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Role of Toll-like Receptors in Spontaneous Commensal-Dependent Colitis

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Summary

Inflammatory bowel disease (IBD) is thought to result from a dysregulated interaction between the host immune system and its commensal microflora. Heterogeneity of disease susceptibility in humans and rodents suggest that multiple mechanisms are responsible for the etiology of IBD. In particular, deficiencies in anti-inflammatory and immune-suppressive mechanisms play an important role in the development of IBD. However, it is unknown how the indigenous microflora stimulates the immune system and how this response is regulated. To address these questions, we investigated the role of Toll-like receptor (TLR) signaling in the development of spontaneous, commensal-dependent colitis in interleukin (IL)-2- and IL-10-deficient mice. We report that colitis was dependent on TLR signaling in *Il10*$^{-/-}$ mice. In contrast, *Il2*$^{-/-}$ mice developed intestinal inflammation in the absence of TLR signaling pathways. These results demonstrate a differential role of innate immune recognition by TLRs in the development of commensal-dependent colitis.

Introduction

The intestines of complex metazoans are colonized by trillions of microorganisms, including hundreds of different species of bacteria and viruses (Savage, 1977; Sonnenburg et al., 2004). This diverse group of microbes, collectively referred to as the indigenous intestinal microflora, is essential for host physiology and survival. Microbial colonization of the intestine confers both local and systemic benefits spanning diverse processes such as host defense from infection, energy and nutrient metabolism, and tissue development and repair (Berg, 1996; Hooper and Gordon, 2001).

However, in certain circumstances the presence of the commensal microflora can become detrimental to the host. It is thought that the intestinal flora plays a major role in the pathogenesis of inflammatory bowel disease (IBD), in particular Crohn's disease (CD) (Podolsky, 2002). It is believed that the excessive inflammatory and immune responses in the intestine may be due to a breach in physiological immune homeostasis between the commensal microflora and the host immune system (Strober et al., 2002).

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Studies in rodents have revealed that several mechanisms contribute to the maintenance of this immune homeostasis in the colon (Powrie, 2004). Mice deficient in interleukin-10 (IL-10) and IL-2 develop spontaneous inflammation of the large intestine (Kuhn et al., 1993; Sadlack et al., 1993), a process that is T cell dependent (Davidson et al., 1996; Ma et al., 1995) and dominated by a pathologic T helper type 1 (Th1) immune response (Ehrhardt et al., 1997; Strober et al., 2002).

Importantly, intestinal inflammation and colitis in IL-10- and IL-2-deficient mice are dependent on the presence of the commensal microflora. These animals are predisposed to develop severe colitis when reared in specific pathogen-free conditions but are completely protected from intestinal disease when raised in germ-free environments (Strober et al., 2002). This indicates that at the steady state, pathologic consequences of activation of the immune system by the commensal microflora are constitutively inhibited by IL-2- and IL-10-dependent anti-inflammatory and immune-suppressive mechanisms (Strober et al., 2002).

However, several essential questions regarding our understanding of host-commensal interactions in the intestine have remained unexplored. A fundamental unanswered question is how the indigenous microflora activates the host immune system. Also, despite similar disease phenotypes, such as the development of pathologic Th1-mediated responses, it is unknown by what mechanisms IL-10 and IL-2 prevent commensal-dependent colitis. In particular, it is unknown whether dominant immunoregulatory factors such as IL-10 and IL-2 play redundant roles in preventing deleterious activation of the immune system by the indigenous microflora, or whether they function nonredundantly by inhibiting different aspects of commensal activation of the immune system. In the latter case, similar disease could develop because of a convergent pathological process, as may be the case in the heterogeneous disease susceptibility observed in human IBD (Bouma and Strober, 2003).

Toll-like receptors (TLRs) play an essential role in the initiation of the immune responses by detecting conserved microbial macromolecules (Takeda et al., 2003). These structures, such as the TLR4 ligand lipopolysaccharide (LPS), present on gram-negative bacteria, are ubiquitous to all microorganisms of a given class, both commensal and pathogenic. Microbial recognition by TLRs is essential for the induction of inflammation and plays an instructive role in the development of the adaptive immune response, in particular the Th1 response (Iwasaki and Medzhitov, 2004).

Given the fundamental role of TLRs in microbial recognition, we examined whether commensal-induced activation of TLRs is responsible for the development of colitis in IL-10- and IL-2-deficient animals. We find that development of colitis in IL-10-deficient mice is completely dependent on an intact TLR-MyD88 signaling pathway. In contrast, mice deficient in IL-2 develop colitis even in the complete absence of the TLR signaling pathways. These results demonstrate distinct and nonredundant mechanisms of negative regulation of

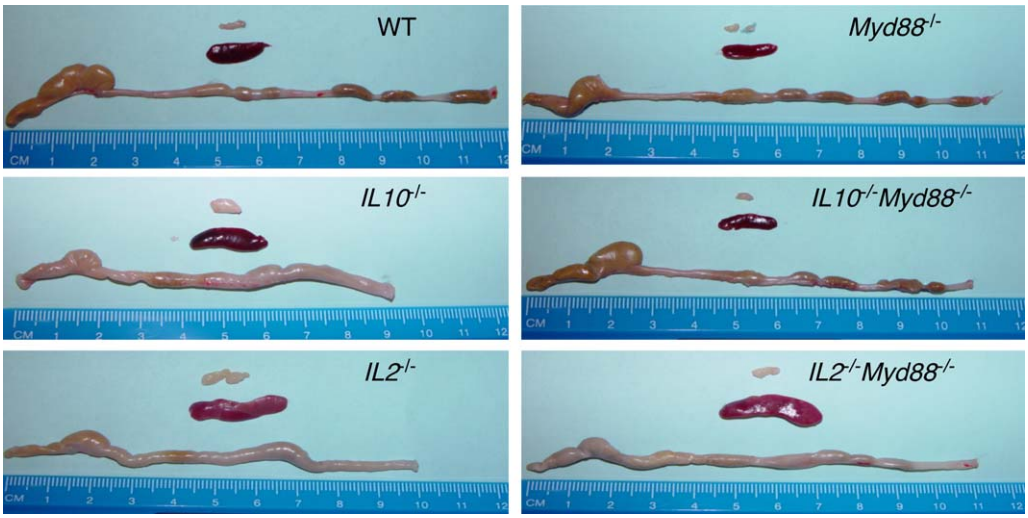

Figure 1. Absence of MyD88 Prevents Gross Pathological Changes in IL-10- but Not IL-2-Deficient Mice

Photographs of representative mesenteric lymph nodes, spleens, and colons from wt, *Myd88*$^{-/-}$, *Il10*$^{-/-}$, *Il10*$^{-/-}$*Myd88*$^{-/-}$, *Il2*$^{-/-}$, and *Il2*$^{-/-}$ *Myd88*$^{-/-}$ mice.

commensal microflora-induced intestinal inflammation mediated by IL-10 and IL-2.

