Immunity
Article

Double-Stranded RNA of Intestinal Commensal but Not Pathogenic Bacteria Triggers Production of Protective Interferon-β

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SUMMARY

The small intestine harbors a substantial number of commensal bacteria and is sporadically invaded by pathogens, but the response to these microorganisms is fundamentally different. We identified a discriminatory sensor by using Toll-like receptor 3 (TLR3). Double-stranded RNA (dsRNA) of one major commensal species, lactic acid bacteria (LAB), triggered interferon-β (IFN-β) production, which protected mice from experimental colitis. The LAB-induced IFN-β response was diminished by dsRNA digestion and treatment with endosomal inhibitors. Pathogenic bacteria contained less dsRNA and induced much less IFN-β than LAB, and dsRNA was not involved in pathogen-induced IFN-β induction. These results identify TLR3 as a sensor to small intestinal commensal bacteria and suggest that dsRNA in commensal bacteria contributes to anti-inflammatory and protective immune responses.

INTRODUCTION

The innate immune system is capable of recognizing a wide variety of microbial signals and of rapidly inducing a range of antimicrobial and inflammatory responses. Among them, Toll-like receptors (TLRs) play a critical role in innate immunity by recognizing structurally conserved bacterial and viral components (Akira et al., 2006). Whereas TLR2 and TLR4 recognize bacterial cell wall components at the cell surface, TLR3, TLR7, and TLR9 recognize bacterial or viral nucleic acids in endosomes after phagocytosis of bacteria or virus (Akira et al., 2006). Dendritic cells (DCs) and macrophages express TLRs and their recognition of microbial components contributes to both rapid antipathogen responses and maintenance of homeostatic protective immunity in healthy individuals (Medzhitov and Janeway, 1998; Beutler et al., 2007).

Gut-associated lymphoid tissues (GALTs) are a major site of host encounter with exogenous antigens and pathogens. Based on a number of observations, including the poor immune response of germ-free mice unless they are colonized with one or more numbers of strains of commensal bacteria (Wu et al., 2010; Schwarzer et al., 2011), it is now well recognized that the interaction of GALT with microbiota regulates both the quality and quantity of systemic immune responses. Moreover, oral administration of selected strains of commensal bacteria was shown to be effective to intervene in immune responses even in conventionally reared mice (Atarashi et al., 2011; Jeon et al., 2012).

The fermentative lactic acid bacteria (LAB) are aerobic and abundant in the environment and food and they consequently compose a major part of our small intestinal commensal flora (Suzuki et al., 2004; Hao and Lee, 2004), and therefore they affect maturation of host immune cells and maintenance of intestinal

immune homeostasis under normal steady-state conditions (Shida and Nanno, 2008). Oral administration of some strains of LAB has been shown to stimulate the innate immunity at mucosal sites and to enhance systemic immune responses against pathogenic bacteria or viruses (Corr et al., 2007; Kawashima et al., 2011; Kosaka et al., 2012). However, the mechanisms by which such commensal bacteria are beneficial for protective immunity and anti-inflammatory conditions and the manner in which the immune system distinguishes these organisms from potential pathogens that cause opportunistic and/or immediate infection remain elusive.

We investigated the role of TLRs in the recognition of LAB by the host immune system. TLRs are expected to play major roles not only in the recognition of pathogenic microorganisms but also in the interactions between indigenous bacteria and host immune cells (Rakoff-Nahoum and Medzhitov, 2008). Although pathogenic strains such as *Salmonella* Typhimurium and *Escherichia coli* are known to stimulate TLR4 (Weiss et al., 2004), little is known mechanistically about how LAB or other commensal bacteria are recognized by the host immune system and eventually fortify it. In the case of *Bacteroides fragilis*, a major flora in large intestine, bacterial polysaccharide A signals through TLR2 directly on Foxp3$^+$ regulatory T cells to promote immunologic tolerance (Round et al., 2011). There are a few examples showing a role of TLR2 in the recognition of peptidoglycans and lipoteichoic acid of LAB (Grangette et al., 2005; Asong et al., 2009), but the function of other TLRs remains largely unknown.

We elucidated the role of TLRs in the recognition of LAB and provide evidence that commensal but not pathogenic bacteria induce a robust interferon-β (IFN-β) response by recognition through endosomal TLRs. Unlike IFN-α, IFN-β is not related to systemic disorder such as autoimmune diseases, but rather is dedicated to stabilize protective immunity against infection and inflammation; therefore, the response to commensal bacteria is expected to protect the host through IFN-β production. Furthermore, we have identified bacterial dsRNA as effective TLR3 ligands that serve to discriminate small intestinal commensals from potential pathogens. These endosomal responses to commensal bacteria should be beneficial as natural boosters of gut protective immunity.

RESULTS

LAB Induce IFN-β from DCs In Vitro

The fermentative LAB are a major component of the small intestinal microbiota and very often are contained in regular diet. We started to analyze the immunological property of this group of commensal bacteria in comparison with several pathogenic species and found that IFN-β secretion from bone-marrow-derived DCs (BMDCs) in response to LAB was generally much stronger than that to pathogenic bacteria regardless of strain difference (Figure 1A). Because IFN-β is an important mediator for antiviral protective immunity and anti-inflammation, we assessed the beneficial effect of IFN-β induced in BMDCs by coculturing with a heat-killed LAB, *Tetragenococcus halophilus* strain KK221. We confirmed that a large amount of IFN-β, but not IFN-α, was secreted in response to this bacterium (Figure S1A available online). These results suggest a possible beneficial activity of LAB in innate protective immunity against viral infection. To assess whether KK221 has a protective effect, BMDCs cocultured with or without heat-killed bacteria were infected with vesicular stomatitis virus (VSV), which is known to be sensitive to type I IFNs (Obuchi et al., 2003). As expected, KK221 suppressed VSV replication in the cells. This suppression was mediated through secreted IFN-β as shown by the fact that the addition of neutralizing IFN-β monoclonal antibodies (mAbs) restored viral replication (Figure S1B).

LAB Induce Anti-inflammatory Effects through IFN-β In Vivo

IFN-β is also a key anti-inflammatory cytokine in the settings of experimental colitis (Katakura et al., 2005). With the dextran sodium sulfate (DSS)-induced colitis model, we found that oral administration of KK221 alleviated colonic inflammation. The ameliorating effects were evident in a histological analysis of the colon, by a reduced infiltration of inflammatory cells such as neutrophils, eosinophils, and macrophages in lamina propria. The inflammatory infiltration in the KK221-fed group was as severe as that of saline-fed group after treatment with the neutralizing Ab against IFN-β (Figures 1B, 1C, S1C, and S1D). Oral administration of LAB prevented the inflammatory signs indicated by the colon length and myeloperoxidase (MPO) activity, a marker for neutrophil infiltration into tissue (Figures 1D and 1E).

Expression of genes encoding inflammatory mediators such as tumor necrosis factor-α (TNF-α), interleukin-6 (IL-6), Cxcl1, and IL-17 in the inflamed colon (Alex et al., 2009) was also suppressed by oral administration of KK221 (Figure 1F). As expected, these protective effects of oral LAB administration were significantly reversed by injection of IFN-β mAb (Figures 1B–1F). Of importance, gene expression of IFN-β in CD11c$^+$ lamina propria cells was equally enhanced in mice with or without the neutralizing Ab (data not shown). These results suggest that the anti-inflammatory and protective effects observed in the DSS-induced colitis model are mediated by IFN-β production in the intestine upon recognition of LAB.

