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# Regulatory mechanisms of immune responses to intestinal bacteria

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The intestinal mucosa allows the residence of trillions of bacteria with which it interacts dynamically. To establish and maintain a mutually beneficial relationship, the mucosal immune system must enforce tolerance toward the vast non-pathogenic microbiota while simultaneously remaining reactive to potentially pathogenic microbes; the disruption of this delicate balance results in inflammatory bowel diseases. In this review, we describe advances in our understanding of regulatory mechanisms of the innate immune system and how these shape adaptive immune systems during steady-state and inflammatory processes in the intestinal mucosa.

## INTRODUCTION

The gastrointestinal tract is composed of very unique tissue with the presence of commensal bacteria.<sup>1</sup> Commensal bacteria colonize the intestinal lumen shortly after birth and comprise approximately 1,000 species with a density of  $10^{12}$  organisms per milliliter of luminal contents in the large intestine.<sup>2–4</sup> The host intestine provides bacteria with a microenvironment rich in nutrients derived from ingested food. Commensal bacteria, in turn, contribute to the digestion of food, provision of essential nutrients, and prevention of propagation of pathogenic microorganisms.<sup>5,6</sup> To maintain these beneficial relationship, the host immune system should remain hypo-responsive to commensal bacteria. However, at the same time, the intestinal immune system has to combat invasive pathogenic bacteria. Thus, the immune system in the intestinal mucosa has very complicated features that are distinct from that in other tissues. The intestinal immune system mounts a strong inflammation against invasive pathogenic bacteria while providing many layers of inhibitory mechanisms not to mount excessive immune response against commensal bacteria. The breakdown of such a delicate balance of the intestinal immune system causes the development of inflammatory bowel diseases (IBDs).<sup>7,8</sup> Accordingly, the regulatory mechanisms of immune responses to intestinal bacteria have recently been a subject of intensive research.

## REGULATION OF TLR SIGNALING IN THE INTESTINE

Among some of the most impressive progress in the field of immunology in the last decade has been the elucidation of the

mechanisms for innate immune recognition of microorganisms.<sup>9</sup> Innate immunity specifically recognizes molecular patterns of microorganisms through Toll-like receptors (TLRs) and other pattern-recognition receptors, and use of pattern-recognition receptors helps regulate the development of antigen-specific adaptive immune responses.<sup>10–12</sup> Functional characterization of TLRs has made us aware that TLR-dependent responses at the intestinal mucosa should be finely regulated. Macrophages isolated from the intestinal lamina propria of healthy humans and mice have been shown to be hypo-responsive to TLR stimulation.<sup>13–16</sup> In contrast, substantial amounts of TLR-dependent cytokine production can be observed in intestinal lamina propria macrophages from hosts with intestinal inflammation.<sup>13,14</sup> For example, macrophages from interleukin (IL)-10-deficient mice and myeloid cell-specific *Stat3*-deficient mice, both of which spontaneously develop intestinal inflammation, are in a state of constitutive and aberrant activation.<sup>17</sup> In these mutant mice, blockade of the TLR signaling by the introduction of deficiency in TLR4 or MyD88 abrogates the development of intestinal inflammation,<sup>18,19</sup> indicating that TLR-dependent excessive inflammatory responses at the intestinal mucosa causes development of colitis. In addition, as with the murine model of intestinal inflammation, a unique subset of macrophages with excess TLR-dependent inflammatory responses were observed in the lamina propria of IBD patients.<sup>20–22</sup> Thus, negative regulation of TLR-dependent responses in intestinal lamina propria macrophages is a prerequisite to prevent intestinal inflammation both in humans and in mice.

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Received 31 December 2008; accepted 30 January 2009; published online 4 March 2009. doi:10.1038/mi.2009.8

Several molecules that inhibit activation of the TLR signaling pathways have been identified.<sup>23</sup> TLR possesses the extracellular domain characterized by the presence of a leucine-rich repeat responsible for the microbial recognition and the intracellular Toll/IL-1 receptor (TIR) domain, and thus constitutes a large TLR/IL-1R superfamily. The single immunoglobulin (Ig) IL-1 receptor-related molecule (SIGIRR, also known as TIR8) represents a unique subgroup of the TLR/IL-1R superfamily, consisting of an extracellular single Ig domain and an intracellular TIR domain.<sup>24</sup> SIGIRR is expressed in dendritic cells and intestinal epithelial cells, and inhibits TLR signaling.<sup>24,25</sup> Indeed, mice lacking *Sigirr* show high susceptibility to intestinal inflammation induced by dextran sodium sulfate (DSS) and increased colitis-associated tumor incidence.<sup>26</sup> Intestinal epithelial cells from *Sigirr*-deficient mice show increased commensal bacteria-dependent cytokine production. Intestinal epithelium-specific expression of SIGIRR reduces the sensitivity to DSS-induced intestinal inflammation and tumorigenesis in *Sigirr*-deficient mice. These findings suggest the crucial role of SIGIRR in maintenance of intestinal homeostasis through negative regulation of TLR signaling in the intestinal mucosa. The inhibitory effect of SIGIRR is shown to be exerted through ligand-dependent interaction with TLR and the signaling molecule tumor necrosis factor (TNF)-receptor-associated factor 6 (TRAF6).<sup>24</sup>

The IL-1R-associated kinase (IRAK) family of kinases consists of four members, namely IRAK1, IRAK2, IRAK-M, and IRAK4. IRAK1, 2, and 4 are shown to mediate the TLR signaling by bridging TIR domain-containing adaptors to TRAF6.<sup>27</sup> In contrast to other IRAK family members that are ubiquitously expressed, IRAK-M expression is limited to monocytes; it negatively regulates the TLR signaling by inhibiting formation of the IRAK1-TRAF6 complex.<sup>28</sup> *Irak3* (the gene encoding IRAK-M)-deficient mice show severe inflammatory response in the intestine after infection with *Salmonella typhimurium*,<sup>28</sup> indicating its role in the modulation of TLR-dependent intestinal inflammatory responses. Although there is no evidence suggesting an association between IBDs and single nucleotide polymorphisms in IRAK-M,<sup>29</sup> further studies are needed to determine whether an impaired negative regulation of the TLR signaling pathway by IRAK-M might be partly responsible for the development of IBD.