Results

Development of Colitis in *Il10*$^{-/-}$ but Not in *Il2*$^{-/-}$ Mice Is Dependent on the MyD88 Signaling Pathway

To investigate the role of TLRs in commensal-dependent colitis, mice deficient in the TLR signaling adaptor, MyD88 (Takeda et al., 2003), were crossed to IL-10- and IL-2-deficient mice. In all experiments, compound mutants were compared to their single mutant or wild-type (wt) littermates. As expected, *Il10*$^{-/-}$ mice had an early onset of rectal prolapse and mild to moderate weight loss (see Figure S1A in the Supplemental Data available with this article online) followed eventually by mortality. In contrast, *Il10*$^{-/-}$*Myd88*$^{-/-}$ double deficient mice were completely free of all signs of intestinal disease throughout the entire observation period of more than 1.5 years (Figure S1B). However, similar kinetics of rectal prolapse, wasting, and mortality in both *Il2*$^{-/-}$ and *Il2*$^{-/-}$*Myd88*$^{-/-}$ mice were observed (Figures S1A and S1B).

Upon macroscopic analysis, colons of *Il10*$^{-/-}$ and *Il2*$^{-/-}$ mice showed several signs of severe colitis including pronounced thickening of the bowel wall, shortened colonic length, and unformed or absent stool (Figure 1). In contrast, colons from *Il10*$^{-/-}$*Myd88*$^{-/-}$ mice appeared healthy and resembled that of wt and *Myd88*$^{-/-}$ animals (Figure 1). In mice doubly deficient in IL-2 and MyD88, however, this was not the case: large intestines from these animals revealed gross signs of colitis similar to *Il2*$^{-/-}$ mice (Figure 1).

Histopathologic analysis revealed that MyD88 deficiency completely abolished all signs of colitis in *Il10*$^{-/-}$ but not in *Il2*$^{-/-}$ mice (Figure 2A). Representative photomicrographs demonstrate that the multifocal, transmural leukocytic infiltrate, severe epithelial hyperplasia, and destruction of crypt architecture present in *Il10*$^{-/-}$ and *Il2*$^{-/-}$ colons are absent in *Il10*$^{-/-}$*Myd88*$^{-/-}$ mice, yet persist in *Il2*$^{-/-}$*Myd88*$^{-/-}$ mice (Figure 2B).

It was possible that the development of colitis in *Il10*$^{-/-}$ *Myd88*$^{-/-}$ mice may have been delayed rather than completely blocked. To address this, histopathologic analysis of colons from very old (1.5 years of age) *Il10*$^{-/-}$*Myd88*$^{-/-}$ mice was performed. In these mice, there were no signs of abnormal (compared to wt and *Myd88*$^{-/-}$ controls) leukocytic infiltrate or other histopathological changes (Figure 2A).

Collectively, these results demonstrate that IL-10- but not IL-2-dependent colitis is mediated by the MyD88-dependent signaling pathway, indicating that IL-10 and IL-2 differentially regulate commensal-mediated activation of the host immune system.

IL-10 Regulates MyD88-Dependent DC Accumulation and T Cell Activation

We next investigated the basis of differential regulation of colitis by IL-10 and IL-2. The pathogenesis of colitis in both IL-10- and IL-2-deficient mice is dependent on the activation of the adaptive immune system by commensals, as shown by the fact that in both models, these microflora-dependent colitides are prevented in the absence of T cells (Davidson et al., 1996; Ma et al., 1995).

Absence of IL-10 and IL-2 led to aberrant activation of CD4$^+$ T cells in the mesenteric lymph nodes of colitic mice (Figures 3A and 3B). We observed significant upregulation of CD25 and moderate but significant upregulation of CD44 in CD4$^+$ cells from *Il10*$^{-/-}$ mesenteric lymph nodes (MLN) (Sellon et al., 1998). However, in the absence of MyD88, the increased CD4$^+$ T cell activation seen in *Il10*$^{-/-}$ mice was completely abolished (Figures 3A and 3B). As IL-2 is required for the upregulation of CD25 on T cells, CD25 may not be used as an activation marker in mice deficient in IL-2. However, other markers, such as CD44, can be used to determine T cell activation in the absence of IL-2. In contrast to the reversal of pathologic T cell activation in *Il10*$^{-/-}$ mice upon MyD88 deficiency, *Il2*$^{-/-}$*Myd88*$^{-/-}$ mice had similar levels of

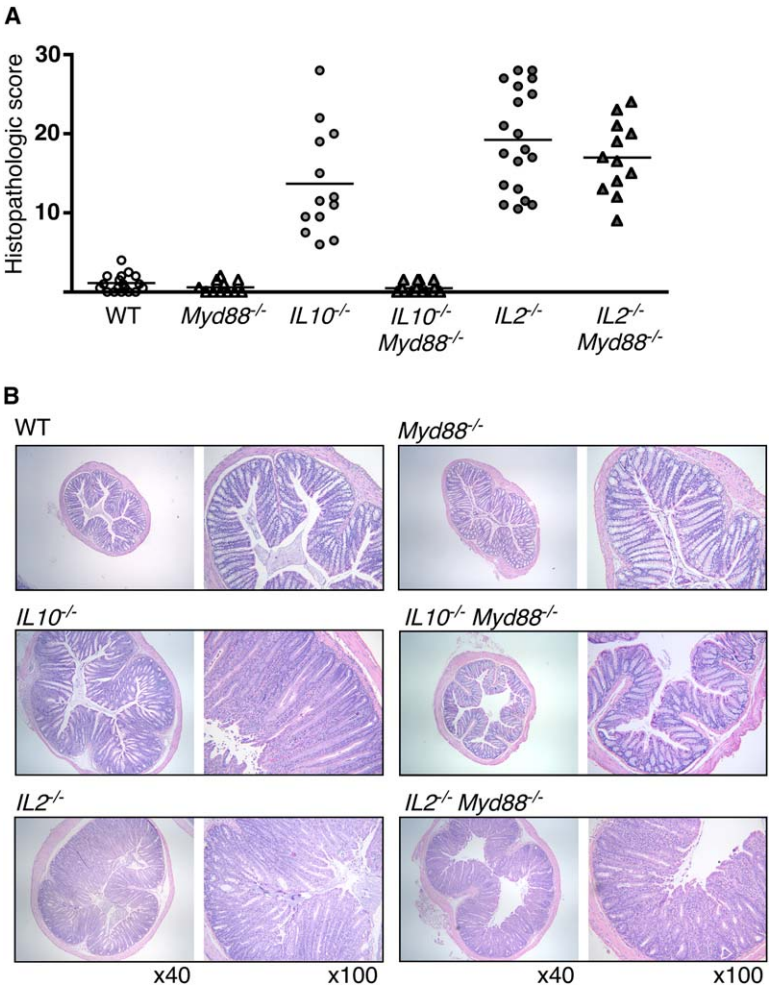

Figure 2. The Development of Spontaneous Colitis in the Absence of IL-10, but Not IL-2, Is Completely Dependent on Signaling through MyD88

(A) Histopathological scoring of colons of wt (n = 19; ranging from 16 weeks to 1.5 years old), *Myd88*$^{-/-}$ (n = 11; ranging from 16 weeks to 1.5 years of age), *Il10*$^{-/-}$ (n = 13; ranging from 16 to 22 weeks of age), *Il10*$^{-/-}$*Myd88*$^{-/-}$ (n = 19; ranging from 16 weeks to 1.5 years of age), *Il2*$^{-/-}$ (n = 19; ranging from 16 to 24 weeks of age), and *Il2*$^{-/-}$*Myd88*$^{-/-}$ (n = 12; ranging from 16 to 24 weeks of age) mice. Scores are based on epithelial hyperplasia and mononuclear and polymorphonuclear infiltrate of the proximal, middle, and distal colon.

