-</sup> cells.

## **Retinoids and Immune Regulation**

Regulatory T cells, in particular FoxP3<sup>+</sup> cells, suppress the immune system to prevent overactive response and inflammation induced by effector T cells, and other non-T cells. FoxP3<sup>+</sup> cells arise in the thymus (nTregs), and in parallel induced Tregs, may arise from naïve CD4<sup>+</sup> T cells in the periphery. The factors that determine the development of Tregs—DCs, cytokines, tissue-derived factors, including retinoids—recently have received much attention. RA can be produced from vitamin A by DCs in the intestine by a metabolic pathway involving enzymes such as RALDH1/2 (retinal dehydrogenase). RA, particularly all trans retinoic acid (ATRA), binds to a retinoid nuclear receptor that acts as a transcription factor on many genes. Retinoids induce expression of gut homing receptor in DCs; B and T cells induce IgA switch, reduce TNF $\alpha$  and IL-1 concentration in serum, and increase IL-10. Consequently, retinoids are important regulators of mucosal immunity.<sup>6</sup>

Both human and mouse FoxP3<sup>+</sup> Tregs cells induced by RA and TGF- $\beta$  express CCR9 and  $\alpha_4\beta_7$ , which engage CCL25 and MadCAM1, respectively, and determine gut homing tropism. These cells also lose the expression of CD62L, which guides them to lymphoid nodes. In addition, RA and TGF- $\beta$  induce CD103 ( $\alpha_E$ ), which allows interaction with E-cadherin.<sup>6</sup> These

changes can occur during the induction of antigen-specific Tregs, as was demonstrated in animal models using ovalbumin (OVA)-specific transgenic mice.<sup>3,7</sup>

### **Th17: A Central Player in Intestinal Antimicrobial/Inflammatory Response?**

In the normal intestine, Th17 cells contribute to maintaining the epithelial barrier integrity and inhibiting bacterial colonization by producing defensins, whereas in chronic intestinal inflammation high levels of IL-23 produced by DCs may activate their aggressive pathogenic program. In this context, production of other inflammatory cytokines (IL-1, IL-6, TNF $\alpha$ ) exacerbates the production of IL-17 by Th17 cells. IL-17A, the most studied member of the IL-17 family, is important in host defense against extracellular bacteria and fungi by stimulating the production of chemokines that recruit and activate granulocytes and macrophages. However, IL-17A also promotes an inflammatory cascade, which can drive autoimmune diseases.

In mice, Th17 cell differentiation is induced by TGF- $\beta$  plus IL-6. Th17 cells produce IL-21, which acts as an autocrine positive regulator, and among other effects, induces IL-23R. IL-23 supports further differentiation. Since mouse Th17 cells express IL-23R only after induction by IL-6, IL-23 is not involved in the initiation of the Th17 differentiation. On the other hand, human Th17 cell differentiation is driven by IL-6, IL-1, and possibly by IL-23.

Th17 cell differentiation depends heavily on the transcription factors ROR $\gamma$ T and ROR $\alpha$  in mouse and human, respectively. IL-6, IL-21, and IL-23 activate the transcription factor STAT3, which directly binds to IL-17 and IL-21 genes. STAT3 is also a positive signal for the receptor ROR $\gamma$ T and ROR $\alpha$ .

Because of the potent inflammatory function of IL-17, Th17 cell differentiation is regulated by negative factors such as IFN $\gamma$ , IL-4, IL-2, and RA. Recently, IL-27 was identified as an important negative regulator of Th17 differentiation.<sup>6</sup>

In contrast, in humans it is possible to find IL-17 producing cells as well as IL-17 plus IFN $\gamma$  “double producer” cells—two populations that seem to be characterized by the expression of CCR6, CCR4 and CCR6, and CXCR3, respectively.<sup>8,9</sup>

There is not a unique link between Th17 and IL-17. Not all the functions of Th17 are in parallel with IL-17 activity. Other cytokines such as IL-21 and IL-22 are involved in the pathogenic cascade that can drive a chronic disorder. IL-21R is expressed on T and B cells, DCs, and macrophages, as well as on epithelial cells and natural killer (NK) cells; its activation drives many different effects. In addition to Th17 cells, other non-T cells such as monocytes and macrophages can produce IL-17.

