Recognition of gut microbiota by NOD2 is essential for the homeostasis of intestinal intraepithelial lymphocytes

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NOD2 functions as an intracellular sensor for microbial pathogen and plays an important role in epithelial defense. The loss-of-function mutation of NOD2 is strongly associated with human Crohn's disease (CD). However, the mechanisms of how NOD2 maintains the intestinal homeostasis and regulates the susceptibility of CD are still unclear. Here we found that the numbers of intestinal intraepithelial lymphocytes (IELs) were reduced significantly in Nod2−/− mice and the residual IELs displayed reduced proliferation and increased apoptosis. Further study showed that NOD2 signaling maintained IELs via recognition of gut microbiota and IL-15 production. Notably, recovery of IELs by adoptive transfer could reduce the susceptibility of Nod2−/− mice to the 2,4,6-trinitrobenzene sulfonic acid (TNBS)–induced colitis. Our results demonstrate that recognition of gut microbiota by NOD2 is important to maintain the homeostasis of IELs and provide a clue that may link NOD2 variation to the impaired innate immunity and higher susceptibility in CD.

Pattern recognition receptors, including NOD-like receptors (NLRs), TLRs, and RIG-I–like receptor et al., play a key role in the innate immune response by recognizing pathogen-associated molecular patterns derived from a diverse collection of microbial pathogens (Janeway and Medzhitov, 2002; Meylan et al., 2006). NOD2, a family member of NLRs, functions as an important intracellular sensor for intracellular bacteria and can detect peptidoglycan through the recognition of muramyl dipeptide (MDP; Meylan et al., 2006; Elinav et al., 2011). After binding with MDP, NOD2 recruits adaptor protein RIP2 to activate NF-κB and initiate a proinflammatory response (Meylan et al., 2006). Furthermore, NOD2 was the first identified gene strongly associated with susceptibility to Crohn's disease (CD; Hugot et al., 2001; Ogura et al., 2001), which affects over one million people in North America and Europe and is characterized by diarrheic colitis and chronic, relapsing inflammation. Although it has been suggested that the loss-of-function mutation of NOD2 leads to reduced antimicrobial resistance and impaired innate immunity in CD (Comalada and Peppelenbosch, 2006), how NOD2 mutation causes compromised host defense and contributes to the pathogenesis of this disease remains elusive.

The intestinal intraepithelial lymphocytes (IELs) are mostly T cells dispersed as single cells within the epithelial cell layer and located at the interface between outside and the body. Unlike lymphocytes in spleen, blood, or lymph node, IELs comprise >70% of CD8+ T cells and include greater numbers of TCRγδ+ T cells (Cheroutre, 2004). In addition, a large fraction of these cells...
express a CD8αα homodimer, which is essentially absent from the circulation. IELs display an “innate” or “memory-like” phenotype and play an important role in the homeostasis of intestinal mucosa (Cheroutre, 2004), and loss of them results in impaired intestinal barrier function and more severe colitis in mice (Hoffmann et al., 2001; Chen et al., 2002; Olivares-Villagómez et al., 2008; Li et al., 2011), suggesting the protective role of IELs in inflammatory bowel diseases. The impaired intestinal barrier function and increased susceptibility to colitis have also been shown in Nod2−/− mice (Barreau et al., 2007; Penack et al., 2009). However, whether NOD2 affects intestinal IELs in sections of small intestine. (Fig. 2, D and E). These data thus indicate a critical and selective role for Nod2 in the homeostasis of TCRγδ+ and CD8αα+ TCRαβ+ IELs.

**RIP2 is a critical adaptor protein for NOD2 to initiate downstream signaling.** To confirm the role of Nod2 signaling in the maintenance of intestinal IELs, we examined Rip2−/− mice. Similar to the results observed in Nod2−/− mice, the TCRγδ+ and CD8αα+ TCRαβ+ IELs were also dramatically reduced in Rip2−/− mice as compared with control mice (Fig. 3, A and B). Collectively, these results indicate that Nod2 signaling plays a critical and selective role for the maintenance of IELs in the intestine.