(B) Representative photomicrographs (magnifications ×40 and ×100; haemotoxylin and eosin staining) of colons from wt, *Myd88*$^{-/-}$, *Il10*$^{-/-}$, *Il10*$^{-/-}$*Myd88*$^{-/-}$, *Il2*$^{-/-}$, and *Il2*$^{-/-}$ *Myd88*$^{-/-}$ mice.

aberrant CD4^{+} T cell activation as seen in *Il2*$^{-/-}$ mice (Figure 3B).

T cell activation in lymph nodes is dependent on DC maturation and migration from peripheral tissues. We investigated whether there was increased frequency and/or number of DCs in the draining lymph nodes of the intestine, the MLN, of mice deficient in IL-10 or IL-2. Frequencies of CD11c^{+} MHC Class II^{hi} cells were slightly, but consistently and significantly, increased in MLN of *Il10*$^{-/-}$ mice (Figure 3C, left; Figure S2A) and profoundly increased in *Il2*$^{-/-}$ mice, compared to wt animals (Figure 3C, left; Figure S2A). Analysis of the absolute number (Figure 3D, left; Figure S2A) of CD11c^{+} MHC Class II^{hi} cells as determined by multiplying the frequency of CD11c^{+} MHC Class II^{hi} cells (Figure 3C, left) by the total number of cells at the MLN (Figure 3E, left) revealed a significant increase in the number of CD11c^{+} MHC Class II^{hi} cells in both IL-10- and IL-2-deficient mice. The increased presence of CD11c^{+} MHC Class II^{hi} cells in the MLN was completely abolished upon MyD88 deficiency in *Il10*$^{-/-}$*Myd88*$^{-/-}$ but not *Il2*$^{-/-}$*Myd88*$^{-/-}$ mice (Figures 3C and 3D, left; Figure S2A). We did observe a trend of lower frequencies of CD11c^{+} MHC Class II^{hi} cells in *Il2*$^{-/-}$*Myd88*$^{-/-}$ MLN compared to that of *Il2*$^{-/-}$, but it was not statistically significant. Analysis of MHC Class II and CD86 expression on CD11c^{+} cells at the MLN (Figures S2A and S2B) demonstrated that the level of expression of these DC activation markers is similar among all genotypes regardless of colitis or colitic potential. Thus, while all DC present at the MLN have a mature phenotype, the difference between colitic and noncolitic mice appears to be the increased accumulation of DC in the former.

We examined whether the increased frequency of CD11c^{+} MHC Class II^{hi} cells observed in *Il10*$^{-/-}$ mice was a consequence of colitis or preceded the development of histopathologic changes in the intestine. In most, but not all (data not shown), precolitic *Il10*$^{-/-}$ mice, we observed frequencies of CD11c^{+} MHC Class II^{hi} cells similar to that of wt mice (Figure 3C, left), indicating that the absence of IL-10 alone is not sufficient to induce the aberrant DC accumulation and maturation. Observation of increased CD11c^{+} MHC Class II^{hi} cells in some precolitic *Il10*$^{-/-}$ mice suggests that dysregulated DC accumulation to the MLN may play an important role in the initiation of MyD88-dependent colitis.

We next asked whether the increased frequencies and numbers of DCs in the MLN observed in *Il10*$^{-/-}$ and *Il2*$^{-/-}$ mice was due to a unique feature of the intestinal compartment drained by the MLN, such as the presence of the commensal microflora. To this end, we investigated the DC accumulation and maturational status in the axillary lymph nodes (AxLN). In contrast to the increases in the total number of cells (Figure 3C, left), frequency of

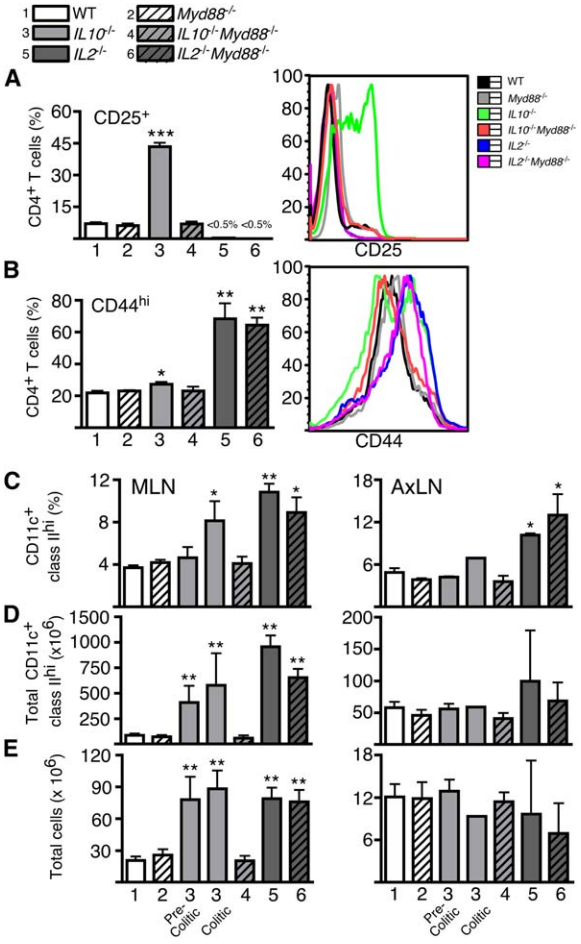

Figure 3. Regulation of MyD88-Dependent and -Independent T Cell Activation and DC Migration by IL-10 and IL-2

Mesenteric lymph node cells from wt, *Myd88*$^{-/-}$, *Il10*$^{-/-}$, *Il10*$^{-/-}$ *Myd88*$^{-/-}$, *Il2*$^{-/-}$, and *Il2*$^{-/-}$*Myd88*$^{-/-}$ mice were stained for (A) CD4 and CD25 and (B) CD4 and CD44. (C) % CDIIc^{+} MHC ClassII^{hi}, (D) total number of CDIIc^{+} MHC ClassII^{hi}, and (E) total number of cells per animal isolated from mesenteric (left) and axillary (right) lymph nodes. Data are the percentage of live cells as determined by forward and side scatter. Data are of four individual experiments with 2–4 mice per genotype per experiment. Error bars are ±SEM. *p < 0.05, **p < 0.01, ***p < 0.001 (compared to wt) according to the Student's test.