A20 is a ubiquitin-modifying enzyme that is rapidly induced by TLR or TNF- $\alpha$  stimulation and terminates TLR-dependent responses by removing ubiquitin moieties from the signaling molecule TRAF6.<sup>30</sup> *Tnfrsf17* (the gene encoding A20)-deficient mice exhibit severe inflammation of multiple organs, including the intestine. Spontaneous inflammation in *Tnfrsf17*-deficient mice is rescued by the introduction of the deficiency of MyD88. In addition, depletion of intestinal bacteria by treatment with broad-spectrum antibiotics diminishes the inflammation in *Tnfrsf17*-deficient mice.<sup>31</sup> Thus, A20 is a key negative regulator of intestinal bacteria- and TLR-dependent inflammation.

The suppressor of cytokine signaling-1 (SOCS1) was identified as a potent negative regulator of signaling pathway of various cytokines, including interferon- $\gamma$  and IL-4.<sup>32</sup> SOCS1 has subsequently been shown to inhibit the TLR-induced inflammatory responses. Although early lethality owing to severe

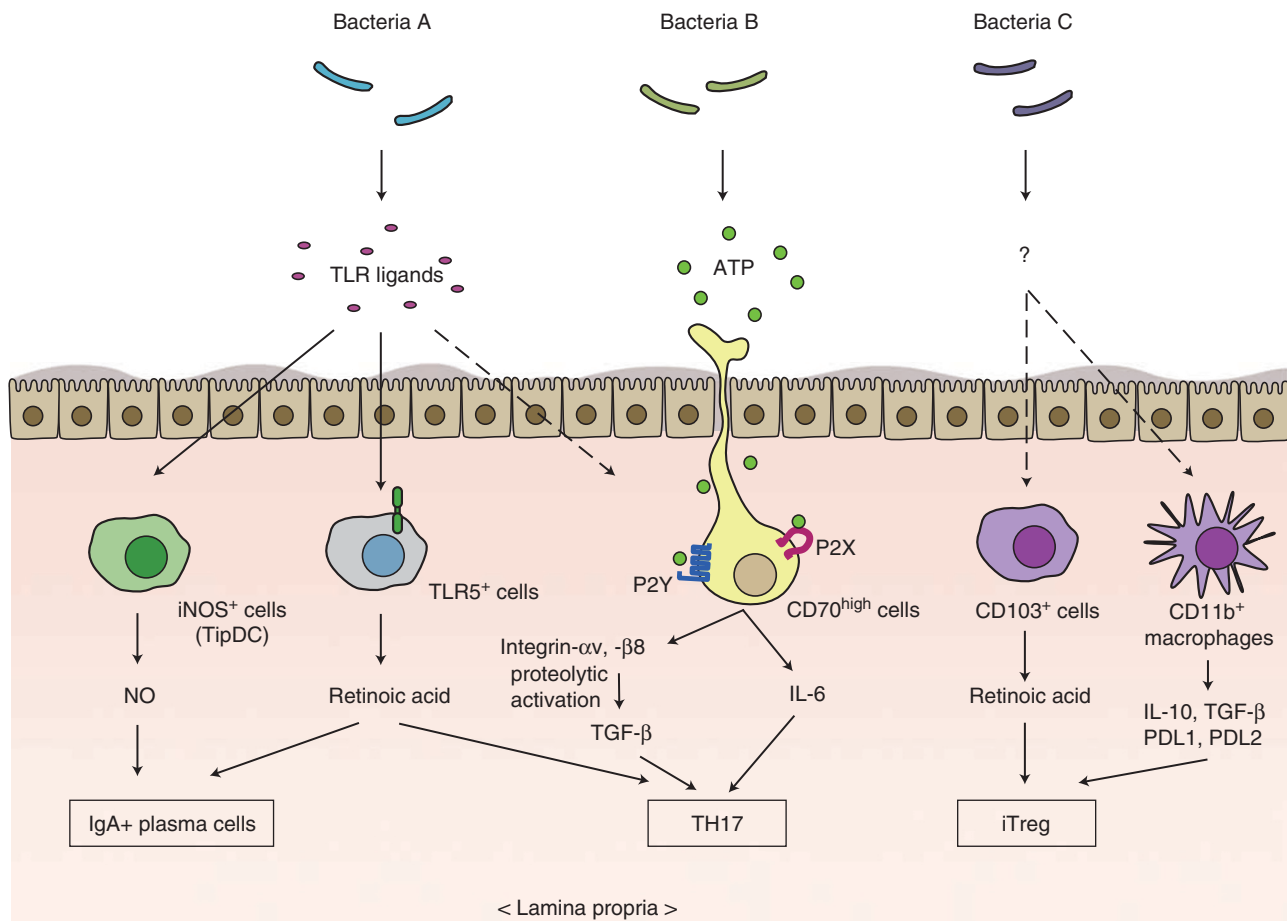
inflammation of multiple organs in *Socs1*<sup>-/-</sup> mice can be prevented by the blockade of interferon- $\gamma$  signaling, macrophages from these mice still show increased cytokine production in response to TLR stimulation.<sup>33,34</sup> The *Socs1* deficiency exacerbates the severity of colitis in mice lacking a T-cell receptor chain or DSS-treated mice presumably owing to excess TLR signaling.<sup>35,36</sup> However, severe colitis does not develop when these mutant mice are kept in a germ-free environment.<sup>32</sup> Thus, SOCS1 is also a key negative regulator to prevent excessive intestinal bacteria- and/or TLR-dependent inflammatory responses in the intestine.