TLR3 and TLR9 Are Involved in IFN-β Secretion by BMDCs in Response to LAB but Not Pathogenic Bacteria

To identify the components of LAB responsible for the protective effect and the nature of their recognition by host cells, BMDCs from mice deficient in various TLRs were analyzed. IFN-β secretion by BMDCs upon coculture with KK221 was significantly reduced in the absence of TLR3 and TLR9, whereas deficiency of TLR2, TLR4, and TLR7 had no effect on IFN-β production (Figure 2A). Neither IL-6 nor TNF-α production was impaired by each TLR deficiency (Figures S2A and S2B), indicating that the endosomal TLRs (TLR3 and TLR9) are specifically involved in IFN-β secretion upon recognition of LAB by BMDCs. Moreover, the mRNA expression of both TLRs was enhanced after exposure to KK221 (Figure S2C).

Having clarified the nature of the response to LAB, we next analyzed responses to a panel of LAB and pathogenic bacteria strains by using *Tlr3*$^{-/-}$ or *Tlr9*$^{-/-}$ mice. First, we analyzed IFN-β production by TLR3-deficient BMDCs stimulated with various strains and species of heat-killed LAB as well as pathogenic bacteria (Table S1). We found that the IFN-β response was induced in a TLR3-dependent manner by a large proportion (five out of

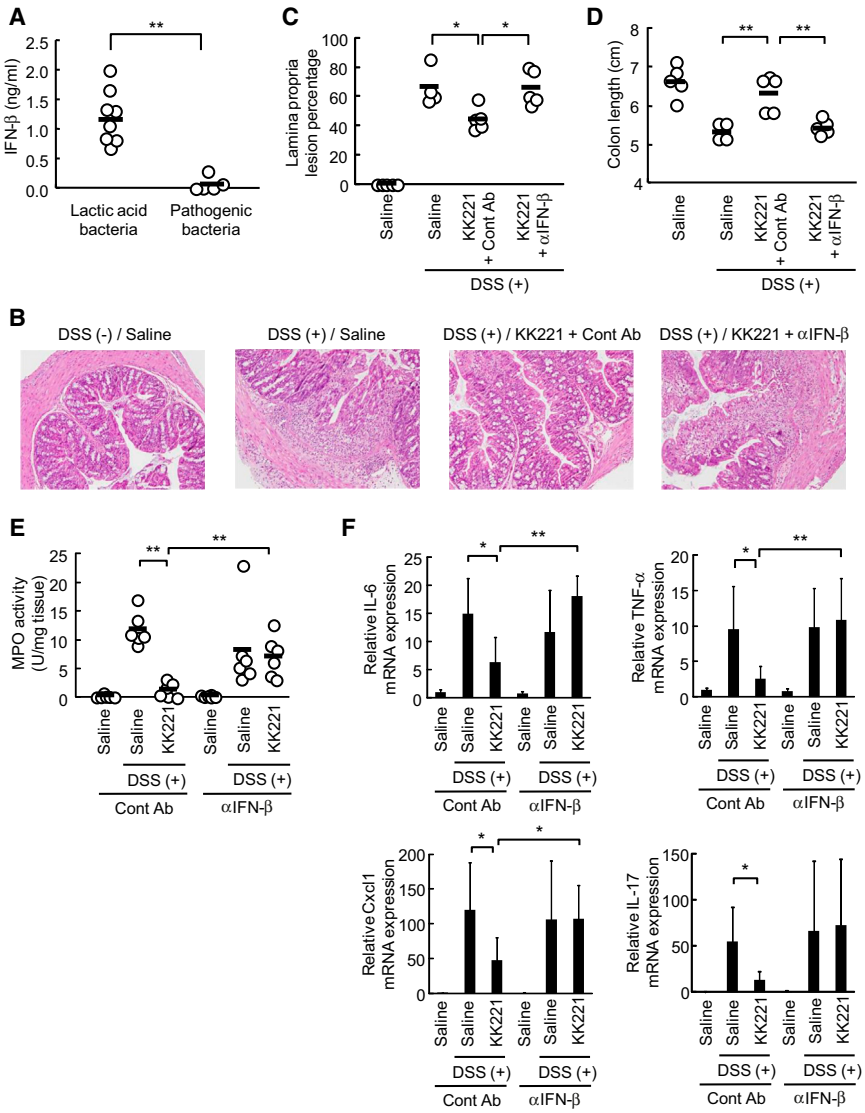

Figure 1. LAB Stimulates IFN-β Secretion by BMDCs, Resulting in Anti-inflammatory Immunity

(A) BMDCs from C57BL/6 mice were stimulated with heat-killed bacteria (Table S1) for 6 hr. IFN-β concentration was measured by ELISA. Data are representative of three independent experiments.

(B–D) KK221-fed mice were treated with i.v. injection of control Ab (Cont Ab) or IFN-β mAb (αIFN-β) in DSS-induced colitis.

(B) Colon tissue sections were stained with H&E (200×).

(C) Lamina propria lesion percentage in colon was quantified.

(D) Colon length was measured.

(E and F) Mice were treated with i.v. injection of Cont Ab or αIFN-β in DSS-induced colitis. Colonic myeloperoxidase (MPO) activity (E) and colonic mRNA expression (F) were measured. Data are represented as mean ± SD relative to saline-fed, Cont Ab-injected, and DSS-untreated group (F).

Bars represent mean (A, C–E). *$p < 0.05$, **$p < 0.01$ (Student's t test). See also Figure S1 and Table S1.

eight) of LAB (Figure 2B). In contrast, the lower responses to all pathogenic bacteria tested, including *Listeria monocytogenes* and *Staphylococcus aureus*, were intact in the absence of TLR3 (Figure 2B).

Next, we determined the importance of the TLR9 pathway in the recognition of various bacteria by using TLR9-deficient BMDCs. The IFN-β responses to all LAB strains were significantly impaired by TLR9 deficiency (Figure 2C). In contrast, the response to all pathogenic bacteria strains tested was unaltered by TLR9 deficiency (Figure 2C).

Thus, the response of BMDCs to LAB differs from that to pathogenic bacteria in two important aspects: they produce higher amounts of IFN-β production and the response is dependent on TLR3 and TLR9 (Figures 2B and 2C).

Endosomal Processing Is Required for IFN-β Secretion in Response to LAB but Not Pathogenic Bacteria

Differential usage of endosomal TLRs to discriminate between LAB and pathogenic bacteria was further confirmed by pharmacological inhibition of endosomal function. Bacteria are digested in endosomes after phagocytosis, and their components are recognized by endosomal TLRs (Akira et al., 2006). NH_4Cl treatment neutralizes the acidic endocytic compartments and/or delays endosomal maturation without inducing nonspecific inhibitory effects (Hotta et al., 2006), thereby impairing LAB-induced IFN-β production from BMDCs (Figure 3A). Further, consistent with the results in Figure 2A, this endosomal pathway was specific to IFN-β production as indicated by the fact that the response of IL-6 and TNF-α was intact by the same treatment (Figure S3A). In contrast, IFN-β production in response to pathogenic bacteria was much lower and unaffected by NH_4Cl (Figure 3A). Indeed, in contrast to KK221, heat-killed *Salmonella* Typhimurium induced IFN-β production by BMDCs only via TLR4 (Figure S3B). A major dependency of this pathogenic strain to TLR4 was also apparent in the production of other inflammatory cytokines (Figure S3B). The distinct recognition manners reflect the fact that IFN-β response to poly(I:C) and CpG DNA was impaired by NH_4Cl^- treatment, whereas the response to LPS was intact (Figure 3B). To confirm the contribution of TLR3 and TLR9, BMDCs from *3d* mutant mice bearing a deficiency of *Unc93b1,* which encodes an endoplasmic reticulum-resident protein required for the trafficking of TLR3, TLR7, and TLR9 (Tabeta et al., 2006), were utilized. The *3d* mutation completely abolished IFN-β production by BMDCs in response to LAB (Figure 3C). Thus, endosomal processing as well as endosomal TLRs are requisite for the recognition of LAB and IFN-β secretion.