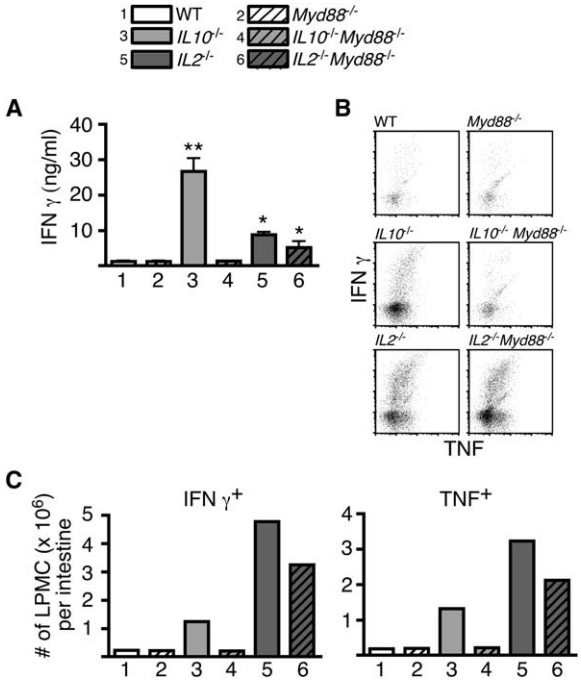

Figure 4. MyD88 Is Required for the Generation of Pathologic Th1 Responses in the Absence of IL-10, but Not IL-2

Lamina propria mononuclear cells were isolated from the colons of wt, *Myd88*$^{-/-}$, *Il10*$^{-/-}$, *Il10*$^{-/-}$*Myd88*$^{-/-}$, *Il2*$^{-/-}$, and *Il2*$^{-/-}$*Myd88*$^{-/-}$ mice. Freshly isolated cells were (A) cultured with plate bound anti-CD3 antibody at 2 × 10^{6} cells/ml for 48 hr, after which supernatants were collected and assayed for the presence of IFN-γ by ELISA or (B and C) assayed for intracellular cytokine production of IFNγ and TNF. Total number of cytokine-positive LPMC intestine {% cytokine^{+} × average number of LPMC per genotype in pooled experiment}. Data are of two individual experiments in which 2–4 colons per genotype were pooled. Error bars are ±SEM. *p < 0.05, **p < 0.01 (compared to wt) according to the Student's test.

CD11c^{+} MHC Class II^{hi} cells (Figure 3C, left), and total number of CD11c^{+} MHC Class II^{hi} cells (Figure 3D, left) observed in *Il10*$^{-/-}$ MLN, there were similar levels of these parameters in the *Il10*$^{-/-}$ AxLN compared to that of wt mice (Figures 3C–3E, right). This suggests that the immune deviations present at the MLN in *Il10*$^{-/-}$ are commensal mediated, consistent with the fact that the only spontaneous defect found in the absence of IL-10 is commensal-dependent colitis in the absence of autoimmunity in other organs (Kuhn et al., 1993). In the absence of IL-2, we observed increased frequencies of CD11c^{+} MHC Class II^{hi} cells in both the MLN (Figure 3D, left) and AxLN (Figure 3D, right) compared to wt controls. However, we did not observe an increase in the total number of CD11c^{+} MHC Class II^{hi} cells in the AxLN (Figure 3D, right), as was observed in the MLN (Figure 3D, left), because lymph node hyperplasia was specific to the MLN in the absence of IL-2 (Figure 3E). Because IL-2 deficiency leads to a systemic autoimmunity in germ-free mice, it is difficult to determine whether the increased MLN hyperplasia and frequency of CD11c^{+} MHC Class II^{hi} cells found in *Il2*$^{-/-}$ and *Il2*$^{-/-}$ *Myd88*$^{-/-}$ mice is commensal dependent but MyD88 independent or commensal independent.

Differential Requirement for the MyD88 Signaling Pathway in Th1 Polarization in IL-10- and IL-2-Deficient Mice

Similar to the pathogenesis of CD in humans (Bouma and Strober, 2003), the development of commensal-dependent colitis in IL-10- and IL-2-deficient mice is characterized by pathologic Th1 immune responses (Berg et al., 1996; Davidson et al., 1998; Ehrhardt et al., 1997). Therefore, the requirement for MyD88 for Th1 polarization of T cells in the colonic lamina propria (LP) in the absence of IL-2 or IL-10 was examined.

Increased production of IFNγ (Figure 4A) and increased frequencies (Figure 4B) and numbers (Figure 4C) of TNF- and IFNγ-producing CD4^{+} T cells (Figure 4B) from lamina propria mononuclear cells (LPMC) isolated from large intestines of *Il10*$^{-/-}$, compared to wt mice (Figure 4), were observed. Similarly, colonic LPMC from

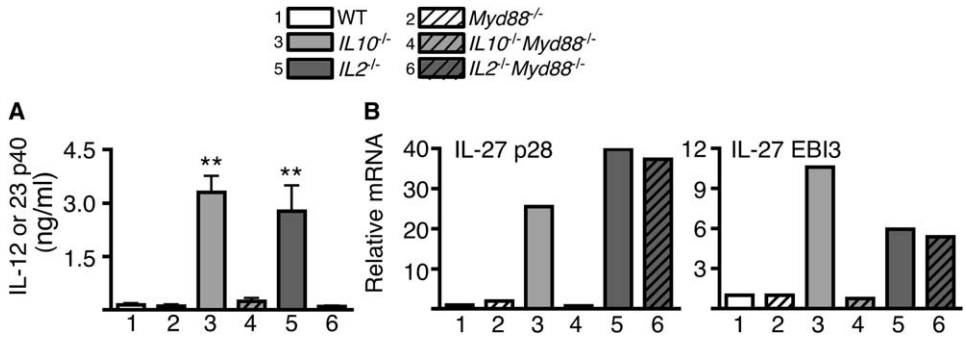

Figure 5. IL-12- or -23-Independent Intestinal Th1 Pathology in the Absence of IL-2

(A) Whole colons of wt, *Myd88*$^{-/-}$, *Il10*$^{-/-}$, *Il10*$^{-/-}$*Myd88*$^{-/-}$, *Il2*$^{-/-}$, and *Il2*$^{-/-}$*Myd88*$^{-/-}$ mice were cultured for 24 hr in serum-free media. Concentration of IL-12 or -23 p40 in supernantant was determined by ELISA. n = 5–8 mice per genotype.

(B) RNA was prepared from isolated colonic lamina propria mononuclear cells of wt, *Myd88*$^{-/-}$, *Il10*$^{-/-}$, *Il10*$^{-/-}$*Myd88*$^{-/-}$, *Il2*$^{-/-}$, and *Il2*$^{-/-}$*Myd88*$^{-/-}$ mice. cDNA was prepared and quantitative polymerase chain reaction was performed. Expression of IL-27 p28 and IL-27 EBI3 was normalized to HPRT and relative induction compared to colonic LPMC from wt mice. Data are of 5 individual experiments of 4–5 mice per genotype. Error bars are ±SEM. **p < 0.01, ***p < 0.001 (compared to wt) according to the Student's test.

IL-2$^{-/-}$ mice, compared to wt mice, had augmented production of IFNγ in vitro (Figure 4A) and increased frequencies (Figure 4B) and numbers (Figure 4C) of IFNγ^{+} and TNF^{+} CD4^{+} Th1 cells.

