TLR9 Turns the Tide on Treg Cells

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In this issue of *Immunity*, Hall et al. (2008) demonstrate that commensal bacterial DNA can suppress Treg cell conversion via TLR9-mediated activation of lamina propria dendritic cells and thus potentially disrupt intestinal homeostasis.

Commensal bacteria express a range of Toll-like receptors (TLR) and NOD-like receptor (NLR) ligands, but normally do not induce excessive inflammation and are not eliminated by host immune effector mechanisms. In contrast, protective innate and adaptive immune responses are generated against pathogenic enteric bacteria. This paradox has been explained on the basis of regulatory T (Treg) cell-mediated tolerance to commensal bacteria and preferential development of host effector responses to pathogenic bacteria. However, commensal bacteria have also been implicated in promoting inflammatory bowl disease (Strober et al., 2007). The mechanism is thought to involve either a breakdown of immunological tolerance because of defective mucosal Treg cells, overactive effector T cells, or differences in the composition of the gut microflora in susceptible individuals. The paper by Hall et al. (2008) in this issue of *Immunity* adds another twist to this story by reporting that activation of TLR9 on lamina propria dendritic cells (LpDCs) helps to promote intestinal inflammation by suppressing Treg cells. The study demonstrates that activation of TLR9 by gut flora DNA can disrupt intestinal homeostasis, promoting T helper 1 (Th1) and Th17 responses, while inhibiting conversion of Treg cells, thorough activation of LpDCs. The findings support a growing number of reports that TLR agonists can directly or indirectly influence the induction or function of Treg as well as effector T cells and provide evidence to consolidate the proposed role of commensal bacteria in promoting intestinal inflammation.

Activation of TLR and NLR signaling pathways by pathogen-derived molecules plays a major role in activating innate immune responses and in host defense against infection. TLR agonists promote the induction of Th1 cells, by enhancing DC maturation and induction of interleukin 12 (IL-12) production. Furthermore, TLR and NLR agonists synergize to induce the production of IL-1, IL-6, TGF-β, and IL-23 by DCs, which drive the differentiation and expansion of Th17 cells. It has also been demonstrated that TLR agonists, including LPS and CpG, can stimulate IL-10 production from DCs and thereby promote the induction of IL-10-producting Treg cells, simultaneously with Th1 cells and Th17 cells (Jarnicki et al., 2008). Finally, it has been demonstrated that TLR agonists can induce production of transforming growth factor-beta (TGF-β), indolamine 2,3-dioxygenase, interferon-alpha (IFN-α), and prostaglandin E2, which have been implicated in peripheral conversion or expansion of $CD4^+Foxp3^+$ T cells (Conroy et al., 2008). Therefore, TLR agonist interaction with their receptors on DCs has the capacity to induce or expand Treg, as well as effector and pathogenic T cells. This may reflect a protective mechanism of the host to limit excessive inflammation and collateral tissue damage during infection. However, the role of TLR in the induction of Treg cells is still controversial, because there is also evidence that TLR agonists can have direct and indirect suppressive effects on Treg cells, leading to a breakdown in tolerance to self antigens (Conroy et al., 2008). It has been reported that TLR-induced IL-6 production by DCs blocks the suppressive function of $CD4^+CD25^+$ Treg cells (Pasare and Medzhitov, 2003). Conversely, TLR4-activated DCs have been shown to indirectly enhance the suppressive function of Treg cells through IL-1 and IL-6 production (Kubo et al., 2004).

The paper by Hall et al. (2008) in this issue adds further complexity by showing that TLR9 agonists can suppress Treg cell conversion in intestinal tissues through interaction with LpDCs. The study demonstrates that *Tlr9*$^{-/-}$ mice have enhanced $CD4^+FoxP3^+$ T cells in the Peyer's patches and lamina propria, with a concomitant reduction in the frequency of IFN-γ- and IL-17-secreting $CD4^+$ T cells. They went on to demonstrate that TLR9 signaling had a more profound effect on the balance of effector to regulatory T cells in the gut than in the periphery. Antigen-specific Th1 responses induced in response to the intracellular parasite *Encephalitozoon cuniculi* were similar in *Tlr9*$^{-/-}$ and WT mice infected intraperitoneally, but Th1 and Th17 responses and parasite clearance was substantially reduced in *Tlr9*$^{-/-}$ mice infected by the oral route. Furthermore, intestinal effector T cell responses induced by oral immunization with a model antigen and mucosal adjuvant were reduced in *Tlr9*$^{-/-}$ mice.