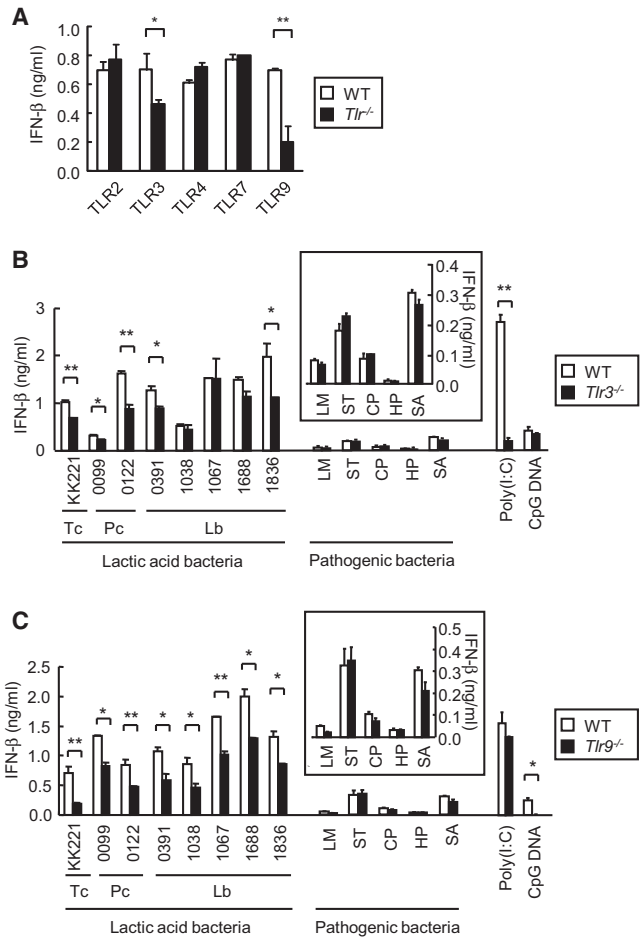

Figure 2. TLR3 and TLR9 Are Involved in IFN-β Secretion by BMDCs in Response to LAB but Not Pathogenic Bacteria In Vitro

(A) WT and TLR-deficient BMDCs were stimulated with heat-killed KK221 for 6 hr.

(B and C) WT and *Tlr3*$^{-/-}$ (B) or *Tlr9*$^{-/-}$ (C) BMDCs were cultured with heat-killed bacteria (Table S1) for 6 hr.

IFN-β concentration was measured by ELISA. Data are represented as mean ± SD and representative of three independent experiments. *$p < 0.05$, **$p < 0.01$ (Student's t test). Abbreviations are as follows: Tc, *Tetragenococcus*; Pc, *Pediococcus;* Lb, *Lactobacillus*; LM, *L. monocytogenes*; ST, *Salmonella* Typhimurium; CP, *C. perfringens*; HP, *H. pylori*; SA, *S. aureus*. See also Figure S2 and Table S1.

dsRNA in LAB Induces IFN-β Secretion by BMDCs

Despite both TLR3 and TLR9 being well-established receptors for nucleic acids, recognition of bacteria via TLR3 has rarely been described. The canonical TLR3 ligand is viral dsRNA, and therefore the requirement of TLR3 for LAB recognition and induction of IFN-β response suggests that bacterial dsRNA may be physiological ligands in this innate immune response. We tested this possibility by treating heat-killed KK221 with RNase A under conditions in which the enzyme exhibits differential substrate specificities. RNase A digests only ssRNA in the presence of 0.3 M NaCl and digests both ssRNA and dsRNA in the absence of NaCl (Ausubel et al., 1994). We also used S1 nuclease, an endonuclease that catalyzes specific degradation of ssDNA and ssRNA. RNase A treatment of KK221 in the absence of NaCl (Figure S4A) but not digestion of ssRNA (0.3 M NaCl condition) resulted in a significant reduction of IFN-β production (Figures 4A and 4B). This finding is consistent with the results that TLR7-deficient BMDCs are impaired in ssRNA recognition yet still mount a robust IFN-β response to KK221 (Figure 2A). As expected, dsRNA depletion of KK221 lowered the amount of induced IFN-β in WT BMDCs to the amount comparable to TLR3-deficient cells (Figure 4C). IFN-β response to RNase A (0 M NaCl)-treated KK221 was abolished in TLR9-deficient cells (Figure 4C), indicating that the combination of TLR3 and TLR9 totally accounts for the IFN-β production from BMDCs. In contrast, IFN-β production from BMDCs triggered by *Salmonella* Typhimurium or *Helicobacter pylori* was not altered after RNase A-treatment of those pathogenic bacteria (Figure 4D).

To further clarify the characteristics of bacterial RNA, dsRNA extracted from various bacteria was quantified by ELISA via mAb specific for dsRNA (Schönborn et al., 1991). In line with our findings, LAB strains contained higher amounts of dsRNA than the pathogenic bacteria examined (Figures 4E and S4B). Treatment of the samples with RNase III, which digests dsRNA, completely abolished the ELISA signals (Figure S4B). We have used heat-killed bacteria on the assumption that a considerable amount of LAB in the gut is not alive under physiological condition, and such heat-killed LAB as probiotics is applicable to intervene in immune responses. We found that heat-killing treatment preserves the total amount of dsRNA in bacteria such as *Salmonella* Typhimurium and KK221 whereas major RNA components were significantly decreased, consistent with the observation of Sander et al. (2011) (Figures S4C and S4D). Thus stimulatory function of bacterial dsRNA should remain intact even after heat-killing treatment. In fact, the amount of dsRNA in heat-killed KK221, which is increased by high-salt stress during bacteria culture, was correlated with the enhanced IFN-β secretion from BMDCs (Figures S4E and S4F).

We observed that heat-killed KK221 induces a considerable amount of cytokines compared to live bacteria. Reduction of the IFN-β response is comparable to that of IL-6 and TNF-α, indicating that heat killing caused denaturation of the ligands for receptors other than TLR3 (Figure S4G).