It is unknown how the indigenous intestinal microflora drives the Th1 polarization in *Il2*$^{-/-}$ and *Il10*$^{-/-}$ mice. Signaling via MyD88 has been shown to be crucial to the induction of systemic Th1 responses (Takeda et al., 2003). We investigated whether Th1 induction in *Il2*$^{-/-}$ and *Il10*$^{-/-}$ mice was MyD88 dependent. Th1 polarization was completely abolished in LPMC of *Il10*$^{-/-}$*Myd88*$^{-/-}$ animals (Figure 4). In contrast, *Il2*$^{-/-}$*Myd88*$^{-/-}$ animals developed pathologic Th1 polarization in the colonic LP similar to that seen in *Il2*$^{-/-}$ mice (Figure 4). No evidence of aberrant Th2 polarization, as determined by measurement of IL-4 expression by whole colons or colonic LPMC, was observed in IL-10- or IL-2-deficient mice, regardless of the absence or presence of functional MyD88 (data not shown), consistent with previous studies showing that colons of *Il10*$^{-/-}$ and *Il2*$^{-/-}$ have pathologic Th1, but not Th2, responses (Berg et al., 1996; Ehrhardt et al., 1997; McDonald et al., 1997). These results indicated that the commensal microflora can induce pathologic intestinal Th1 polarization via both MyD88-dependent and -independent pathways. Thus, in the absence of IL-10, MyD88 signaling pathway is required for the development of commensal-driven Th1 responses. However, in IL-2-deficient mice, a MyD88-independent signaling pathway is sufficient for the development of commensal-driven Th1 responses in the large intestine.

Differential Control of Intestinal Th1 Responses in *Il10*$^{-/-}$ and *Il2*$^{-/-}$ Mice

The generation of Th1 immune response is dependent on the production of polarizing cytokines by antigen-presenting cells such as DCs and macrophages. The heterodimeric cytokines IL-12 and IL-23, which share the p40 subunit, play a critical role in Th1 differentiation in a variety of settings (Trinchieri, 2003). We found increased production of IL-12 or -23 p40 in the colons of *Il10*$^{-/-}$ and *Il2*$^{-/-}$ mice compared to wt mice (Figure 5A), consistent with previous reports (Ehrhardt et al., 1997; Sellon et al., 1998). Thus, deficiencies in IL-10 or IL-2 result in the failure to regulate IL-12 or -23 p40 production in the colon. However, it is unknown how the indigenous microflora stimulates the production of IL-12 or -23 p40 in the large intestine. Analysis of colons from both *Il10*$^{-/-}$*Myd88*$^{-/-}$ and *Il2*$^{-/-}$*Myd88*$^{-/-}$ mice demonstrated that the increased production of IL-12 or -23 p40 in the absence of IL-10 and IL-2 was completely dependent upon MyD88 (Figure 5A). This suggests that in both models of colitis, the commensal bacteria induce the aberrant production of IL-12 and IL-23 through stimulation of TLR-MyD88 signaling in the colon. The aberrant IL-12 or -23 p40 expression at the colon (Figure 5A) and the MLN (Figure S3), but not in the other inflamed organs, such as the kidney, lung, and liver of *Il2*$^{-/-}$ mice (Figure S4), indicates that MyD88-dependent regulation of IL-12 or -23 p40 at the intestine is due to activation of TLR via the resident commensal flora.

Myeloid cells such as macrophages and DC are the best-known producers of IL-12 and IL-23 (Trinchieri, 2003). In the intestine, lamina propria DC may be the sole producer of IL-12 or -23 p40 (Becker et al., 2003). This suggests that IL-10- and IL-2-dependent mechanisms negatively regulate TLR recognition of the commensal microflora by macrophages and DC in the large intestine and certain consequences of this recognition such as the colitogenic (in *Il10*$^{-/-}$ mice) production of IL-12 or -23 p40 and perhaps other factors (Kullberg et al., 2001) (see below). We have previously reported that TLR-mediated recognition of the commensal microflora occurs at the steady state in the colon and that such recognition may be important for maintaining intestinal epithelial homeostasis and protection from injury (Rakoff-Nahoum et al., 2004). To determine which cell types (bone marrow [BM] or non-BM-derived) were responsible for both TLR-mediated protection from intestinal injury and recognition of the commensal microflora at the steady state, we made BM chimeras to create mice with MyD88 deficency in BM-derived cells and MyD88 sufficiency in non-BM-derived cells and vice versa. We find that MyD88-dependent signaling on BM-derived cells is responsible for protection from DSS-induced mortality and morbidity (Figures S5A and S5B).

In addition, we find that BM-derived cells recognize TLR ligands of the commensal microflora at the steady state (Figure S6), in a MyD88-dependent (Rakoff-Nahoum et al., 2004; Figure S6), commensal microflora-dependent (Rakoff-Nahoum et al., 2004), and TLR2- and TLR4-mediated manner (Figure S6). Together, these data suggest that IL-10- and IL-2-dependent mechanisms set a threshold for physiologic recognition of the commensal microflora by TLRs by bone marrow-derived cells. The absence of IL-12 and IL-23 in *Il10*$^{-/-}$ *Myd88*$^{-/-}$ mice correlated well with the absence of pathologic intestinal Th1 responses and the protection from colitis. However, the absence of MyD88-dependent IL-12 or -23 p40 production in *Il2*$^{-/-}$*Myd88*$^{-/-}$ mice revealed that MyD88-independent, IL-12- or -23-independent factor(s) were sufficient to drive Th1 immune responses in the IL-2-deficient mice.

Several studies have shown that, in addition to IL-12/IL-23, other cytokines induced upon microbial recognition, including type I interferons (Takeda et al., 2003) and the IL-6/12 superfamily member IL-27 (Becker et al., 2005), may play a role in the development of Th1 responses (Trinchieri et al., 2003). We hypothesized that IL-2 deficiency may result in aberrant expression of such factors and drive the commensal-dependent Th1 polarization observed in the colons of *Il2*$^{-/-}$ and *Il2*$^{-/-}$*Myd88*$^{-/-}$ mice. No changes in the relative expression of IFNα4 or IFNβ in the colons of all mice examined were detected (data not shown). However, we found an increase in the expression of both subunits of IL-27, p28 and EBI3, in the colonic LPMC and colons of both *Il10*$^{-/-}$ and *Il2*$^{-/-}$ mice, compared to wt (Figure 5B and data not shown). This suggested that IL-10- and IL-2-dependent mechanisms, in addition to negatively regulating IL-12 or -23 production, also inhibit the expression of IL-27. Interestingly, the increased expression of both subunits of IL-27 observed in *Il10*$^{-/-}$ mice was abolished in *Il10*$^{-/-}$*Myd88*$^{-/-}$ mice. However, in contrast to the MyD88-dependent regulation of IL-12 or -23 p40 (Figure 5A), *Il2*$^{-/-}$*Myd88*$^{-/-}$ mice produced aberrant levels of IL-27 p28 and EBI3, similar to that found in *Il2*$^{-/-}$ mice (Figure 5B). This indicated that the expression of IL-27 is induced via both MyD88-dependent (in *Il10*$^{-/-}$ colitis) and -independent (in *Il2*$^{-/-}$ colitis) mechanisms and that IL-10- and IL-2-dependent mechanisms differentially and nonredundantly regulate the induction of IL-27.