TLR-mediated immune response can be divided into two phases, early and late. During the early phase, nuclear factor- $\kappa$ B (NF- $\kappa$ B) is swiftly recruited to constitutively and immediately accessible promoters of genes, such as TNF- $\alpha$ , CXCL2, and CXCL1 (also known as KC). In the later phase of the TLR response, genes for which induction requires stimulus-dependent chromatin modifications become accessible to NF- $\kappa$ B and other transcription factors, and are strongly induced. It can be noted, many of the late inducible genes, such as IL-6, IL-12p40, and IL-18, are known to be involved in the activation of T cells. It has been shown that the induction of late inducible genes is regulated, at least in part, by early inducible genes, which include members of nuclear I $\kappa$ B proteins, I $\kappa$ B $\zeta$  and I $\kappa$ BNS. The induced I $\kappa$ B $\zeta$  interacts with p50 subunit of NF- $\kappa$ B and enhances the induction of late inducible genes through trimethylation of histone H3K4 in their promoters.<sup>37,38</sup> On the other hand, I $\kappa$ BNS is selectively expressed in the lamina propria macrophages during early phase of TLR signaling.<sup>13</sup> In sharp contrast to the case of I $\kappa$ B $\zeta$ , I $\kappa$ BNS inhibits the induction of the late inducible genes.<sup>39</sup> The *Nfkbid* (the gene encodes I $\kappa$ BNS)-deficient mice are highly susceptible to intestinal inflammation, such as DSS-induced colitis.<sup>39</sup> Thus, the selective expression of I $\kappa$ BNS may provide a mechanistic basis for the hypo-responsiveness of intestinal lamina propria macrophages to TLR stimulation.

Peroxisome proliferator-activated receptor- $\gamma$  (PPAR $\gamma$ ), a member of the nuclear receptor family, is highly expressed in the intestinal epithelial cells;<sup>40</sup> it has been shown to suppress the expression of a subset of TLR-dependent genes.<sup>41</sup> *Bacteroides thetaiotaomicron*, a prevalent commensal anaerobic bacterium of the human intestine, attenuates inflammatory responses by limiting duration of NF- $\kappa$ B activity. PPAR $\gamma$  has been shown to be responsible for *B. thetaiotaomicron*-dependent nuclear export of RelA subunit of the NF- $\kappa$ B in intestinal epithelial cells.<sup>42</sup> Thus, PPAR $\gamma$  is also proposed as a therapeutic target of IBDs.<sup>43</sup>

#### TLR SIGNALING REQUIRED FOR INTESTINAL HOMEOSTASIS

So far, we have discussed that when TLR-dependent activation of innate immunity is in excess, intestinal inflammation ensues. However, a growing body of evidence indicates that TLR-dependent recognition of commensal bacteria is required for the maintenance of the intestinal homeostasis. Mice lacking TLR signaling components (TLR2, TLR4, or MyD88) are highly susceptible to DSS-induced intestinal inflammation.<sup>44,45</sup>



**Figure 1** A hypothetical model for intestinal bacteria-mediated differentiation of adaptive immune cells in the lamina propria. The intestinal microflora is composed of a variety of bacteria with different characters; each of them produces stimulatory factors for host immune system. A subset of commensal bacteria (Bacteria A) produces TLR ligands, which activate specific subsets of lamina propria APCs, TipDCs, and TLR5<sup>+</sup> DCs, thereby inducing the differentiation of IgA<sup>+</sup> plasma cells and TH17 cells. Another subset of intestinal bacteria (Bacteria B) produces and secretes high amounts of extracellular ATP. CD70<sup>high</sup>CX3CR1<sup>+</sup> dendritic cells extend their dendrites into the lumen, sense the extracellular ATP through P2X and P2Y receptors, and produce IL-6 and TGF- $\beta$ -activating enzymes to promote differentiation of TH17 cells. A subset of intestinal commensal bacteria (Bacteria C) produce an as-yet unknown factor that activate CD103<sup>+</sup> DCs or CD11b<sup>+</sup> macrophages to produce retinoic acid and other factors to induce differentiation of iT<sub>reg</sub>. TLR, Toll-like receptor; APC, antigen-presenting cell; IgA, immunoglobulin A; TipDC, TNF- $\alpha$ /iNOS-producing dendritic cell subset; IL-6, interleukin-6; TGF- $\beta$ , transforming growth factor- $\beta$ ; DC, dendritic cell; iT<sub>reg</sub>, inducible T<sub>reg</sub>.

Moreover, treatment of broad-spectrum antibiotics also result in high susceptibility to DSS-induced intestinal inflammation.<sup>44</sup> This is revealed owing to the defective expression of tissue-protective factors in the intestinal epithelial cells or defective TLR-dependent control of tight junction-associated intestinal epithelial barrier integrity.<sup>44,46</sup> TLR9-dependent signal in intestinal epithelial cells has also been shown to maintain the intestinal homeostasis.<sup>47</sup> Thus, these studies show that TLR-dependent recognition of commensal bacteria under normal steady-state conditions is crucial for the maintenance of intestinal homeostasis. The importance of the interaction of TLRs and commensal bacteria is also shown in other studies. Colonization of germ-free mice with *B. thetaiotaomicron* induces epithelial expression of genes involved in several intestinal functions, including nutrient absorption, mucosal barrier fortification, angiogenesis, and postnatal intestinal maturation.<sup>48</sup> In addition, systemic immunological defect as evidenced by the imbalance of TH1/TH2 cell populations in germ-free mice is recovered by colonization with a commensal bacterium *Bacteroides fragilis*. A polysaccharide

of *B. fragilis* is sufficient for the proper development of systemic immunity.<sup>49</sup> Modulation of TH1/TH2 balance by the polysaccharide of *B. fragilis* is further shown to be dependent on TLR2.<sup>50</sup> Thus, commensal bacteria-derived factors critically modulate intestinal immune responses, at least in part, through TLRs.