Belkaid and colleagues had previously shown that oral exposure to antigen can induce conversion of $CD4^+Foxp3^-$ T cells to $CD4^+Foxp3^+$ T cells, mediated by LpDCs, via TGF-β and retinoic acid. Consequently, in the current manuscript they examined the influence of commensal bacterial DNA on Treg cell activation. LpDCs stimulated with CpG (a TLR9 agonist), but not by TLR2, TLR4, or TLR5 agonists, suppressed conversion of Treg cells (Hall et al., 2008). This was accompanied by enhancement of Th1 and Th17 cells by CpG-activated LpDCs. The IL-17 induction was explained on the basis of IL-6 production by the CpG-activated LpDCs, because anti-IL-6 blocked Th17 activation. However, they did not examine IL-1, TGF-β, or IL-23, which have also been shown to be required for induction and expansion of Th17 cells. Furthermore, they did not provide a definitive mechanism to explain suppression of

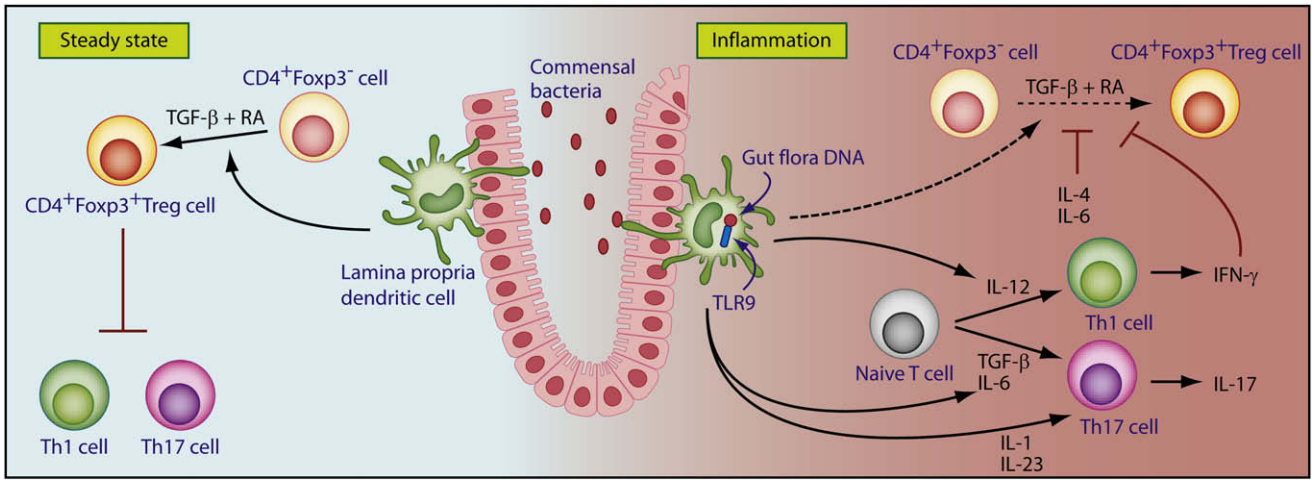

Figure 1. Gut Flora DNA Can Disrupt Intestinal Homeostasis
In steady-state conditions, intestinal homeostasis is maintained through Treg cell suppression of effector T cells, and lamina propria dendritic cells (LpDCs) play a role by enhancing conversion of $CD4^+Foxp3^-$ to $CD4^+Foxp3^+$ Treg cells, through retinoic acid (RA) and TGF-β. However, in susceptible individuals (e.g., with polymorphisms in key innate receptors or with altered gut microflora composition), LpDCs, which can sample bacteria across the intestinal epithelium, are likely activated by the commensal bacterial DNA through TLR9. After TLR9 activation, the LpDCs secrete IL-6 and probably other cytokines that promote differentiation and expansion of Th17 and Th1 cells. In addition, the TLR9-activated LpDCs suppress Treg conversion, through negative feedback by Th1-derived IFN-γ, in combination with IL-4 and IL-6. Thus, TLR9-activated LpDCs can alter the balance of effector over regulatory T cells and thereby promote intestinal inflammation.

Treg by TLR9-activated LpDCs. There was evidence to suggest that this may in part be mediated by IL-4, IFN-γ, and IL-6, but the cellular source of the suppressive cytokines was not clear.

Having shown that CpG can block Treg conversion by interacting with TLR9 on LpDCs, Hall et al. (2008) demonstrated that DNA enriched from gut flora could mediate the same effect. Furthermore, gut flora DNA was capable of replacing commensal bacteria (removed with antibiotics) in inducing effector Th1 and Th17 responses and clearance of *E. cuniculi*. These findings support the hypothesis that commensal bacteria may in some cases help to dysregulate the balance of intestinal effector over regulatory T cells, leading to intestinal inflammation (Figure 1). However, the study also poses a number of questions. First, what is so special about TLR9? TLR2, TLR4, and TLR5 are expressed on LpDCs or epithelial cells in the gut, and agonists for these receptors are also produced by gut microflora, so why do they not also inhibit Treg cells? Second, why LpDCs and why do they behave differently from DCs in other lymphoid tissues? Finally, if commensal bacteria are able to suppress Treg responses allowing uncontrolled inflammation, why do we all not get colitis? Clearly, genetic and environmental factors have a major role in determining susceptibility to intestinal inflammation. Individuals with polymorphisms in NOD2 gene have increased susceptibility to Crohn's disease (CD) and there is also evidence that CD is associated with polymorphisms in TLRs, including TLR9. Although NOD2 and TLR9 agonists can synergize to activate innate inflammatory cytokines that promote Th1 and Th17 responses, it has been suggested that NOD2 activation has a protective role against intestinal inflammation, in part by inhibiting TLR signaling pathways (Strober et al., 2007).