**Nod2 signaling is not required for the development of IELs in the thymus.** Because mice with Nod2 deletion lack IELs, especially TCRγδ+ and CD8αα+ TCRαβ+ IELs, we investigated whether Nod2 signaling had an impact on the development of IELs in the thymus. First, our results showed that the percentages of T and B cells were normal in the thymus or spleen of Nod2−/− mice (Fig. 4 A), suggesting that Nod2 deletion has no impact on the development of conventional lymphocytes in the thymus. Despite early studies suggesting that IELs can develop extra-thymically, more recent studies indicate that the majority of them are derived from thymic precursors, at least in the presence of thymus (Cheroutre and Lambolez, 2008). We then examined the frequencies of TCRγδ+ T cells in the spleen, and no differences were observed in Nod2−/− mice as compared with control mice (Fig. 4 B). Recent evidence suggests that CD8αα+ TCRαβ+ IELs are derived from CD4−CD8− (double negative [DN]) NK1.1+ TCRαβ+ thymocytes, which can give rise to CD8αα+ TCRαβ+ IELs when transferred into Rag2−/− mice (Gangadharan et al., 2006). We therefore investigated whether this putative IEL precursor population was affected by Nod2 deletion. The analysis showed that DN NK1.1+ TCRαβ+ thymocytes were present in normal frequencies in Nod2−/− mice (Fig. 4 C). These results indicate that Nod2 deficiency has no effect on the development of TCRγδ+ IELs.

**RESULTS**

**Selectively reduced IEL subsets in Nod2−/− mice**

To investigate whether NOD2 signaling has an impact on the homeostasis of IELs, we first examined the numbers and subpopulation percentages of IELs in Nod2−/− mice. The total number of IELs in Nod2−/− mice was reduced significantly in the small intestine and colon (approximately fourfold; Fig. 1, A and B) as compared with wild-type or Nod1−/− mice. We further examined whether Nod2 deletion affected the lymphocytes in other immune organs, and the results showed that the total numbers were normal in the thymus or spleen of Nod2−/− mice (Fig. 1, C and D), suggesting that Nod2 deletion specifically affects the maintenance of intestinal IELs.

IELs are simply classified as TCRγδ+, CD8αα+ TCRαβ+, CD8αβ+ TCRαβ−, and CD4+ TCRαβ− IELs. TCRγδ+ and CD8αα+ TCRαβ+ IELs are regarded as unconventional T cells bearing innate-like phenotypes (Cheroutre, 2004; Cheroutre and Lambolez, 2008). Although CD8αβ− TCRαβ+ IELs are conventional T cells, the majority of them exhibit memory-like phenotype (Cheroutre, 2004). Further analysis of small intestinal IELs in Nod2−/− mice revealed that the unconventional TCRγδ+ (approximately eightfold) and CD8αα+ TCRαβ+ IELs (approximately sixfold) were dramatically reduced and the memory-like CD8αβ− TCRαβ+ IEL subset was significantly reduced (approximately threefold), whereas the CD4+ IEL subset was normal (Fig. 2, A and B). Similar results were also observed in the colon of Nod2−/− mice (Fig. 2 C). The loss of CD8+ and TCRγδ+ IELs in Nod2−/− mice was also confirmed by staining of CD8αα and TCRγδ in sections of small intestine (Fig. 2, D and E). These data thus indicate a critical and selective role for Nod2 in the homeostasis of TCRγδ+ and CD8αα+ TCRαβ+ IELs.

**Figure 1.** *Loss of IELs in Nod2−/− mice.* (A and B) The numbers of IELs in the small intestine (A) and colon (B) of mutant mice and individual control mice. **, P < 0.01; ***, P < 0.001. (C and D) The numbers of total thymocytes (C) and splenocytes (D) of mutant mice and individual control mice. Horizontal bars indicate the mean. 20–25 mice per group from three independent experiments.

### References

IELs display reduced proliferation and increased apoptosis in Nod2−/− mice

To further explore the reason for loss of IELs in Nod2−/− mice, we examined whether Nod2 deletion affected the proliferation and apoptosis of IELs. The isolated IELs from Nod2−/− mice were stained with Ki67 and annexin-V as the marker for proliferation and apoptosis, respectively. TCRγδ+, CD8α+TCRαβ+, and CD8β+TCRαβ+ IELs showed poorer proliferation and higher apoptosis in Nod2−/− mice, whereas the CD4+ IELs were normal (Fig. 5, A and B). The poorer proliferation of TCRγδ+, CD8α+TCRαβ+, and CD8β+TCRαβ+ IELs was also confirmed by BrdU incorporation assay (Fig. 5 C). These results were consistent with loss of IEL subsets in Nod2−/− mice, suggesting that Nod2 regulates the homeostasis of IELs by affecting their proliferation and survival.