### **Th17 Cells: Gut Homeostasis and Inflammation**

Chronic inflammatory disorders of the intestinal tract (eg, Crohn’s disease and ulcerative colitis) have unknown etiology, and no cure exists at this time. Disease mechanisms involve a complex multifactorial scenario. It is evident that chronic inflammation is mediated by an aberrant immune response directed against components of the intestinal microflora, but many steps involved in the molecular pathogenesis remain undiscovered. It is recognized that IL-12p40 is involved in Crohn’s disease, leading to the concept that the disease is mainly a Th1 IL-2-driven pathology. Furthermore, anti-IL-2p40 antibody therapy has been used successfully in many patients. However, more recent findings demonstrate that IL-23 is the cytokine implied in the pathology. IL-23 belongs to the IL-12 heterodimeric family, and it is composed of IL-23p19 and

IL-12p40 subunits. Consequently, treatment with anti-IL-12p40 antibodies targets IL-23.<sup>10</sup> In addition, IL-23 selectively potentiates the IL-17 expression by Th17 cells, and its deficiency protects against experimental autoimmune encephalomyelitis (EAE) in a mouse model. Many of the Th1-driven autoimmune diseases may in fact be due to IL-23.

Epithelial cells constitute not just a physical barrier, they also take part in the amplification and maintenance of chronic intestinal inflammation in IBD. Intestinal epithelial cells can synthesize cytokines that control survival and activity of lymphocytes and contribute to generating a chemoattractant milieu that sustains the recruitment of inflammatory cells. They express IL-21R and respond to stimulation with IL-21 by secreting the chemokine MIP-3 $\alpha$ , which can mediate the recruitment of macrophages, but also of  $\alpha_4\alpha_7$  T lymphocytes.<sup>11</sup>

By a different pathway, epithelium is involved in inflammatory processes. Epithelial cells can produce IL-32 after induction by a Th1 environment such as IL-12 and IFN- $\gamma$  and stimulate TNF $\alpha$  and IL-8. In addition, IL-32 synergizes with signaling derived of Nod protein activation to produce IL-1 $\beta$  and IL-6. All these mediators largely contribute to amplification of the inflammatory process.

Moreover, lamina propria fibroblasts play an important role in tissue remodeling and fibrosis. They secrete collagen, but in inflammatory conditions also matrix metalloproteinases (MMPs), which are neutral endopeptidases secreted in the inactive form and activated in the extracellular environment. Their activity is controlled by specific tissue inhibitor of MMPs (TIMPs). Imbalance between the activity of MMPs and TIMPs contributes to tissue damage and fibrosis. Fibroblasts have IL-21R and respond to IL-21 by secreting large amounts of MMPs, but not TIMPs, from internal deposits. TNF $\alpha$  and IL-1 $\beta$  are inducers of IL-21R expression on fibroblasts, driving the initial steps in the inflammatory process.

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## Q/A

Q: Dr Chirido, in most of your studies, dendritic cells were isolated from the lamina propria. You also mentioned that there were differences between T cells and cells in the lymph nodes. What about those in Peyer's patches? Are there many differences between these dendritic cells?

Apparently, they function the same way.

Dr Chirido: It is difficult to answer the question precisely, but there are some differences in the function of dendritic cells in the lamina propria and Peyer's patches. Subset analysis shows that the composition of the whole cell population is different. The percentage of conventional dendritic cells in the lamina propria differs from those in Peyer's patches. To me, however, the most important difference is that in the Peyer's patches, some cells are located in specific places—some are in the dome region, and some are in the interfollicular region. In comparing the whole subset, not too many differences are seen, but specific cells may function only at that specific location. In the lamina propria, however, cells are not restricted to a specific location. Also, lamina propria dendritic cells do nothing in priming; they must migrate to the mesenteric region. In the Peyer's patches, however, the dendritic cells are able to use priming, and this is a different situation.

Q: Did you say that the cells underneath the epithelium and in the villi are plasmacytoid DCs (pDCs)?

Dr Chirido: That has been published [Wendland M et al: *PNAS* 2007;104(15):6347-6352].

Q: Are the majority pDCs, or all of them?

Dr Chirido: The authors claim that a large part of the population is close to the epithelium, although not in the epithelial compartment. They estimate that most of the cells are close to the basal membrane.