Contents in the Small Intestine and Commensal Bacteria Stimulate TLR3-Mediated IFN-β Secretion by BMDCs

To examine the role of TLR3 in response to fresh gut microflora, intestinal contents including commensal bacteria were harvested from specific-pathogen-free mice and applied to BMDC culture. The TLR3 dependency was observed for the ileum contents, as well as highest production of IFN-β, whereas the amount of IL-6 and TNF-α was not affected by TLR3 deficiency (Figure 5A). It is important that TLR3 seems to have a physiological role in small intestine where LAB is a major component of gut flora. These results are consistent with the observation that ileal contents have the highest amount of dsRNA among jejunum, ileum, cecum, and colon (Figure S5A). The colon, which has the second highest amount of dsRNA in the intestine, seems related to neither higher IFN-β production nor TLR3 dependency, suggesting the presence of *Salmonella* Typhimurium-type bacteria whose IFN-β production is more dependent on TLR4 (Figures S3B and S5B). This may also explain the different state

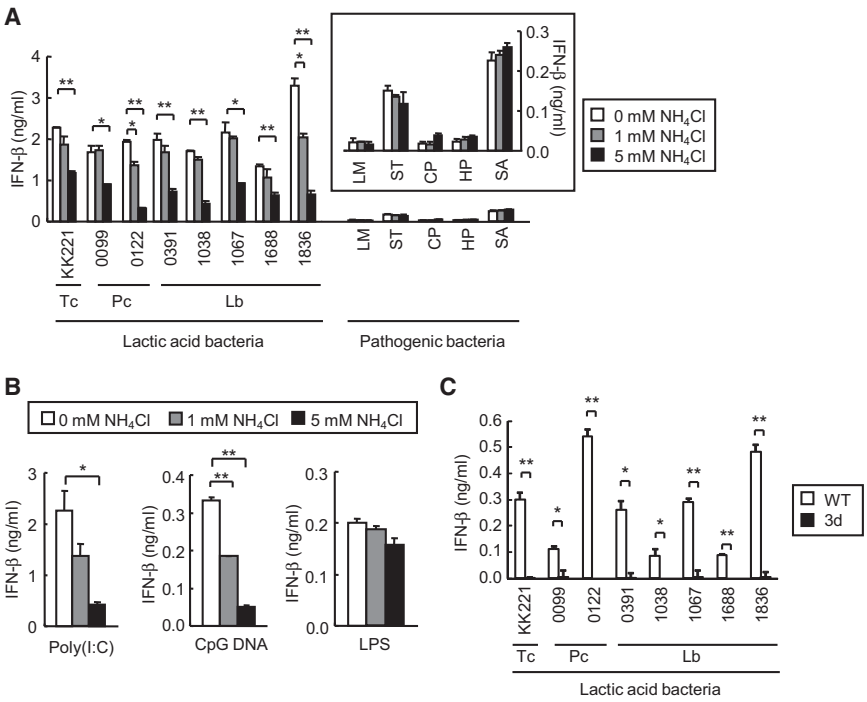

Figure 3. Requirement of Endocytic Processing for IFN-β Response to LAB by BMDCs

(A and B) BMDCs from BALB/c mice were cultured with heat-killed bacteria (Table S1) (A) and TLR ligands (B) in the presence of NH_4Cl for 6 hr.

(C) WT and *Unc93b1*^{3d} (3d) BMDCs were stimulated with heat-killed LAB for 6 hr.

IFN-β concentration was measured by ELISA. Data are represented as mean ± SD and representative of at least two independent experiments. *p < 0.05, **p < 0.01 (Student's t test). Abbreviations are as in Figure 2. See also Figure S3 and Table S1.

for small versus large intestine: in the contrast between LAB and opportunistic pathogenic bacteria as components of their respective commensal flora, and further, its relevance to an anti-inflammatory feature of small intestine.

In the present study, we isolated small intestinal commensal LAB and cocultured with BMDCs (as described in Table S2). Out of 11 strains, 8 induced significant amount of IFN-β, with prominent dependency on TLR3 for most of highly efficient inducers (5/8: *Lactobacillus jonsonii* No. 1-17, No. 2-13; *Lactococcus lactis* No. 3-2, No. 4-4, *Lactobacillus intestinalis* No. 3-8) (Figure 5B). Interestingly, the frequency was similar to that obtained with heat-killed LAB from established culture collections (Figure 2B). The importance of endosomal recognition for these small intestinal LAB was confirmed by using *Unc93b1*^{3d} BMDCs (Figure 5C).

These results suggest that an appreciable amount of commensal bacteria in the ileal microflora enhances the level of IFN-β expression through TLR3 under physiological steady-state conditions.

TLR3 Is Involved in Anti-inflammatory Effects of Oral Administration of LAB

When IFN-β mRNA expression in intestinal epithelial cells (IECs) and lamina propria cells were compared, the amount in lamina propria was much higher than that in IECs and was significantly decreased by TLR3 deficiency (Figure S5C). We confirmed that TLRs other than TLR3 were equally expressed in lamina propria cells of WT and *Tlr3*$^{-/-}$ mice (Figure S5D). This finding is consistent with our observation with BMDCs showing that induction of IFN-β in the coculture with contents of small intestine was reduced by TLR3 deficiency (Figures 5A and S5C). Moreover, although IFN-β expression in CD11c^{+} cells was enhanced in WT mice by oral administration of KK221 (oral KK221), it was not the case for *Tlr3*$^{-/-}$ mice in both intestinal and systemic organs (Figures 6A–6C). These results indicate that TLR3 is important for IFN-β production through activation of CD11c^{+} cells in vivo.

Accordingly, luminal environment in *Tlr3*$^{-/-}$ mice may be less anti-inflammatory with reduced IFN-β response, and indeed *Tlr3*$^{-/-}$ mice were more susceptible to DSS-induced colitis (Figure 6D). Moreover, infiltration of inflammatory immune cells was prominent in *Tlr3*$^{-/-}$ mice even after oral KK221, which is otherwise protective in WT mice. Although KK221 treatment reversed the intensity of the lesions in WT mice, it failed to attenuate the lesions in the *Tlr3*$^{-/-}$ group (Figures 6D and 6E). All other inflammatory signs indicated by colon length, MPO activity, body weight, and expression of soluble mediators support these observations (Figures 6F–6I).

dsRNA in LAB Plays a Central Role to Alleviate Inflammation in DSS-Induced Colitis

Nucleic acids are strong stimulator of innate immune system and we observed LAB trigger the production of IFN-β via TLR3 and TLR9 in vitro (Figure 4C), suggesting that both dsRNA and DNA are possibly protective components of LAB. However, TLR3 deficiency canceled out the anti-inflammatory effect of oral KK221 in vivo (Figures 6D–6I). Therefore, it is important to assess the contribution of dsRNA to the protective effect under physiological conditions. dsRNA-free LAB was prepared by treating KK221 with RNase A (0 M NaCl) and orally administered to mice. The results from experimental colitis clearly showed that dsRNA in KK221 is required to exert the anti-inflammatory effect (Figures 7A–7D and S6A), confirming the central role for dsRNA in vivo. Oral KK221 partially protected *Tlr9*$^{-/-}$ mice in terms of colon length and MPO activity of which effect was again erased in the case of RNase A (0 M NaCl)-treated KK221 (Figures 7A and 7B).