Nod2 signaling maintains IELs via recognition of gut microbiota

NOD2 is an intracellular sensor not only for microbial but also for commensal bacteria (Kobayashi et al., 2005; Petnicki-Ocwieja
et al., 2009). Nod2 expression in the intestine is dependent on the presence of gut microbiota, and Nod2−/− mice have an increased load of commensal resident bacteria (Petnicki-Ocwieja et al., 2009). Moreover, the commensal bacteria in the gut are also required for the maintenance of IELs (Kawaguchi et al., 1993), but the mechanisms remain unclear. Thus, we investigated whether the gut microbiota maintained IELs via NOD2 signaling. To deplete gut microbiota in the intestine of mice, we fed mice with a cocktail of antibiotics after birth (Rakoff-Nahoum et al., 2004). After 6 wk, the load of bacteria was reduced significantly in antibiotic-treated mice (Fig. 6 A). Consistent with the results observed in germ-free mice, depletion of the gut microbiota reduced the IEL numbers in the intestine but had no effect on the lymphocytes from other immune organs, including thymus, spleen, and liver (Fig. 6 B). Importantly, the total numbers of IELs could be recovered significantly by supplementation of NOD2 agonist MDP in drinking water (Fig. 6 C). In contrast, supplementation of iEDAP, an agonist for NOD1, did not increase the IELs in gut microbiota–depleted mice (Fig. 6 C). Further analysis of IEL subsets in MDP-supplemented mice revealed that NOD2 activation by MDP treatment selectively and significantly recovered TCRγδ+ and CD8α+TCRαβ+ IELs, whereas it didn’t have an effect on CD8αβ+TCRαβ+ IELs (Fig. 6, D and E). We also analyzed the subpopulations of lymphocytes, including B, CD4+ T, CD8+ T, NKT, and NK cells in the spleen of gut microbiota–depleted mice, and no differences were observed (Fig. 6 F). Collectively, these results indicate that NOD2 signaling maintains the IELs via sensing of gut microbiota.

**Nod2 signaling in the hematopoietic system–derived APCs maintains IELs**

To further investigate how Nod2 signaling maintains IELs, we then determined whether IEL-intrinsic or -extrinsic mechanisms were responsible for the specific reduction in IELs. Adoptive transfer of BM cells from Nod2+/+ mice and Nod2−/− mice both reconstituted IELs in Rag1-deficient hosts (Fig. 7, A and B), suggesting that IEL-intrinsic Nod2 signaling is not required for the homeostasis of IELs. This was consistent with the results showing that no expression of Nod2 was detected

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**Figure 3. Loss of IELs in Rip2−/− mice.** (A) Quantification of total numbers of small intestinal IELs, liver lymphocytes, splenocytes, and thymocytes in wild-type mice and Rip2−/− mice. **, P < 0.01. (B) The absolute numbers of the indicated IEL subsets in wild-type mice and Rip2−/− mice. ***, P < 0.001. Five mice per group. Representative of three experiments. Error bars indicate SEM.