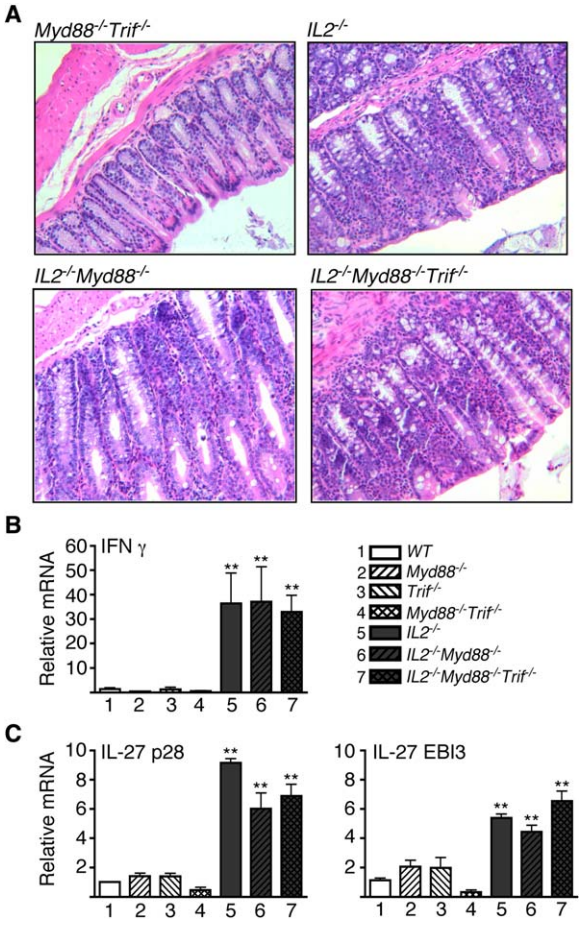

Figure 6. Commensal-Dependent Colitis in the Absence of IL-2 Is Completely Independent of TLR Signaling

(A) Representative microphotos of large intestine from *Myd88*$^{-/-}$ *Trif*$^{-/-}$, *Il2*$^{-/-}$, *Il2*$^{-/-}$*Myd88*$^{-/-}$, *Il2*$^{-/-}$*Myd88*$^{-/-}$ *Trif*$^{-/-}$ age-matched littermates. Magnification ×200.

(B and C) IFNγ (B) and IL-27 p28 (C) and EBI3 expression in colons. RNA was prepared from colons of wt, *Myd88*$^{-/-}$, *Trif*$^{-/-}$, *Trif*$^{-/-}$ *Myd88*$^{-/-}$, *Il2*$^{-/-}$, *Il2*$^{-/-}$*Myd88*$^{-/-}$, and *Il2*$^{-/-}$*Myd88*$^{-/-}$TRIF$^{-/-}$ mice. cDNA was prepared and quantitative polymerase chain reaction was performed. Expression of IFNγ, IL-27 p28, and EBI3 was normalized to HPRT and relative induction compared to colons from wt mice. Data are of duplicate experiments of 2–4 mice per genotype. Error bars are ±SEM. **$p < 0.01$ (compared to wt) according to the Student's test.

Commensal-Dependent Colitis in the Absence of IL-2 Is Independent of Commensal Recognition by TLRs

The above results revealed a divergence in the regulation of immune responses to the commensal microflora in the development of commensal-dependent colitis. Thus, in *Il10*$^{-/-}$ but not in *Il2*$^{-/-}$ mice, colitis is dependent on the TLR-MyD88 signaling pathway. This left us with the question of whether the commensal-dependent colitis, which develops in *Il2*$^{-/-}$ mice, occurs in a TLR-dependent/MyD88-independent manner or is completely independent of signaling via TLRs. While most TLRs are completely dependent on the MyD88 signaling pathway (Takeda et al., 2003), MyD88-independent signaling downstream of TLR4 and TLR3 occurs via the signaling adaptor TRIF (Hoebe et al., 2003; Yamamoto et al., 2003). Thus, to definitively address the role of TLR signaling in the development of commensal-dependent colitis in *Il2*-deficient mice, we generated mice triply deficient in IL-2, MyD88, and TRIF. In these mice, all classical TLR-dependent signaling, such as activation of NF-κB, MAP kinases, and the production of inflammatory mediators, is abolished (Yamamoto et al., 2003). Analysis of the colons of *Il2*$^{-/-}$*Myd88*$^{-/-}$ *Trif*$^{-/-}$ mice revealed that even in the absence of all classical TLR-mediated signaling, chronic intestinal inflammation (Figure 6A) still occurs in IL-2-deficient mice. This chronic inflammation in triply-deficient mice was Th1 polarized (Figure 6B). In addition, we observed increased expression of both IL-27 p28 and EBI3 in *Il2*$^{-/-}$ mice, independent of MyD88 and TRIF-dependent signaling (Figure 6C), indicating that the induction of IL-27

occurs in a TLR-independent manner in the context of IL-2 deficiency.

IL-2 deficiency leads to a systemic autoimmunity manifested by autoimmune hemolytic anemia, splenic lymphoplasia, and inflammation of the kidney, liver, lung, and stomach, which occurs independently of the commensal microflora (Ma et al., 1995; Sadlack et al., 1993) and MyD88 as determined by investigation of systemic autoimmunity of *Il2*$^{-/-}$*Myd88*$^{-/-}$ mice (Figure S7). This systemic and organ-specific autoimmunity is most likely driven by autoantigens and defects in regulation of the adaptive immune system. However, the colitis that develops in the absence of IL-2 is dependent on the presence of the commensal microflora, indicating that host self antigens are not sufficient to drive intestinal inflammation. Barring the formal possibility that IL-2 deficiency on a MyD88- or TRIF-deficient background affects the role of the commensal flora in disease pathogenesis, our studies have determined that classical TLR-mediated pattern recognition of the commensal microflora is not required for the development of colitis in the absence of IL-2. Thus, it remains to be determined whether commensal-dependent colitis in *Il2*$^{-/-}$ mice is driven by commensal non-self antigens or non-TLR-mediated innate immune recognition of commensal microbial products. To address this, we looked for signs of activation of other known non-TLR microbial pattern-recognition receptors. Activation of the inflammasome and processing and secretion of IL-1β by caspase-1 is perhaps the best-known function and affect of activation of PRRs such as NALP1, NALP3, and NAIP5 (Meylan et al., 2006). We find IL-1β to be aberrantly secreted in both *Il10*$^{-/-}$ and *Il2*$^{-/-}$ colons (Figure S8). However, in both of these models, we find IL-1β secretion to be completely dependent on MyD88, despite full-blown colitis in *Il2*$^{-/-}$*Myd88*$^{-/-}$ mice (Figure S8). These data support the hypothesis that the primary, colitogenic defect in IL-2 deficiency is due to defective regulation of antigen recognition rather than pattern recognition of the commensal microflora, in addition to demonstrating that the role of MyD88 in *Il10*$^{-/-}$ colitis is due to TLR recognition and not IL-1β production secondary to non-TLR-mediated recognition, although this factor (as well as IL-18) may play a role in TLR-initiated disease pathogenesis.

Thus, in contrast to *Il10*$^{-/-}$ colitis, the commensal-dependent colitis in the absence of IL-2 occurs independently of TLR signaling, revealing fundamentally different mechanisms of microflora-induced disease development and pathologic Th1 responses in these two models.

Discussion

Negative regulation of the host immune response to the indigenous microflora maintains steady-state intestinal immune homeostasis. Deficiencies in IL-10 or IL-2 lead to commensal-induced chronic, T cell-mediated intestinal inflammation. However, how the intestinal microflora initiates and drives IBD and how this process is regulated by IL-10 and IL-2 is enigmatic.