We cannot exclude the role of TLR9 in the full protective function downstream of TLR3 (dsRNA) because the suppression level was less significant in *Tlr9*$^{-/-}$ mice and such effect of KK221 was completely abolished in *Unc93b1*^{3d} mice (Figures 7A–7D) as it was in *Tlr3*$^{-/-}$ mice (Figures 6D–6I). In connection with it, we observed in vitro that expression of IFN regulatory factor 7 (IRF7), an essential molecule for IFN-β induction (Honda

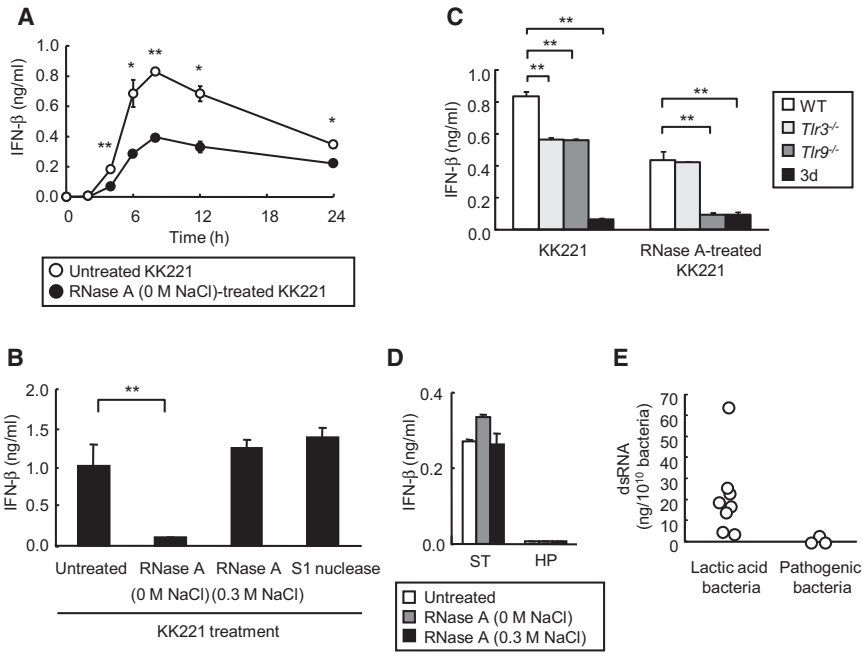

Figure 4. dsRNA of LAB Induces IFN-β Secretion by BMDCs in Response to LAB

(A) BMDCs from BALB/c mice were cultured with untreated or RNase A (0 M NaCl)-treated heat-killed KK221 for the indicated period.

(B) BMDCs from BALB/c mice were cultured with heat-killed KK221 treated with RNase A (0 or 0.3 M NaCl) or S1 nuclease for 6 hr.

(C) WT, *Tlr3*$^{-/-}$, *Tlr9*$^{-/-}$, and *Unc93b1*^{3d} (3d) BMDCs were stimulated with untreated or RNase A (0 M NaCl)-treated heat-killed KK221 for 6 hr.

(D) BMDCs from BALB/c mice were cultured with untreated or RNase A-treated (0 or 0.3 M NaCl) heat-killed *Salmonella* Typhimurium (ST) and *H. pylori* (HP) for 6 hr.

IFN-β concentration was measured by ELISA. Data are represented as mean ± SD and are representative of three independent experiments.

(E) dsRNA in heat-killed bacteria was detected by a sandwich ELISA. dsRNA concentration was quantified by ELISA. Tested LAB are described in Table S1A and tested pathogenic bacteria are ST, HP, and *S. aureus* (SA). Data are representative of two independent experiments.

*p < 0.05, **p < 0.01 (Student's t test). See also Figure S4 and Table S1.

et al., 2005), was enhanced by LAB stimulation but reduced by RNase A treatment (0 M NaCl) of LAB (Figure S6B).

It has been shown that DNA derived from luminal bacteria contributes significantly to the perpetuation of chronic intestinal inflammation via TLR9 and therefore the inflammation of *Tlr9*$^{-/-}$ mice as such was milder compared to WT mice (Obermeier et al., 2005). Thus, under physiological settings, it seems that TLR9 is involved in the induction of large intestinal inflammation whereas TLR3 mainly contributes to the anti-inflammatory mechanism in small intestine through recognition of dsRNA from LAB.

DISCUSSION

We show here that dsRNA from LAB triggers TLR3-mediated IFN-β secretion by DCs in the gut. It was also found that TLR3 is important for recognizing ileal luminal contents and commensal bacteria, thereby affecting IFN-β production in small intestine under physiological conditions.

Unlike IFN-α, IFN-β is not related to autoimmune diseases, but rather is involved in enhancing protective and Th1 cell immunity. IFN-α, which is most probably produced by plasmacytoid DCs, has been shown to contribute to the pathogenesis of inflammatory conditions such as autoimmune systemic lupus erythematous, multiple sclerosis, and skin wounds (Banchereau and Pascual, 2006; Lande et al., 2008; Gregorio et al., 2010). In contrast, IFN-β is suggested to stabilize immune homeostasis (Teige et al., 2003). It has been reported that subcutaneous injection but not oral administration of the TLR3 ligand, poly(I:C), which activates IFN-β production, suppressed DSS-induced colitis (Vijay-Kumar et al., 2007). Consistently, we showed here that DSS-induced colitis was suppressed by oral administration of LAB through IFN-β induction. That oral LAB has the advantage over oral poly(I:C) is probably reasoned by the nature of LAB to resist digestion in the gut and by their spheroidal or rod shape to be easily phagocytosed by DCs.

Bacteria recognition through TLR3 pathway directly contributes to IFN-β production, thereby enhancing both protective and anti-inflammatory response. We identified dsRNA, a component of commensal bacteria, as natural ligands for TLR3 to induce IFN-β. This TLR3-dependent pathway seems to involve activation of TLR7 and TLR9 for sufficient IFN-β induction (Gautier et al., 2005). More specifically, stimulation of TLR7 and/or TLR9 is required for phosphorylation of IRFs, which is essential for the full induction of IFN-β (Honda et al., 2005). We demonstrated that IRF7 expression required for TLR9 signaling was enhanced by LAB stimulation and that RNase A-treated LAB suppressed this enhancement. These observations suggest that TLR3 contributes to enhancing TLR9-mediated IFN-β production through augmenting IRF response, and in turn, TLR9 ligands are important costimulators for the IFN-β production initiated by dsRNA via TLR3, as well as being potent IFN-β inducers themselves.

The cooperative and additive effects of dsRNA as TLR3 ligands and DNA as TLR9 ligands in vitro are also clarified with *Unc93b1*^{3d} mice, which are defective in TLR3, TLR7, and TLR9 signaling. In this mutant mouse, IFN-β production by BMDCs was abolished, indicating that endosomal TLRs are essential for this activity. Because TLR7 is less involved in our experimental system, it is clear that an additive and/or sequential role of TLR3 and TLR9 pathways represent major effects of this LAB-induced IFN-β production. Therefore, DNA should partially activate BMDCs to produce IFN-β and explains the residual amount of IFN-β production after digestion of dsRNA in vitro.

However, TLR9 deficiency caused less pathology in a DSS-induced colitis model, suggesting that the role of TLR9 in the intestine is as a danger-sensing machinery that potentially