Another interesting aspect of TLR signaling in the intestinal mucosa is the finding that TLR-dependent signaling can induce intestinal tumorigenesis under certain circumstances.<sup>51</sup> Thus, TLRs at the intestinal mucosal surfaces have a variety of functions, such as induction of inflammation, maintenance of homeostasis, and suppression of tumorigenesis. Further investigation is needed to learn how these yin and yang properties of TLRs are regulated in the intestine.

#### NLR-DEPENDENT RESPONSES IN THE INTESTINE

There are also TLR-independent mechanisms for microbial recognition by cytoplasmic molecules, including nucleotide-binding and oligomerization domain (NOD)-like receptors (NLRs),<sup>52–54</sup> which are a large family comprising over 20

members of proteins.<sup>55</sup> NLRs have been implicated in the recognition of several bacterial components as well as of environmental danger signals, such as adenosine 5'-triphosphate (ATP), urate crystal, and silica.<sup>56</sup> Among these NLR family members, NOD1 and NOD2 have been characterized to be involved in immune responses at the intestinal mucosa.<sup>53</sup>

Involvement of NOD proteins in the intestinal inflammation was first shown in studies in which NOD2 gene mutation associates with increased susceptibility to Crohn's disease in Western (but not Asian) populations.<sup>57,58</sup> The mutations are located in the C-terminal leucine-rich repeat region responsible for ligand recognition. Although *in vitro* assays suggest that the NOD2 mutations result in a loss of function,<sup>59</sup> whether these mutations predispose to Crohn's disease *in vivo* through a loss or gain of function remains a matter of debate. NOD2 is expressed in Paneth cells that are a critical source of various antimicrobial peptides.<sup>60</sup> *Nod2*-deficient mice are susceptible to oral infection of *Listeria monocytogenes*, with defective expression of anti-microbial peptides in the small intestine.<sup>61</sup> Furthermore, the expression of the antimicrobial peptides  $\alpha$ -defensins has been shown to be reduced in Crohn's disease patients with the NOD2 mutation.<sup>62</sup> Thus, these results support the notion that the Crohn's disease-associated NOD2 mutations render the molecule hypo-responsive to the NOD2 ligands. Another study using mice carrying the NOD2 mutation that corresponds to the Crohn's diseases-associated NOD2 mutation shows that this type of the NOD2 mutation results in increased production of IL-1 $\beta$ , leading to the development of intestinal inflammation.<sup>63</sup> This study discusses that this type of mutation in mice, unlike that in humans, causes hyper-responsiveness to NOD2 stimulation.<sup>59</sup> Another mechanism in which NOD2 is a negative regulator of TLR2 signaling is also proposed.<sup>64</sup> In this mechanism, TLR2-dependent production of IL-12 by dendritic cells is increased in the absence of NOD2-mediated suppression, leading to aberrant TH1 cell development, which causes intestinal inflammation.<sup>65</sup> In any case, NOD2 mutation presumably results in a marked change in the components of intestinal microflora, which play a critical role in promoting disease pathogenesis.

Involvement of NOD1, expressed in the intestinal epithelial cells, in the intestinal immune responses has also been shown. *Nod1*-deficient mice are highly susceptible to infection with *Helicobacter pylori*, indicating that NOD1 is critical to the innate immune response in the intestinal epithelia to bacterial infection.<sup>66</sup> In addition, NOD1 gene polymorphisms are shown to be associated with susceptibility to inflammatory diseases such as Crohn's diseases and ulcerative colitis.<sup>67</sup> Thus, NOD1 and NOD2 are both critical constituents of innate immune system in the intestinal mucosa.

#### REGULATION OF ADAPTIVE IMMUNE SYSTEM BY COMMENSAL BACTERIA

The intestinal mucosa also has a unique and extremely complicated immune system composed of a variety of adaptive immune cell populations, including IgA-producing plasma cells,  $\gamma\delta$ T cells, and CD4<sup>+</sup> T cells dominated by a TH1 or a TH2 phenotype. In addition, recent studies have revealed that CD4<sup>+</sup>

T cells in the intestinal mucosa comprise significant numbers of IL-17-producing cells (TH17 cells) and regulatory T cells (T<sub>reg</sub> cells). Furthermore, very recent reports showed the presence of IL-22-producing natural killer-like cells (NK-22 cells) in the intestinal mucosa, which are positive for NK cell markers but do not have killing activity of MHC class I-negative cells. All of these cells are particularly abundant in the intestinal mucosa and are present even at steady state. The mechanism of how the intestinal mucosa can accomplish the equipment of diverse sets of adaptive immune cell populations is not fully understood. The most commonly accepted view is that such a large variety occurs as a result of interaction between commensal bacteria, each with a distinct character, and antigen-presenting cell (APC) populations, each has a different functional bias (Figure 1). We discuss below how commensal bacteria and APCs provide a particular environment for the unique and well-balanced development of adaptive immune cells.

#### DEVELOPMENT OF IGA-PRODUCING CELLS

Among the adaptive immune cell populations in the intestinal mucosa, the best-characterized cells are the IgA-producing plasma cells. Normal intestinal mucosa contains abundant IgA-secreting plasma cells, and the secreted IgA plays a critical role in the host defense against pathogenic bacteria. More importantly, IgA regulates the ecological balance of commensal bacteria; in other words, IgA regulates the composition and character of intestinal microflora. For example, the lack of activation-induced cytidine deaminase (AID), which results in the defect of class-switch recombination, and thereby lack of IgA-producing plasma cell in the intestine, leads to excessive anaerobic expansion in the small intestine, particularly the segmented filamentous bacteria (SFB).<sup>68</sup> Conversely, the addition of IgA rescues the aberrant SFB expansion in the small intestine of AID<sup>-/-</sup> mice.<sup>68</sup> Furthermore, it has been shown that IgA does not affect the growth rate of *B. thetaiotaomicron*, but affects the expression of bacterial molecules.<sup>69</sup> As a result, in the absence of IgA, *B. thetaiotaomicron*, which is otherwise commensal to the host, elicits a more robust innate immune response, including induction of inducible nitric oxide synthase (iNOS).<sup>69</sup> Thus, mucosal IgA can determine the nature of bacteria and is required for keeping commensal bacteria to be "commensal" to the host.