We demonstrate that IL-10 mediates intestinal immune homeostasis by regulating the consequences of innate recognition of commensals by TLRs via the MyD88-dependent signaling pathway. A contribution of TLR4 in the pathogenesis of spontaneous colitis has been shown in studies of mice with myeloid-specific deletion of STAT3, which functions downstream of several cytokines, including IL-10 (Kobayashi et al., 2003). When these mice were crossed to TLR4-deficient animals, colitis was delayed, yet not prevented, and aberrant IFNγ production by T cells was apparent even at the very early age of 16 weeks (Kobayashi et al., 2003). Notably, PRRs other than TLR4 can recognize commensal bacteria. Our studies show that *Il10*$^{-/-}$ mice deficient in MyD88 are free from any signs of colitis or aberrant immune responses for more than 1.5 years. This unequivocally demonstrates that IL-10 negatively regulates MyD88-dependent, commensal-induced inflammation and that MyD88-dependent signaling is the cause of colitis in the absence of IL-10.

In contrast to the colitis in *Il10*$^{-/-}$ mice, development of colitis in *Il2*$^{-/-}$ mice was independent of TLR signaling, even though both forms of colitis are dependent on the commensal microflora. While it remains formally possible that some nonclassical TLR-dependent signaling, independent of MyD88 and TRIF, is involved in *Il2*$^{-/-}$ colitis, our results demonstrate that IL-2- and IL-10-dependent mechanisms nonredundantly regulate commensal-induced activation of the immune system. It is not fully understood why IL-2 deficiency leads to commensal-independent systemic autoimmunity and commensal-dependent colitis. In vitro studies have indicated that IL-2 may be important in activation-induced T cell death (Van Parijs et al., 1999). Emerging in vivo studies suggest that the immune abnormalities observed in IL-2 deficiency may be due to the observed deficiency in naturally occurring CD4$^+$ CD25$^+$ regulatory T cells (T_{reg}) (Malek et al., 2002). IL-2 has recently been described to act as a growth/maintenance factor required for the survival of T_{reg} (Fontenot et al., 2005; Setoguchi et al., 2005). Therefore, it is likely that commensal-dependent colitis in *Il2*$^{-/-}$ mice is due to the deficiency in T_{reg} function or maintenance.

A surprising finding of the present study is the differential control of Th1 responses to the intestinal microflora. It is believed that instructive signals induced upon innate recognition of microbes are essential for the initiation of a Th1 response. Indeed, we find that the aberrant intestinal Th1 response in the absence of IL-10 is dependent on MyD88 and correlates with the production of MyD88-dependent IL-12 or -23 p40, which has been demonstrated to play an important, yet incomplete, role in microbe-driven IL-10-deficient colitis (Kullberg et al., 2001). In contrast, we found that pathologic intestinal Th1 responses in the absence of IL-2 were independent of TLR (MyD88 and TRIF) and, surprisingly, also of IL-12/IL-23 p40, which seems to be strictly regulated in a MyD88-dependent manner, even in the absence of IL-2. A role for IL-12 or -23 p40 in a hapten-induced form of colitis in *Il2*$^{-/-}$ mice has been suggested (Ehrhardt et al., 1997); however, the role of this factor in spontaneous, commensal-dependent colitis has not been addressed. Indeed, our results suggest that commensal-dependent colitis in the absence of IL-2-mediated immunoregulation does not require IL-12 or -23 p40. This situation is reminiscent of the long-standing paradox of pathogenesis in celiac disease in which there are aberrant Th1 responses and T_{bet}

expression (Monteleone et al., 2004), yet no detectable abnormalities in IL-12 or STAT4 (Nilsen et al., 1998). IL-12 or -23 p40-independent Th1 response to microbial pathogens (Jankovic et al., 2002; Sato and Iwasaki, 2004; Schijns et al., 1998) have been demonstrated, suggesting that a MyD88-independent factor(s) other than IL-12 and IL-23 may drive a Th1 response. We identified IL-27 as one candidate cytokine for this function because of its previously described role in both initiating and maintaining Th1 responses (Villarino et al., 2005) and its high expression in IBD lesions (Becker et al., 2005). We find IL-27 to be positively associated with commensal-dependent, Th1-polarized colitis in both *Il10*$^{-/-}$ and *Il2*$^{-/-}$ models. Interestingly, there appears to be both MyD88-dependent and TLR- (and MyD88-) independent regulation of IL-27 because its expression is MyD88 dependent in the absence of IL-10 but TLR independent in the absence of IL-2. It is also possible that IL-27 does not play a causative role in the development of colitis, but rather is expressed as a result of colitis. This latter possibility is supported by evidence suggesting that IL-27, or at least its receptor WSX-1, plays a role in negatively regulating immune responses (Villarino et al., 2005). In this regard, it is possible that the Th1 polarization in *Il2*$^{-/-}$ colons is IL-27 independent, but that this expression is a consequence of dysregulated adaptive immune responses and is not driven by innate immune recognition. Future studies will be required to determine the relative contribution of IL-27 in these models of commensal-dependent colitis and also in physiologic Th1 responses in the intestine, such as to intestinal pathogens.

Recent studies have demonstrated that activation of the TLR-MyD88 signaling pathway due to commensal recognition plays an essential role in intestinal tissue protection from injury and repair (Araki et al., 2005; Cario et al., 2004; Fukata et al., 2005; Pull et al., 2005; Rakoff-Nahoum et al., 2004). Importantly, these responses occur in mice sufficient in IL-10 and IL-2. Thus, IL-10- and IL-2-dependent regulatory mechanisms do not completely extinguish the activation of the immune system by the commensal microflora but rather "fine-tune" (Madara, 2004) commensal-TLR interactions in the intestine. A major challenge is to understand how IL-10- and IL-2-dependent mechanisms prevent the deleterious consequences of commensal-induced immune responses, while allowing for beneficial, physiological interactions between commensals and the host. Loss-of-function mutations in TLRs, resulting in either negative (Gewirtz et al., 2006) or positive (Braat et al., 2005; Brand et al., 2005; Franchimont et al., 2004; Gazouli et al., 2005; Oostenbrug et al., 2005) associations with IBD, the beneficial role of TLRs in mucosal tissue repair (Araki et al., 2005; Cario et al., 2004; Fukata et al., 2005; Jiang et al., 2005; Pull et al., 2005; Rakoff-Nahoum et al., 2004), and protection from mucosal pathogens (Hawn et al., 2003) may be indicative of these functional and evolutionary constraints.

Collectively, our study demonstrates the existence of two distinct and nonredundant mechanisms that control intestinal Th1 responses and the development of commensal-dependent colitis. It appears that the anti-inflammatory cytokine IL-10 regulates the pathologic consequences of TLR recognition of the commensal microflora, which occur via MyD88. However, while IL-2 regulates TLR-MyD88 signaling in the intestine (such as the regulation of IL-12 or -23 p40), recognition of the commensal microflora by TLRs is not responsible for commensal-dependent colitis in IL-2$^{-/-}$ mice. This suggests that the colitis in IL-2 deficiency is driven by some other aspect of the commensal microflora. Indeed, commensal-dependent immune responses can be caused by either commensal stimulation of the innate immune system or by the recognition of commensal-derived antigens by the adaptive immune system. T_{reg} are potent regulators of adaptive immune responses and can act on both T cells (Shevach, 2002) and DC (Malmstrom et al., 2001). It is likely, therefore, that a defect in regulation of the adaptive immune response to the plethora of commensal non-self antigens in the context of T_{reg} deficiency is responsible for the development of colitis in *Il2*$^{-/-}$ mice, although a role of non-TLR innate recognition cannot yet formally be ruled out.