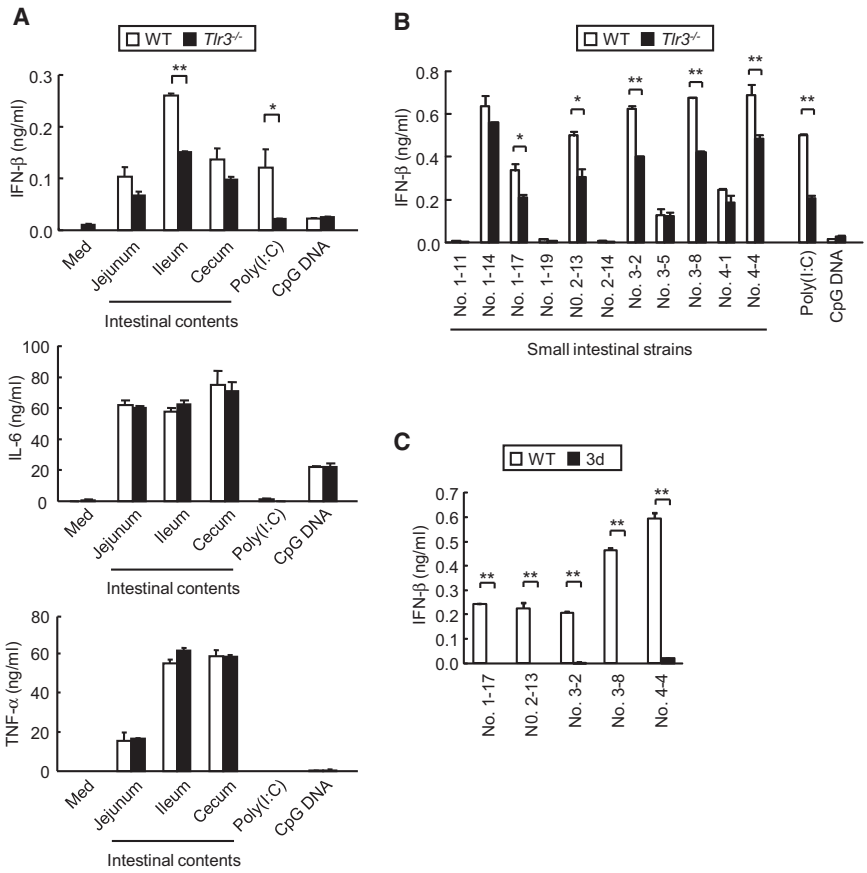

Figure 5. The Intestinal Contents of Mice and Commensal LAB Isolated from Small Intestine Stimulate TLR3-Mediated IFN-β Secretion by BMDCs

(A) WT and *Tlr3*$^{-/-}$ BMDCs were stimulated with the intestinal contents for 6 hr.

(B) WT and *Tlr3*$^{-/-}$ BMDCs were stimulated with commensal LAB isolated from the small intestine of mice (Table S2) for 6 hr.

(C) WT and *Unc93b1*^{3d} BMDCs were stimulated with commensal LAB for 6 hr.

IFN-β, IL-6, and TNF-α concentrations were measured by ELISA. Data are represented as mean ± SD and are representative of two independent experiments. *$p < 0.05$, **$p < 0.01$ (Student's t test). See also Figure S5 and Table S2.

promotes inflammation. In contrast, deletion of TLR3 aggravates the disease. Thus, our findings indicate the discrete roles of TLR3 and TLR9 in vivo, despite their additive and cooperative effects for IFN-β production from BMDCs in vitro.

In connection with the anti-inflammatory nature of TLR3 activation in vivo, TLR3 ligands seem less efficient to induce inflammatory cytokines such as IL-6 and TNF-α as compared with IFN-β, ensuring the anti-inflammatory cytokine milieu in the site where the TLR3 pathway is active. Our finding that ileum contents stimulate the TLR3-mediated pathway most efficiently may partially contribute to the anti-inflammatory feature of small intestine.

Endosomes were identified as the intracellular sites of LAB recognition. In addition to TLR3, two RNA helicases have been shown to be cytosolic sensors of dsRNA: retinoic acid-inducible gene I (RIG-I) and melanoma differentiation-associated gene 5 (MDA5) (Kato et al., 2006). Certain viruses or dsRNA activate a TLR-independent pathway through RIG-I or MDA5, which are recruited by IFN-β promoter stimulator 1 (IPS-1), an adaptor molecule localized on the mitochondrial membrane, thereby stimulating IRF3 and IRF7 (Kumar et al., 2006). However, we found that IPS-1 deficiency had no effect on IFN-β induction by heat-killed LAB (data not shown), thus suggesting that TLR3 and TLR9 in endosomes, but not the cytoplasmic innate receptors, are critically involved in the recognition of commensal bacteria for IFN-β induction. Therefore, considering that the RIG-I pathway is known to be a major route of viral RNA recognition by conventional DCs, it is possible that TLR3 is specifically involved in bacterial dsRNA recognition toward induction of protective immunity such as Th1 cell responses, and at the same time support the maintenance of immune homeostasis via anti-inflammatory cytokines. It has been also shown that TLR3 facilitates cross-priming by conventional DCs during virus infection by phagocytosis of infected cells (Schulz et al., 2005). The TLR3 pathway may promote cellular protective immunity whereas RLRs are more efficient in immediate recognition of virus infection.

The contents in the small intestine induced IFN-β secretion by BMDCs, suggesting that CD11c^{+} DCs in lamina propria or Peyer's patches may also be activated to produce IFN-β by commensal bacteria, especially by LAB. We confirmed this speculation by isolating strains of LAB from small intestine and observed that they induced BMDCs to secrete IFN-β through TLR3. In the gut, it has been shown that bacteria translocate to lamina propria via M cells (Jang et al., 2004) or that some CD11c^{+} cells express tight junction proteins and penetrate gut epithelial monolayers to sample bacteria directly (Rescigno et al., 2001). Phagocytosis of bacteria by intestinal DCs and digestion of cell body in endosome may then occur subsequently. This pathway should contribute to physiological homeostasis of the mucosal immune system. In this regard, mice at weaning, a time when they are experiencing profound changes in the intestinal microenvironment and microbial flora, undergo a surge of IFN-β production (S. Rakoff-Nahoum and R. Medzhitov, personal communication).

In the present study, we also demonstrate a contrast between LAB and pathogenic bacteria in their ability to induce IFN-β production through TLR3. Our findings define a role for TLR3 in the small intestine, not just as a sensor of RNA viruses (Kawai and Akira, 2009) and necrotic self (Karikó et al., 2004) but also as a sensor of commensal bacteria, where it responds by amplifying IFN-β production. These findings indicate a unique characteristic of small intestine: symbiotic bacteria stimulate CD11c^{+} cells by dsRNA under normal steady-state conditions. There have been