The development of IgA-secreting cells is regulated, in turn, by the presence of intestinal microflora. The germ-free mice exhibit severe reductions in their fecal IgA levels and in the numbers of lamina propria IgA-positive cells.<sup>5,70-72</sup> The specific pathogen-free (SPF) mice treated with antibiotics, such as vancomycin and metronidazole, show similar reductions of the levels of IgA to those in germ-free mice. Thus, commensal bacteria play a critical role in the provision of a particular environment for the development of IgA-producing cells. Using gnotobiotic mice where only certain known strains of bacteria are present, it has been shown that a set of bacterial species, such as SFB and clostridia, specifically stimulate the development of IgA-producing cells.<sup>71,72</sup> IgA concentrations of ileal contents or feces are much higher in SFB- or clostridia-monoassociated

mice than in mice associated with whole intestinal flora (conventionalized mice).<sup>73</sup> Thus, certain bacterial species promote differentiation of IgA-producing cells, whereas others do not or may inhibit it.

In conventional views, IgA-positive B cells are mainly generated in a T cell- and CD40 ligand (CD40L)-dependent manner in the germinal centers of organized follicular structures of Peyer's patches (PPs), where antigens of commensal bacteria are taken up by M cells located in the follicle-associated epithelium of PPs. After generation in the PPs, IgA<sup>+</sup> B cells migrate into the draining mesenteric lymph nodes, where they differentiate into IgA-secreting cells, which then home to the intestinal lamina propria. In contrast to this T-cell- and PP-dependent class-switch model, an emerging model is that a large proportion of the intestinal IgA is locally induced in the intestinal lamina propria by local factors in a T-cell-independent manner.<sup>74</sup> Commensal bacteria are directly taken up by epithelial cells through Fc receptor Rn or by dendritic cells at mucosal sites.<sup>75,76</sup> In parallel, commensal bacteria provide molecular patterns that activate TLR or other innate immune receptors in lamina propria cells. Microbial TLR ligands, such as lipopolysaccharide (LPS), can substitute for CD40L and act directly on B cells along with transforming growth factor- $\beta$  (TGF- $\beta$ ) to initiate IgA class switching.<sup>77</sup> In addition to engaging TLRs on B cells, microbial products stimulate the release of B-cell-activating factor (BAFF; also known as BLyS) and a proliferation-inducing ligand (APRIL) by dendritic cells. BAFF and APRIL are functionally related to CD40L and promote AID expression and IgA class switching in a CD40L-independent manner. Recently, it has been shown that BAFF and APRIL are co-expressed with iNOS in CD11c<sup>lo</sup> dendritic cells in the intestinal lamina propria.<sup>78</sup> The iNOS<sup>+</sup> dendritic cells express gaseous nitric oxide, which induces BAFF and APRIL by an unknown mechanism. The iNOS<sup>+</sup> dendritic cells are also positive for TNF- $\alpha$ ; thus, they are called as a naturally occurring TNF- $\alpha$ /iNOS-producing dendritic cell subset (TipDC). As the number of TipDC is much reduced in the lamina propria of germ-free, *Myd88*<sup>-/-</sup>, and *Tlr2*<sup>-/-</sup> *Tlr4*<sup>-/-</sup> *Tlr9*<sup>-/-</sup> mice, the expression of iNOS in TipDC is mediated by microbial products and signaling through TLR.<sup>78</sup> It is likely that TipDCs are "IgA-inducing DCs" that recognize specific species of commensal bacteria and preferentially promote B-cell differentiation into IgA-producing cells in the intestinal lamina propria *in situ* (Figure 1).

In the context of lamina propria dendritic cells that preferentially mediate IgA<sup>+</sup> cell differentiation, there is another subset of lamina propria dendritic cells in the small intestine, that is, TLR5<sup>+</sup>CD11c<sup>hi</sup>CD11b<sup>hi</sup> cells that mediate IgA<sup>+</sup> cell differentiation through the production of retinoic acid.<sup>79</sup> Upon stimulation by a TLR5 ligand flagellin, TLR5<sup>+</sup> dendritic cells express retinal dehydrogenase 2 (Raldh2), which enzymatically converts vitamin A into retinoic acid. Retinoic acid then acts on naive B cells to differentiate into IgA-producing plasma cells (Figure 1). As *Tlr5*<sup>-/-</sup> mice still have normal numbers of IgA<sup>+</sup> cells in the intestinal lamina propria, this mechanism is not essential during steady state. However, TLR5<sup>+</sup> dendritic cells might play a critical role during infections by pathogenic bacteria, because

flagella are usually observed in pathogenic bacteria, but are rarely seen in intestinal commensal bacteria. Further investigation is required to decipher the relationship between TipDC and TLR5<sup>+</sup> dendritic cells for IgA class switching during health and disease.

Recently, much attention has been focused on isolated lymphoid follicles (ILFs) in the lamina propria as the site where T-cell-independent induction of IgA class switching of B cells takes place.<sup>80</sup> It was shown that although *Tcrb*<sup>-/-</sup> *Tcrd*<sup>-/-</sup> mice fail to generate IgA<sup>+</sup> B cells within PP follicles, they have an almost normal level of luminal IgA.<sup>80</sup> Instead of PP, a considerable number of IgA<sup>+</sup> plasma cells can be observed in the lamina propria of the small intestine, particularly in ILFs.<sup>80</sup> These facts support the idea that the T-cell-independent development of IgA<sup>+</sup> cells predominantly takes place in ILF. Interestingly, the formation of ILFs is dependent on the presence of commensal bacteria, as is evident by the analysis of germ-free mice in which ILF is defective.<sup>81</sup> To induce the genesis of ILFs, peptidoglycan from Gram-negative commensal bacteria, such as *Bacteroides* genus, is required. Peptidoglycan is recognized by the NOD1 receptor in epithelial cells, resulting in the induction of chemokines and recruitment of cells required for the formation of ILFs. Conversely, in the absence of ILFs, the composition of the intestinal bacterial community is profoundly altered: *Clostridiales* and *Bacteroides* markedly expand, whereas the Gram-negative *Lactobacillaceae* are reduced in ILF-deficient mice.<sup>81</sup> Thus, it is highly likely that the steady and well-balanced state of the intestinal mucosa is achieved by a dynamic equilibrium between immune system, including ILF-IgA system, and intestinal microflora.