Dr Brandtzaeg: We have to be aware of the important differences between mice and humans in regard to the distribution of various dendritic cell types. I do not think we always are talking about the same cells in mice and humans, particularly when we are dealing with CD103+ DCs and plasmacytoid DCs. To my knowledge, we have not seen IL-3 receptors, CD45A, and CD103 in the lamina propria of humans, except in the periphery of lymphoid aggregates, which are the markers we ordinarily use. You mentioned mesenteric lymph nodes.

Dr Chirido: A recent publication shows them in the mesenteric lymph nodes but not in the lamina propria [Jaensson E et al: *J Exp Med* 2008;205:2139-2149].

Dr Brandtzaeg: I understand from other discussions that those researchers have seen them in the human lamina propria, but these data are not published.

Q: I have seen rodent studies in which vitamin A deficiency decreased asthma severity, while high levels of vitamin A increased asthma severity. Is there evidence that vitamin A levels differ in diets in Western countries compared to those in non-Western diets, and could vitamin A be implicated in the increases in immune disorders over the last few decades?

Dr Chirido: I not know about Western vs non-Western dietary differences in vitamin A, but research findings about the effects of deficient and excess concentrations of vitamin A in tissues are contradictory. Both can cause problems. There are research difficulties in studying this. In vitro, we can calculate exactly what concentration of vitamin A we are using, but we do not know what really happens in tissue.

Dr McSorley: The literature suggests that many nutritional deficiencies in developing countries are associated with increased susceptibility to infection. This is the opposite of the model, whereby retinoic acid suppresses Th17 cells and encourages regulation.

Q: Dr Chirido, you mentioned CX3CR1 cells but not CCR6 dendritic cells expressing the chemokine receptor, which Dr McSorley talked about with regard to the mouse model. Can you comment?

Dr Chirido: In a big study in Europe, researchers are trying to define the relationships between CCR6 positive or negative and CX3CR1 positive or negative. They seem to belong to different subsets. CD103 positive or negative also are different lineages. One does not develop from the other. The problem for developing different lineages in dendritic cells is that no one knows exactly how these cells differentiate each other.

Q: We have talked a lot about DCs in the gut. In terms of expression of chemokine receptors, are these cells very different in the respiratory tract and other mucosal sites?

Dr Chirido: The intestinal cells are best known.

Dr Brandtzaeg: We studied the human nasal mucosa and found that the antigen-presenting cells are quite different from those in the gut. Some cells with a clear macrophage phenotype look like normal dendritic cells. In the human gut, there is a band of antigen-presenting cells below the epithelium where there is a transition between monocyte-derived macrophages and dendritic cells. So it is often hard to say whether cells are true dendritic cells, but small subsets of cells in various layers of the gut are clearly dendritic cells.

Dr Chirido: A recently published paper claimed that all regulatory functions in the intestine are due to macrophages, not to DCs—that the production of IL-10 and induction of Tregs are due to macrophages, not to dendritic cells [Denning TL et al: *Nat Immunol* 2007;8:1086-1094]. This is the opposite of most other research findings. As Dr Brandtzaeg said, we cannot clearly define what a dendritic cell is and what a macrophage is. At some point, we define this population of DCs according to markers, and the markers we use are CD11C and CD11B. They may be

expressed at different levels at the surface, and consequently it is difficult to set the definition of both lineages only on these markers.

Q: Regarding CCR6 and CX3CR1, the data you presented from Chieppa's paper [Chieppa M et al: *J Exp Med* 2006;203:2841-2852] were in direct conflict with Dr McSorley's. Can you reconcile that?

Dr McSorley: I presented my interpretation of the data. Christian Reinecker's group would interpret them slightly differently and say that the CX3CR1 cells are actually involved in the priming of CD4 cells [Neiss JH et al: *Science* 2005;307:254-258]. For various reasons, I do not think that is correct, but he could be right.

Dr Chirido: One of the problems in comparing Reinecker's paper to Chieppa's is that although their ends are the same, their methodologies differ. Reinecker and colleagues used a green fluorescent protein (GFP) under the promoter of CX3CR1, Chieppa's group used the GFP under the promoter of Class II, so they produced different findings.