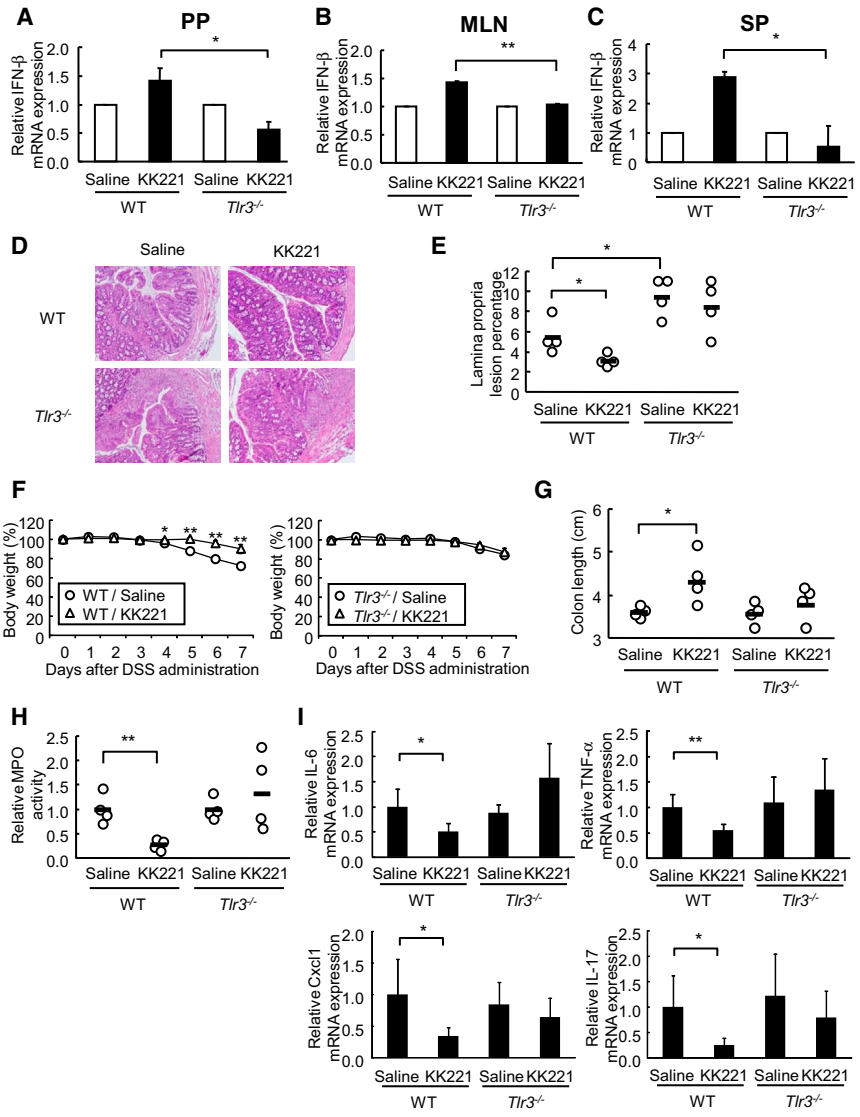

Figure 6. Oral-LAB Alleviates DSS-Induced Colitis through TLR3

(A and B) WT and *Tlr3*$^{-/-}$ mice were treated with heat-killed KK221. After 15 hr, IFN-β mRNA expression in CD11c^{+} cells from Peyer's patches (A, PP) and mesenteric lymph nodes (B, MLN) was measured.

(C) Heat-killed KK221 was orally administered to WT and *Tlr3*$^{-/-}$ mice for 14 days. IFN-β mRNA expression in CD11c^{+} cells from spleens (SP) was measured.

Data are represented as mean ± SD relative to saline-treated controls of two independent experiments.

(D–I) WT and *Tlr3*$^{-/-}$ mice were treated with heat-killed KK221 in DSS-induced colitis.

(D) Colon tissue sections were stained with H&E (original magnification 200×). In *Tlr3*$^{-/-}$ mice, KK221 treatment failed to attenuate an infiltration of neutrophils, eosinophils, and macrophages.

(E–I) Lamina propria lesion percentage in colon (E), body weight (F), colon length (G), colonic MPO activity (H), and colonic mRNA expression (I) were measured. Values are relative to saline-fed group (H, I). Data are represented as mean ± SD (F, I). Bars represent mean (E, G, H).

*$p < 0.05$, **$p < 0.01$ (Student's t test).

some reports that oral administration of LAB enhances protective immunity against viruses and pathogenic bacteria (Corr et al., 2007; Kawashima et al., 2011). Importantly, the anti-inflammatory effect of oral LAB in the DSS-induced colitis model was highly dependent on dsRNA in LAB. LAB can thereby benefit the host immune system and may have coevolved to become major commensal bacteria in the small intestine. We conclude that commensal bacteria-induced IFN-β production by DCs is a physiological response, which stabilize protective immunity. It is also suggested that utilization of dietary supplements, which take advantage of such physiological mechanisms, is a secure way to improve immune responses and immune homeostasis.

The mechanism for the preferential stimulation of the TLR3 pathway by dsRNA from small intestinal commensal bacteria, at least in part, may be attributed simply to be quantitative level: LAB contain much higher levels of dsRNA than the other species including pathogenic bacteria. Our observation that KK221 which had been manipulated to contain more dsRNA induced more IFN-β production supports this idea. However, some of the bacterial strains did not exhibit simple quantitative correlation. In such strains, stimulation by other TLR ligands in the bacteria or digested bacterial components in endosomes could also be involved in IFN-β induction. For instance, TLR4 is preferentially involved in IFN-β production upon stimulation with *Salmonella*, and it has been reported that the resistance of bacterial cell wall components to digestive enzymes in endosomes is related to the induction of inflammatory cytokines (Shida et al., 2006). However, it is stressed that in vivo, dsRNA stimulation via TLR3 played a central role for the protective effect of oral LAB. Mechanisms of predominant role for TLR3 in vivo need to be further clarified.

dsRNA is typically observed in viruses. In bacteria, multiple small noncoding RNAs are known to be involved in posttranscriptional gene regulation, often in response to stress. In *E. coli*, a small RNA (RyhB) is synthesized when iron is limited and binds to target mRNA to form dsRNA (Massé and Gottesman, 2002). Therefore, not only LAB but also other species of bacteria may increase the amount of dsRNA under various conditions. Recently, clustered regularly interspaced short palindromic repeats (CRISPR), whose size is as small as less than 50 bps, has been reported to provide the mechanism for upregulating acquired immunity against viruses and plasmids (Horvath and Barrangou, 2010). In fact, multiple CRISPR families have been identified within LAB species including *Lactobacillus* (Horvath et al., 2009). Considering that TLR3 can bind to 40–50 bp segments of dsRNA (Leonard et al., 2008; Liu et al., 2008), our findings may suggest that LAB provides such a length of dsRNA,