#### DEVELOPMENT OF TH17 CELLS

TH17 cells constitute a subset of activated CD4<sup>+</sup> T cells that are characterized by the production of IL-17A, IL-17F, IL-21, and IL-22. The produced IL-17 acts on a broad range of immune and non-immune cells and regulates granulopoiesis, neutrophil recruitment, and induction of antimicrobial peptides. IL-22 acts on epithelial cells of the gastrointestinal tract and skin (but not on immune cells that are defective of IL-22 receptor expression) and leads to the upregulation of antimicrobial proteins and cellular proliferation. By producing these cytokines, TH17 cells play critical roles in the regulation of host defense against a variety of infections by fungus and bacteria, such as *Klebsiella pneumoniae*, *Streptococcus pneumoniae*, and *Citrobacter rodentium*.<sup>82-84</sup> However, at the same time, the aberrant TH17 response can lead to severe autoimmune diseases, such as multiple sclerosis, rheumatoid arthritis, and IBDs. Contrary to their inflammatory roles, IL-17 and IL-22 also have a protective role in inflammation. For example, it has been shown that IL-22 counteracts the destructive nature of inflammation during hepatitis: *Il22*-deficient mice are highly sensitive to ConA-induced acute liver inflammation.<sup>85</sup> Thus, depending on the circumstances, TH17 cells may serve as an inflammatory or a protective population to maintain tissue homeostasis.

Specific microbial stimuli detected by innate immune cells initiate TH17 differentiation, where a "master regulator" transcription factor ROR $\gamma$ t is induced by the local cytokine milieu, including

IL-6 and TGF- $\beta$  in the mouse system.<sup>86</sup> It should be noted that all the TH17 cells are not the same and different environments induce TH17 cells with different characteristics.<sup>87</sup> For example, the presence of ligands for aryl hydrocarbon receptor affects the expression of IL-22, but not other TH17 cytokines.<sup>88</sup> Furthermore, although TGF- $\beta$  and IL-6 are essential for the “initiation” of TH17 differentiation,<sup>84</sup> they are not sufficient to induce fully inflammatory TH17 cells. During stimulation with TGF- $\beta$  and IL6, TH17 cells express IL-23 receptor and become responsive to IL-23.<sup>89</sup> In the presence of the pro-inflammatory cytokine IL-23, TH17 cells can expand and mediate a full-blown TH17 response.<sup>87,89</sup> Indeed, despite the fact that the development of TH17 cells in *C. rodentium*-infected mice is promoted by the TGF- $\beta$ -IL-6 pathway, IL-23 is indispensable in these mice for the TH17 response that protects against *C. rodentium*-driven colitis.<sup>84</sup> In the presence of excess amounts of IL-23, TH17 cells become pathogenic and induce autoimmune disease in mice. Indeed, using a T-cell transfer model of experimental allergic encephalomyelitis, a recent study showed that TH17 cells that are expanded in the presence of IL-23, but not with TGF- $\beta$  plus IL-6, induce disease in mice.<sup>90</sup> IL-23 is also shown to mediate intestinal inflammation.<sup>91,92</sup> Moreover, in humans, it has been shown that variants of the IL23R gene are linked to susceptibility to IBDs.<sup>93</sup>

Importantly, the intestinal lamina propria is a unique site for the presence of TH17 cells in healthy animals. TH17 cells are present at high frequencies in the small and large intestinal lamina propria but not in the spleen, mesenteric lymph nodes, or PPs of healthy mice housed in a SPF environment.<sup>86,94,95</sup> In the small and large intestines, about 10% of CD4<sup>+</sup> cells are IL-17<sup>+</sup> cells; and 80–90% of the IL-17<sup>+</sup> cells are CD4<sup>+</sup>TCR $\alpha\beta$ <sup>+</sup> TH17 cells.<sup>95</sup> In contrast, in extra-intestinal sites, only a very small proportion of CD4<sup>+</sup>TCR $\alpha\beta$ <sup>+</sup> T cells express IL-17 at steady state (fewer than 1%), and < 10% of IL-17<sup>+</sup> cells are CD4<sup>+</sup> cells (NK cells, CD8<sup>+</sup> T cells, and TCR $\gamma\delta$ <sup>+</sup> T cells also express IL-17 and ROR $\gamma$ t in extra-intestinal sites).<sup>95</sup> The number of TH17 cells in the intestinal lamina propria increases with age, correlating with the development of intestinal microflora.<sup>94</sup> Furthermore, in germ-free mice or antibiotics-treated mice, the numbers of lamina propria TH17 cell are greatly reduced.<sup>94,95</sup> Therefore, similar to the case of IgA-producing plasma cells, the development of TH17 cells is dependent on the stimulation by intestinal commensal bacteria. Interestingly, mice obtained from different commercial vendors have marked differences in the number of TH17 cells in their lamina propria: C57BL/6 mice from the SPF facilities of Taconic Farms (Hudson, NY) have higher numbers of TH17 cells than those from the Jackson Laboratory (Bar Harbor, ME).<sup>95</sup> The difference in the presence of intestinal TH17 cells is correlated with the presence of members of the cytophaga–flavobacter–bacteroides phylum.<sup>95</sup> Thus, it is likely that TH17 cells are induced in the lamina propria in response to specific components of the commensal microflora, which presumably activate innate signaling pathways in subsets of APCs in the lamina propria. In sharp contrast to this notion, a recent report showed that commensal bacteria may inhibit TH17 differentiation through IL-25.<sup>96</sup> Further investigative studies will need to clarify these discrepant controversies.