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**Figure 4. Normal subsets in the thymus and spleen of Nod2−/− mice.** (A) The thymocytes and splenocytes from Nod2+/+ or Nod2−/− mice were stained as indicated. (B) The splenocytes from Nod2−/− mice were stained as indicated. (A and B) Numbers in the dot plots indicate the percentage of cells represented in the quadrant. (C) Analysis of putative thymic IEL precursors in Nod2+/+ and Nod2−/− mice. Total thymocytes were stained as indicated. DN thymocytes were analyzed for NK1.1 and TCRβ expression. Numbers in the quadrants indicate percentages. (D) The splenocytes from Nod2−/− mice were stained as indicated. The percentages of memory CD8+ T cells are shown. Five mice per group. Representative of two or three experiments.
in IELs (Fig. 7 C). As Nod2 was expressed in parenchymal cells, including intestinal epithelial cells (IECs), and some hematopoietic system–derived APCs, such as macrophages and DCs (Fig. 7 C), we then determined whether Nod2 signaling in hematopoietic or parenchymal cells is responsible for the maintenance of IELs. When BM cells from CD45.1 WT mice were transferred into lethally irradiated Nod2+/+ or Nod2−/− mice, after 8 wk, there was no difference for the reconstitution of IELs (Fig. 7, D and E), suggesting that Nod2 signaling in parenchymal cells, including IECs, is not responsible for the homeostasis of IELs. When BM cells from Nod2+/+ or Nod2−/− mice were transferred into lethally irradiated CD45.1 WT mice, after 8 wk, TCRγδ and CD8ααTCRαβ in mice transfected with Nod2−/− BM cells were poorly reconstituted compared with mice transfected with Nod2+/+ BM cells (Fig. 7, D and E), suggesting that Nod2 signaling in hematopoietic cells is required for the homeostasis of IELs. Because IEL–intrinsic Nod2 signaling was not involved (Fig. 7, A and B), these results indicate that Nod2 signaling in the hematopoietic system–derived APCs, such as macrophages or DCs, might be critical for the homeostasis of IELs.

Impaired IL-15 expression results in the loss of IELs in Nod2−/− mice

IL-15 is an NF-κB target gene, and more importantly, the IL-15 enrichment environment in the gut is critical for the survival and homeostasis of IEL subsets (Cao et al., 1995; Washizu et al., 1998). We thus investigated whether Nod2 signaling affected the proliferation and apoptosis of IELs via IL-15. First, exogenous IL-15 administration recovered the IEL loss in Nod2−/− mice partially (Fig. 8 A), suggesting that the impaired expression of IL-15 might be responsible for the loss of IELs in Nod2−/− mice. Consistent with the results in Nod2−/− mice, IL-15−/− mice showed reduced TCRγδ+CD8αβTCRαβ+ and CD8ααTCRαβ+ IELs but normal CD4+ IELs (Fig. 8 B). Importantly, MDP supplementation could not recover the IEL loss in Nod2−/− mice. We then examined the expression of IL-15 in IECs, intestinal macrophages, and DCs and found that macrophages and DCs expressed higher IL-15 and in the basal condition (Fig. 8 C). As expected, the basal or MDP-induced expression of IL-15 in macrophages was decreased when Nod2 was absent (Fig. 8 D). Furthermore, our results showed that MDP treatment up-regulated IL-15 expression in macrophages in a Nod2- and Rip2-dependent manner (Fig. 8 E). Because MDP supplementation rescued the loss of IELs in gut microbiota–depleted mice, we also examined whether MDP could recover the IL-15 expression. As expected, gut microbiota–depleted mice showed lower IL-15 expression in macrophages, whereas MDP treatment restored the expression of IL-15 (Fig. 8 F), suggesting that Nod2 signaling might maintain the expression of IL-15 via recognition of microbiota. These results indicate that the reduced IL-15 expression contributes to the loss of IELs in Nod2−/− mice.

IEL loss contributes to the high susceptibility to TNBS-induced colitis in Nod2−/− mice

IELs inhabit the intestinal epithelial barriers and provide a first line of defense against environmental challenges or pathogen invading via stimulating repair of damaged epithelia and limiting bacterial penetration (Cheroutre, 2004). Loss of TCRγδ+ IELs or CD8ααTCRαβ+ IELs in mice aggravates colitis in several animal models and results in impaired ability to
alleviated significantly (Fig. 9, B–D), suggesting that recovery of IELs in Nod2−/− mice can reduce the high susceptibility of these mice to colitis. Furthermore, consistent with the finding that MDP supplementation restored the IELs partially in gut microbiota–depleted mice, MDP treatment reduced the colitis induced by TNBS in these mice (Fig. 10, A and B). Collectively, these results indicate that the loss of IELs in Nod2−/− mice caused by failing to recognize gut microbiota contributes to the impaired innate immune defense and high susceptibility to colitis in these mice.