The findings by Hall et al. (2008) that TLR9 activation by gut microflora DNA enhances inflammation are backed by reports that enteric bacteria are necessary for the development of spontaneous colitis and that TLR9 agonists can overcome tolerance and precipitate autoimmune diseases in mouse models. However, other investigators have suggested that commensal bacteria and TLR agonists can protect against intestinal inflammation. A study showing that DSS-induced colitis is more severe in *Myd88*$^{-/-}$ mice suggested that TLR activation by commensal bacteria plays a role in the maintenance of intestinal homeostasis (Rakoff-Nahoum et al., 2004). It was also reported that commensal bacteria may reduce the frequency of Th17 cells in the intestine; germ-free mice had enhanced IL-23, IL-12, and IL-17, and commensal induction of IL-25 in conventional mice regulated IL-23-induced IL-17 production (Zaph et al., 2008). Furthermore, s.c. administration of TLR9 agonists have been shown to protect mice from colitis through IFN-α production (Katakura et al., 2005).

The difference between studies suggesting positive and negative roles for TLRs in Treg cell responses may in part reflect the different experimental systems, cell types, and tissues examined. The study by Hall et al. (2008) demonstrates functional differences between LpDCs and splenic DCs and between oral and parental routes of antigen exposure, suggesting that there may be intrinsic difference between DCs in intestinal and secondary lymphoid tissues. The recent paper by Goubier et al. (2008) demonstrating that oral tolerance is mediated by plasmacytoid DCs in the liver provides further evidence that tissue-specific DCs may have distinct functions and that different DC subtypes may mediate tolerance versus effector immune responses.

If the findings in mice (that gut flora DNA enhances rather than inhibits intestinal inflammation) translates to humans, then inhibition of TLR9 signaling may be

a promising approach for the development of new therapeutic interventions against CD, especially if they can be selectively targeted to the gut or lamina propria. Furthermore, the findings might also help to tackle a long-standing problem in the vaccine field. Oral vaccines have considerable attractions in terms of ease of administration but are constrained by poor immunogenicity and induction of tolerance by antigens delivered by this route. Suppression of Treg cell induction has been shown to enhance the efficacy of vaccines delivered by parenteral routes (Jarnicki et al., 2008). Therefore, suppression of Treg cell conversion by commensal DNA or synthetic TLR9 agonists has potential to reverse tolerance and improve the immunogenicity of vaccines delivered by the oral route.

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CMV and the Art of Memory Maintenance

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The CD8$^+$ T cell responses to CMV gradually increase in magnitude over time—so-called memory "inflation." In this issue of *Immunity*, Snyder et al. (2008) examine the dynamics of memory inflation and demonstrate continuous turnover of inflating T cells, drawing on both memory cells and naive cells to replace them.

Cytomegaloviruses are ubiquitous pathogens. Human CMV (HCMV) infects most of the human population, usually asymptomatically, and persists lifelong. The cellular immune responses to HCMV are vigorous and sustained—indeed they tend to increase over time and may come to dominate the peripheral blood of healthy elderly donors (Khan et al., 2002). They are also essential for viral control, given that immunosuppression for transplantation or as a result of human immunodeficiency virus (HIV) infection can lead to viral reactivation and severe disease.

This host-virus balance has been studied by Snyder and colleagues (Snyder et al., 2008, this issue of *Immunity*) with the murine (MCMV) model, in which a key role for specific CD8$^+$ T cell populations can be more precisely defined. MCMV-specific CD8$^+$ T cells may be protective against disease—for example in immunosuppressed mice, as shown by the group of Reddehase (Holtappels et al., 2000). In these experiments it was noted that some CD8$^+$ T cells populations may be sustained or even increase over time in the lung. In further experiments using MHC-peptide tetramers, a continuous accumulation of virus-specific CD8$^+$ T cells over time was noted in all organs—a feature termed "memory inflation" (Karrer et al., 2003). The expansion of CMV-specific T cells in the elderly, which appears to be rather similar, has been implicated in immunosenescence, and thus understanding how memory inflation is established may be important in understanding the negative effects of CMV infection on human longevity (Khan et al., 2002).

CMVs are large viruses, with a number of open reading frames, but memory inflation is only seen for a small subset of epitopes from CMV proteins. Previous work by the group of Hill, preceding the study of Snyder in this issue, has shown that the CD8$^+$ T cell response to acute CMV infection may be broad (Munks et al., 2006). As the virus establishes persistence, the T cell response narrows to focus on a smaller number of immunodominant epitopes, and these T cell populations tend to inflate. In contrast, T cells specific for many of the acute-phase epitopes show a classical memory response,