Two groups independently showed that *Myd88* and *Trif* doubly deficient mice had normal numbers of lamina propria TH17 cells in the small and large intestines, and concluded that the TH17 development in the lamina propria is independent of TLR signaling at least at steady state.<sup>94,95</sup> However, a possible caveat to this interpretation is that these mutant mice might have altered intestinal microflora. Indeed, it was shown that *Myd88* deficiency changes the composition of the distal gut microbiota: *Myd88*<sup>-/-</sup> mice have a significantly lower *Firmicutes/Bacteroidetes* ratio compared with *Myd88*<sup>+/-</sup> mice.<sup>97</sup> Furthermore, another group showed that *Tlr9*-deficient mice have decreased numbers of lamina propria TH17 cells.<sup>98</sup> Thus, to formally conclude the involvement of TLR signaling in TH17 differentiation, gnotobiotic studies using germ-free mice deficient for MyD88 or TLRs colonized with a certain TH17-inducing bacteria needs to be carried out.

Besides TLR ligands, bacteria have been shown to generate and secrete large amounts of extracellular ATP.<sup>99</sup> ATP is a well-known source of intracellular energy transfer; however, it also serves essential roles in extracellular signaling processes. Extracellular ATP binds and activates the cell-surface ionotropic (P2X) and metabotropic (P2Y) purinergic receptors, which deliver intracellular signals through ion channels or G-proteins, respectively.<sup>100,101</sup> In the context of the immune system, extracellular ATP is known to modulate immune cell functions, such as phagocytosis, chemotaxis, and cytokine production.<sup>100,101</sup> Particularly, the induction of ATP signaling through P2X7 receptor is known to be involved in the activation of caspase-1 through the assembly of a cytosolic protein complex that is known as the “inflammasome” and required for the conversion of immature form of IL-1 $\beta$  into the mature form.<sup>102</sup> ATP can be quickly degraded in the extracellular space by ATPases into ADP, AMP, or adenosine forms. However, intestinal commensal bacteria seem to produce high amounts of ATP beyond the capacity of host ATPases. Indeed, ATP concentrations in intestinal luminal contents of SPF mice are very high, but found at very low levels in germ-free mice or antibiotics-treated mice.<sup>94</sup> Moreover, high ATP concentration can be detected in the supernatant of *in vitro*-cultured intestinal commensal bacteria. The addition of the culture supernatant of intestinal commensal bacteria markedly enhances differentiation of IL-17-expressing cells, and this TH17 differentiation is severely inhibited by the presence of ATP-degrading enzyme, apyrase.<sup>94</sup> Furthermore, treatment of germ-free mice with ATP markedly increases the numbers of IL-17-producing CD4<sup>+</sup> cells.<sup>94</sup> Thus, extracellular ATP is one of the critical TH17-inducing factors produced by intestinal commensal bacteria (**Figure 1**).

Lamina propria CD11c<sup>+</sup> cells, particularly CD70<sup>high</sup> cells, express high levels of P2X and P2Y receptors and also express several genes, including IL-6, IL-23, integrin- $\alpha$ V, and integrin- $\beta$ 8, in response to ATP stimulation.<sup>94</sup> Integrin- $\alpha$ V and integrin- $\beta$ 8 are involved in the conversion of latent form of TGF- $\beta$  into the active form through triggering degradation of latency-associated protein binding to TGF- $\beta$ .<sup>103</sup> CD70<sup>high</sup> CD11c<sup>+</sup> cells preferentially induce the differentiation of naive CD4<sup>+</sup> T cells into TH17 cells.<sup>94</sup> There is another report showing that lamina

propria CD11b<sup>high</sup>CD11c<sup>+</sup> cells are a TH17-inducing dendritic cell population.<sup>104</sup> Both CD70<sup>high</sup> and CD11b<sup>high</sup> populations express CX3CR1, a chemokine receptor mediates the extension of cellular dendrites of dendritic cells between the tight junctions of epithelial cells to take up luminal bacteria.<sup>76</sup> Thus, it is likely that CD70<sup>high</sup> and CD11b<sup>high</sup> populations seem to be, at least partially, overlapping with each other and both play a critical role in the TH17 differentiation in the lamina propria *in situ*.

Collectively, the current model for TH17 differentiation in the intestinal lamina propria therefore involves production of high amounts of ATP or other TH17-inducing molecules by a specific subset of intestinal bacteria, followed by the activation of a specific subset of lamina propria dendritic cells expressing CD70 or CD11b and the production of IL-6, IL-23, and TGF- $\beta$  (Figure 1). In inflammatory conditions, the differentiation of intestinal TH17 cells seems to be enhanced by TLR-dependent pathway, as flagellin activates TLR5<sup>+</sup> lamina propria dendritic cells to induce TH17 cell differentiation.<sup>79</sup> It will be important to understand further the specific types of TH17-inducing intestinal bacteria as well as of TH17-inducing APC subsets during steady and disease states.

#### DEVELOPMENT OF INDUCIBLE T<sub>REGS</sub> CELLS

T<sub>regs</sub> play an indispensable role in maintaining immunological unresponsiveness to self-antigens and in suppressing excessive immune responses deleterious to the host.<sup>105</sup> T<sub>regs</sub> express the transcription factor fork-head box P3 (Foxp3), which is critical to initiating a genetic program for the suppressive functions of T<sub>regs</sub>. Besides evidence that natural Foxp3<sup>+</sup> T<sub>reg</sub> arise and mature in the thymus, there is mounting evidence that Foxp3<sup>+</sup> T<sub>reg</sub> can develop extra-thymically under certain conditions, particularly in the intestinal mucosa. Indeed, the peripheral conversion of Foxp3<sup>-</sup> CD4<sup>+</sup> T cells into Foxp3<sup>+</sup> T<sub>regs</sub> can be observed experimentally in the intestinal lamina propria and gut-associated lymphoid tissue (GALT) after oral exposure to antigen.<sup>106,107</sup> These inducible T<sub>reg</sub> (iT<sub>regs</sub>) are believed to mediate peripheral T cell tolerance to antigens derived from commensal flora or of dietary origin. Furthermore, a high percentage of T cells in the small and large intestines spontaneously produce anti-inflammatory cytokine IL-10.<sup>108</sup> IL-10 is not expressed by thymic natural T<sub>regs</sub>, but can be detectable in lamina propria Foxp3<sup>+</sup> CD4<sup>+</sup> T cells. As T<sub>reg</sub>-specific disruption of IL-10 in mice (*Foxp3-Cre* × *IL-10<sup>fllox/flox</sup>*) results in severe autoimmune colitis,<sup>109</sup> T<sub>regs</sub> are the main source of intestinal IL-10, which play a critical role in maintenance of intestinal immune homeostasis.