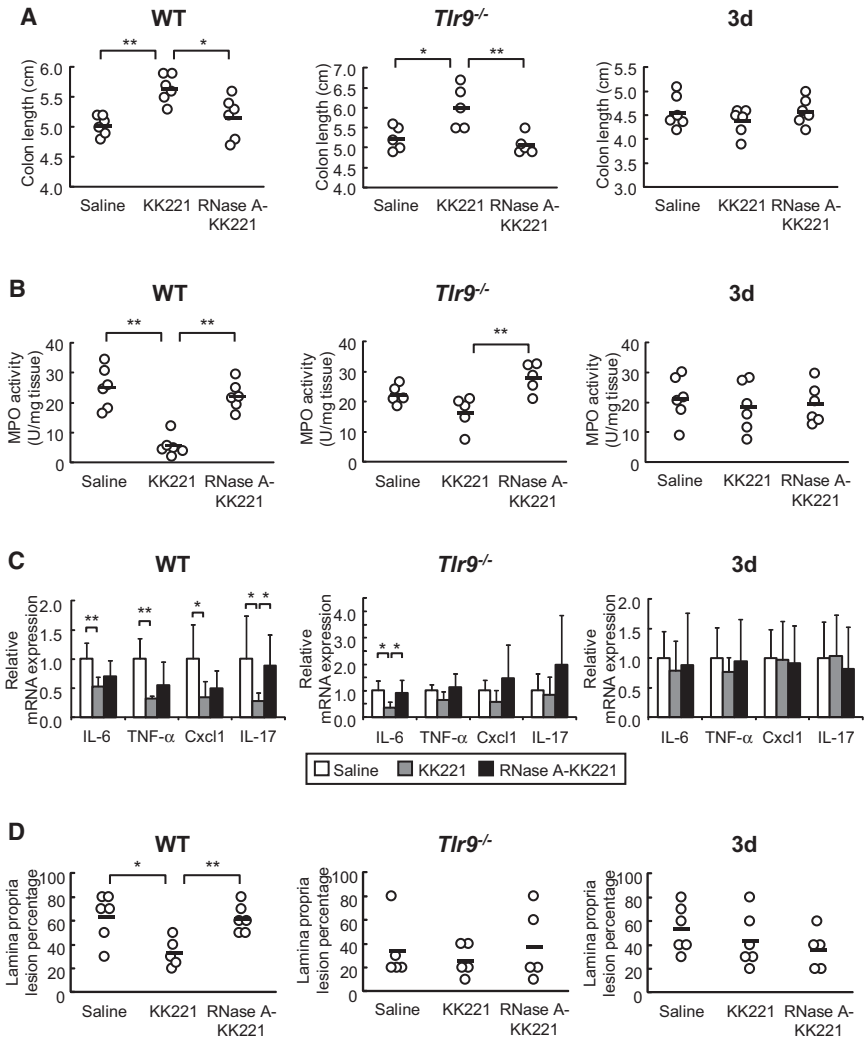

Figure 7. dsRNA in LAB Alleviates Inflammation in DSS-Induced Colitis

Saline-treated, untreated, or RNase A (0 M NaCl)-treated heat-killed KK221 was orally administered to WT, *Tlr9*$^{-/-}$, and *Unc93b1*^{3d} (3d) mice in DSS-induced colitis. Colon length (A), colonic MPO activity (B), and colonic mRNA expression (C) were measured. Lamina propria lesion percentage in colon (D) was quantified. Data are represented as mean ± SD relative to saline-fed group in each strain (C). Bars represent mean (A, B, D). *$p < 0.05$, **$p < 0.01$ (Student's t test).

See also Figure S6.

including CRISPR families, to DCs for IFN-β production. Further analysis of dsRNA may clarify the quantitative difference of dsRNA between LAB and pathogenic bacteria.

On the other hand, in the intestine, stimulation of TLRs other than TLR3 by pathogenic bacteria may cause occasional inflammation and discriminate them from less-inflammatory strains worthy to become cohabitants. Considering that dsRNA-TLR3-mediated activation of DCs contributes to gut homeostasis, there may have been strong selection in the gut for commensals with higher levels of dsRNA. LAB can grow and thrive under low pH conditions (Williams, 2010). By producing lactic acid, LAB protects the gut microflora community from overgrowth of pathogenic bacteria by lowering the pH. Environmental conditions, such as pH or nutrition, seem to affect the dsRNA content of bacteria and may help distinguish between commensal bacteria that stabilize immune homeostasis and pathogenic bacteria that may as well be involved in opportunistic infection and cause inflammatory conditions. Such environmental factors that determine or modify the contents of dsRNA or TLR ligands in bacteria should be clarified and are now under investigation.

EXPERIMENTAL PROCEDURES

Bacteria

Tetragenococcus halophilus KK221was cultured in MRS broth (Difco) containing 10% (w/v) NaCl. Other species of LAB were cultured in MRS broth. Heat-killed pathogenic bacteria were prepared by Hyogo College of Medicine. Strain numbers and names of the tested bacteria are listed in Tables S1 and S2. Nuclease treatment of bacteria and isolation of intestinal LAB are described in the Supplemental Experimental Procedures.

Mice

6- to 10-week-old female BALB/c or C57BL/6 mice were purchased from SLC Japan or Japan Crea. TLR-deficient C57BL/6 mice were provided by Hyogo College of Medicine, RIKEN, The University of Tokyo, or purchased from Oriental Bioservice. *Unc93b1*^{3d} mutant C57BL/6 mice were kindly provided by B. Beutler (Southwestern University). Mice were maintained according to the respective institutional guidelines. Animal experiments were approved by the Animal Welfare Committee of AIST.

Preparation of BMDCs

Bone marrow cells were collected from tibiae and femurs. After red blood cell lysis and depletion of CD4-, CD8-, and I-A/I-E-expressing cells, the cells were cultured in RPMI 1640 (Gibco) containing 10% fetal bovine serum and 10% granulocyte macrophage colony-stimulating factor (GM-CSF). On day 8, nonadherent and loosely adherent cells were harvested (see also the Supplemental Experimental Procedures).

Reagents

A mAb to mouse IFN-β was purchased from Yamasa Co. A rat IgG_1 Ab (eBioscience) was used as the isotype control Ab. Poly(I:C), LPS, and CpG DNA (ODN 1826) were purchased from Invivogen.

Cell Culture

BMDCs were cultured in 96-well flat-bottomed culture plates at 2×10^5 cells/well/200 μl in the presence or absence of 1×10^7 bacteria. For inhibition of acidification in endosomes, BMDCs were preincubated under 1 or 5 mM NH_4Cl. Thereafter 1×10^7 bacteria were added and cocultured for 6 hr. The contents of jejunum, ileum, cecum, and colon were added to cell culture medium, adjusted to 0.25 of absorbance (600 nm), and cocultured for 6 hr. Poly(I:C) (50 μg/ml), LPS (10 μg/ml), and CpG DNA (10 μg/ml) were added as TLR ligands.

Measurement of Cytokines in Supernatants

The cytokine concentrations were determined with the Mouse OptEIA ELISA set (BD Bioscience). The IFN-β concentration was determined with the Mouse IFN-β ELISA kit (PBL Biomedical Laboratories).

In Vivo Studies

To induce experimental colitis to WT, TLR-deficient, and *Unc93b1*^{3d} mutant C57BL/6 mice (n = 4–6), distilled water containing 3% (w/v) DSS (MP Biomedicals) was provided ad libitum for 7 days. Saline or heat-killed KK221 (1×10^9/day) was orally administered for 14 days from 7 days before induction of experimental colitis. Control Ab or IFN-β mAb (50 μg/mouse) was injected intravenously every 2 days. After 7 days of DSS administration, colon tissue was collected from each mouse.

Histological Analysis

Colon tissue sections were stained with H&E. The numbers of fields with lamina propria inflammation in colon were quantified and the percentage calculated with Olympus BX53 Biological Microscope (Olympus Corporation) at 200× original magnification and digital imaging system (Ventana iScan Coreo AW v.3.3.1 and Image Viewer v.3.1; Ventana Medical Systems). Histological analysis is described in detail in the Supplemental Experimental Procedures.

Determination of MPO Activity

Colon tissues were homogenized in 50 mM phosphate buffer (pH 6.0) containing 0.5% hexadecyltrimethyl-ammonium bromide, frozen and thawed three times, and centrifuged. An aliquot of the supernatant was used for determination of enzyme activity as described (Vijay-Kumar et al., 2007).

Quantitative RT-PCR

Total RNA was extracted from the cells or tissue samples with TRIzol reagent (Invitrogen). cDNA obtained by reverse transcription via PrimeScript RT reagent (Takara) was amplified with specific primers (Table S3) and SYBR Premix Ex Taq (Takara). Each mRNA expression level was normalized with β-actin.

Analysis of dsRNA in Bacteria

Nucleic acid was extracted from heat-killed bacteria (see also Supplemental Experimental Procedures). The concentration of bacterial dsRNA was determined by sandwich ELISA with the mAb K1 and the biotinylated mAb J2 (English and Scientific Consulting) for detection (Schönborn et al., 1991) followed by streptavidin peroxidase (Zymed). The concentration of dsRNA was calculated with poly(I:C) as a standard.

Statistical Analysis

Error bars indicate standard deviation. Statistical significance was determined by a two-tailed Student's t test for unpaired data.

SUPPLEMENTAL INFORMATION

Supplemental Information includes Supplemental Experimental Procedures, six figures, and three tables and can be found with this article online at http://dx.doi.org/10.1016/j.immuni.2013.02.024.

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