The etiology of human IBD is unknown, but it is thought to result from dysregulated interactions between the commensal microflora and the host immune system. There are potentially several regulatory mechanisms that may be responsible for this dysregulation given the heterogeneity of disease susceptibility, including malfunction of anti-inflammatory cytokines or regulatory T cells. Depending on the mechanism, different factors may be responsible for the initiation and maintenance of the disease. Our study suggests that TLR-mediated signaling plays a critical role in intestinal inflammation in the context of deficiency in the anti-inflammatory cytokine IL-10, but not in the context of insufficient activity of regulatory T cells. Thus, depending on the pathophysiological mechanism, different aspects of host-commensal interactions should be targeted for optimal therapeutic intervention in IBD.

Experimental Procedures

Mice

Il10$^{-/-}$ and *Il2*$^{-/-}$ (Jackson Lab, Bar Harbor, ME) mice both on a C57BL/6 background were each crossed to *Myd88*$^{-/-}$ mice. Breeding was established so that each litter contained single deficient (*Il10*$^{-/-}$, *Il2*$^{-/-}$, or *Myd88*$^{-/-}$), double deficient (*Il10*$^{-/-}$ *Myd88*$^{-/-}$ or *Il2*$^{-/-}$*Myd88*$^{-/-}$), and wt littermate controls. For establishment of *Il2*$^{-/-}$*Myd88*$^{-/-}$*Trif*$^{-/-}$ mice among *Il2*$^{-/-}$ and *Il2*$^{-/-}$ *Myd88*$^{-/-}$ littermates, *Il2*$^{+/-}$*Myd88*$^{+/-}$ *Trif* $^{+/-}$ mice were intercrossed. *Myd88*$^{-/-}$ and *Trif*$^{-/-}$ mice were kindly provided by Dr. S Akira. Mice were bred and maintained under specific pathogen-free conditions at the animal facility of Yale University School of Medicine. Studies were approved by the Institutional Animal Care & Use Committee of Yale University.

Histological Scoring

Colons were excised and cut into three equal segments: proximal, middle, and distal. Tissue was fixed with 10% neutral formalin, paraffin embedded, sectioned at 3–6 μm, and stained with hematoxylin and eosin. Sections were analyzed in a blinded manner by a trained gastroentero-pathologist. Each segment was given a score of 0–3 (where 0 = none; 1 = mild; 2 = moderate; 3 = severe) for epithelial hyperplasia, mononuclear infiltrate, and polymorphonuclear infiltrate. Histopathologic score is the sum of all three criteria for each segment.

Isolation of Lymph Node and Colonic Lamina Propria Mononuclear Cells

Mesenteric or axillary lymph nodes were isolated and placed on ice in 10% RPMI media. Lymph nodes were cut into fragments and

shaken at 150 rpm at 37°C for 30 min in 400 units/ml collagenase (Roche) and 15 μg/ml DNase (Roche) in 10% complete RPMI media. Digested fragments were spun and incubated for 5 min in 5 mM EDTA/5% FCS/Hank's balanced salt solution (HBSS). Cells were prepared into single-cell suspension by grinding with a 3 ml syringe plunger through 70 μm plastic mesh. Cells were washed twice and filtered once more through a 70 μm strainer. Colonic lamina propria mononuclear cells were isolated via a modification of a protocol of Laky et al. (1997). In brief, colons were excised and cut longitudinally and then laterally into 1.5 cm pieces and washed three times in CMF buffer at room temperature. Tissue was shaken at 37°C for 20 min in CMF plus dithioerythritol (DTE). Intestinal fragments were further degraded by a 25 min incubation with CMF plus EDTA, washed three times with 1× HBSS, and shaken at 37°C for 30–40 min in RPMI supplemented with collagenase, DNase (Roche), and Mg^{2+}/Ca^{2+}. Supernatant was spun and reconstituted in media and passed through nylon wool columns and subjected to fractionation by 44% and 67.5% Percoll (Amersham) gradients for the isolation of mononuclear cell populations. Cells were counted on a Coulter counter.

Cell Culture of LPMC

Flat-bottom, 96-well tissue-culture plates were coated with 10 μg/ml αCD3 (2C11; BD Pharmingen) in PBS overnight at 4°C. 2×10^6 cells/ml were cultured in RPMI complete medium (10% fetal calf serum, 100 u/ml penicillin-streptomycin, 2 mM L-glutamine, 1 mM Sodium Pyruvate, 10 mM HEPES, 50 μM β-mercaptoethanol) supplemented with 40 U/ml recombinant human IL-2 (BD Laboratory Discovery) for 48 hr. Supernatants were harvested and stored at −20°C.

Intracellular Cytokine Staining and FACS

For intracellular cytokine staining, freshly isolated LPMC were cultured for 5 hr in complete media supplemented with 50 ng/ml PMA, 500 ng/ml ionomycin, and 1 μl/ml BD GolgiPlug (BD Pharmingen) in 96-well plates. Cell were harvested and processed with the Intracellular Cytokine Staining kit (BD Pharmingen). Cells were stained with αCD4-APC, αCD8α-PerCP, αIFNγ-PE, and αTNF-FITC. IgG-PE and IgG-FITC were used as isotype controls. For MLN and LPMC phenotypic analysis, freshly isolated cells were stained with antibodies to CD3, CD4, CD8α, CD11b, CD11c, and B220 and analyzed on FACSCalibur flow cyometers (Becton-Dickenson). All antibodies were from BD Pharmingen (San Diego, CA).

Colon Organ Culture

In brief, 1 cm × 1 cm standardized segments of all three parts of the colon were washed in cold PBS supplemented with penicillin and streptomycin (GIBCO). These segments were cultured in 24-well flat-bottom culture plates (Falcon) in serum-free RPMI 1640 medium (GIBCO) supplemented with penicillin and streptomycin. After 24 hr, supernatant fluid was collected and stored at −20°C until analyzed.

Cytokine Measurement by Enzyme-Linked Immunosorbant Assay

Paired antibodies (α-mouse purified and biotinylated) and recombinant standards for IFNγ, IL-6, IL-12 p40 (BD Bioscience Pharmingen), and KC (R&D Systems) were used to quantify factors present in supernatants of whole colon and LPMC cultures.

Isolation of RNA and Quantitative Reverse Transcriptase PCR

For quantitative RT-PCR, tissue was homogenized with a rotor stator in TRIZOL (Life Technologies), and total RNA was isolated according to manufacturer's instructions. RNA was digested with Dnase I and processed with RNAII kit (Clontech). RNA was reverse transcribed by Superscript II (Invitrogen), and cDNAs were used for PCR with Quantitect SYBR Green reagents (Qiagen, Valencia, CA) on a Stratagene MX3000 bioanalyzer (La Jolla, CA). The abundance of each cytokine mRNA was normalized to HPRT expression and compared to levels in wt intestines to calculate the fold induction. Sequences of primers available upon request.

Supplemental Data

Supplemental Data include eight figures and Supplemental Experimental Procedures and can be found with this article online at http://www.immunity.com/cgi/content/full/25/2/319/DC1/.

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