Several subsets of dendritic cells with regulatory properties have been described with the capacity to induce iT<sub>regs</sub> or induce oral tolerance at steady-state conditions. Particularly, there are several reports that indicate that a CD103 (also known as  $\alpha$ E-integrin)-expressing dendritic cell population in the lamina propria preferentially promotes the generation and homing of iT<sub>reg</sub> cells to the intestinal mucosa.<sup>106,107</sup> CD103<sup>+</sup> dendritic cells express retinal dehydrogenase (RALDH) and produce high amounts of the vitamin A metabolite retinoid acid, which mediates the expression of the gut-homing receptors on T cells, and at the same time enhances iT<sub>reg</sub> differentiation in the gut by cooperating with TGF- $\beta$  (Figure 1).

In the context of iT<sub>reg</sub>-inducing APCs in the gut, CD11b<sup>high</sup>CD11c<sup>-</sup> lamina propria macrophages have also been shown to preferentially promote iT<sub>regs</sub> development.<sup>104</sup> The lamina propria macrophages have high expression of genes encoding anti-inflammatory molecules, including IL-10, TGF- $\beta$ 1, TGF- $\beta$ 3, programmed cell death 1 ligand 1 (PDL1), PDL2. As a result, the lamina propria macrophages preferentially induce the differentiation of naive CD4<sup>+</sup> T cells into Foxp3- and IL-10-expressing cells. Collectively, the preferential induction of iT<sub>reg</sub> cells at the mucosal site is attributed, at least in part, to the gut-specific presence of CD103<sup>+</sup> dendritic cells and CD11b<sup>high</sup>CD11c<sup>-</sup> macrophages, both of which have a functional bias for iT<sub>reg</sub> induction (Figure 1).

Interestingly, it was recently reported that the presence of *B. fragilis* in the intestine suppress inflammation through the induction of IL-10-producing T cells.<sup>110</sup> This anti-inflammatory effect by *B. fragilis* is achieved by polysaccharide A, which also prevents TH1 and TH17 effector T-cell-mediated inflammation caused by *Helicobacter hepaticus* in the intestine. On the other hand, DNA from commensal bacteria is shown to limit iT<sub>reg</sub> development through TLR9.<sup>98</sup> These studies suggest that the interaction between a specific subset of intestinal bacteria and a specific subset of APCs positively and negatively regulate iT<sub>reg</sub> differentiation in the intestinal mucosa.

#### CONCLUSION AND PERSPECTIVES

Considerable progress has been made in improving our understanding of the roles of TLRs and NLRs in the induction of inflammation against pathogens: In isolated individual cells, their signaling and consequences look rather simple. Thus, one might envisage that this can be applied to the *in vivo* situation, where the activation of TLRs and NLRs only occurs when intestinal bacteria accidentally invade the mucosa and simply results in inflammation. However, we now know that the actual and entire picture of the intestinal innate immune system is not so simple. Not only pathogenic bacteria but also commensal bacteria continuously provide activation signals to TLRs and NLRs. The continuous activation of host cells, particularly epithelial cells, results in continuous production of tissue-repairing factors for maintaining intestinal homeostasis, without which intestinal barrier injury and bacterial translocation could occur.<sup>111</sup> The innate immune system is also required for the production of antimicrobial peptides and lectins by Paneth cells, which critically control components of intestinal microflora to create a mutually beneficial relationship.<sup>112</sup> Accordingly, the deficiency of innate immune signaling in *Myd88<sup>-/-</sup>* mice or *Nod1<sup>-/-</sup>* mice for example, results in dramatic changes of the composition of gut microbiota.<sup>81,97,112</sup> Furthermore, recent evidences suggest that innate immune signaling plays a role in oncogenesis.<sup>51</sup> Further investigation is clearly required for the innate immune signaling for the maintenance of mucosal homeostasis *in vivo*.

The recent investigations also provide invaluable insight into the development and action of adaptive immune cells. In particular, recent studies have revealed the role of TH17 cell and T<sub>reg</sub> cells in the regulation of intestinal homeostasis and have implicated these subsets in IBDs. Considering the requirement

of TGF- $\beta$  for the differentiation of IgA-producing plasma cells, TH17 cell and iT<sub>reg</sub> cell, the interaction between commensal bacteria and intestinal immune and non-immune cells may provide a specific environment rich in active form of TGF- $\beta$ . It is important to study further the molecular basis for this issue. In addition, accumulating evidence suggest the presence of functionally specified dendritic cell subsets in intestinal mucosa.<sup>113,114</sup> In the future it will be important to understand more details regarding the specific types and features of intestinal bacteria for differential adaptive immune cell differentiation and how they interact with specific intestinal APC subsets during the steady state and also inflammatory processes.

**ACKNOWLEDGMENTS**

This work was supported by Grants-in-Aid for Scientific Research from PRESTO, the Ministry of Education, Culture, Sports, Science and Technology, the Ministry of Health, Labour and Welfare, the Osaka Foundation for the Promotion of Clinical Immunology, the Ichiro Kanehara Foundation, Sumitomo Foundation, Senri Life Science Foundation and the Naito Foundation.

**DISCLOSURE**

The authors declared no conflict of interest.

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