**DISCUSSION**

We show here that Nod2 signaling is important to maintain IELs in the intestine. Mice with Nod2 or Rip2 deletion lacked IELs, especially the unconventional TCRγδ+ IELs and CD8αα+TCRαβ+ IELs, in the small intestine and colon. In contrast, the lymphocytes in thymus, spleen, and liver control bacterial overgrowth (Ninomiya et al., 2000; Hoffmann et al., 2001; Olivares-Villagómez et al., 2008), suggesting a critical role of IELs in the mucosal defense and epithelial homeostasis. Similarly, Nod2−/− mice are susceptible to the colitis induced by TNBS (Barreau et al., 2007; Penack et al., 2009), but the mechanisms are unclear. We thus studied whether the loss of IELs contributed to the high susceptibility of Nod2−/− mice to colitis. To do this, we transferred CD8+ IELs and splenic CD8+ T cells from wild-type mice to Nod2−/− mice and found that adoptive transfer of CD8+ IELs could recover the IEL loss in Nod2−/− mice partially (Fig. 9 A). Consistent with previous studies (Barreau et al., 2007; Penack et al., 2009), challenge of Nod2−/− mice with TNBS resulted in higher mortality and colitis scores as compared with control mice (Fig. 9, B–D). Moreover, when Nod2−/− mice were transferred with CD8+ IELs from wild-type mice to recover the loss of IELs by Nod2 deletion, the mortality and histology damage were alleviated significantly (Fig. 9, B–D), suggesting that recovery of IELs in Nod2−/− mice can reduce the high susceptibility of these mice to colitis. Furthermore, consistent with the finding that MDP supplementation restored the IELs partially in gut microbiota–depleted mice, MDP treatment reduced the colitis induced by TNBS in these mice (Fig. 10, A and B). Collectively, these results indicate that the loss of IELs in Nod2−/− mice caused by failing to recognize gut microbiota contributes to the impaired innate immune defense and high susceptibility to colitis in these mice.
Nod2−/− mice have been reported to be more susceptible to oral infection with *Listeria monocytogenes* (Kobayashi et al., 2005), suggesting the important role of NOD2 signaling in epithelial defense. The current knowledge for the mechanism of defective epithelial defense in Nod2−/− mice is that these mice show impaired production of defensins by Paneth cells (Kobayashi et al., 2005). However, the defensin defect in Nod2−/− mice is limited, and only several, but not all, defensins are modestly decreased, questioning the role of these limited changes on relevant antimicrobial defense in the intestine (Kobayashi et al., 2005). In this study, we show that IELs are dramatically reduced in Nod2−/− mice. IELs play a critical role for maintaining the integrity of the epithelial barrier, and loss of TCRγδ+ IELs results in higher bacterial penetration of the intestinal mucosa (Ismail et al., 2011). The higher permeability remained normal. In Nod2−/− mice, the residual IELs displayed reduced proliferation and increased apoptosis. Furthermore, we found that Nod2 signaling maintained IELs via recognition of gut microbiota because supplementation of NOD2 agonist MDP recovered the IELs in gut microbiota-depleted mice. The loss of IELs in Nod2−/− mice was caused by the impaired expression of IL-15 in APCs, and supplementation of IL-15 rescued the IEL loss caused by Nod2 deletion. Importantly, recovery of IELs by adoptive transfer of IELs to Nod2−/− mice could reduce the susceptibility of the mice to TNBS-induced colitis. Thus, our results demonstrate a previously unrecognized role for Nod2 signaling in the homeostasis of IELs and probably provide a new clue for the observed impaired host defense in Nod2−/− mice and patients with CD.

**Figure 7.** Nod2 signaling in the hematopoietic system–derived APCs maintains IELs. (A and B) FACS analysis of IELs of the indicated BM chimeras of Nod2+/+ or Nod2−/− BM cells into irradiated Rag1−/− mice. The percentages (A) or absolute numbers (B) of the indicated IEL subsets are shown. (C) Isolated IECs, sorted IEL subsets, macrophages, and DCs were determined by quantitative PCR, and the relative expression of Nod2 is shown. (D and E) FACS analysis of IELs of the indicated BM chimeras of CD45.1+ WT BM cells into irradiated Nod2+/+ or Nod2−/− mice, or Nod2+/+ or Nod2−/− BM cells into irradiated CD45.1+ WT mice. The percentages (D) or absolute numbers (E) of the indicated IEL subsets are shown. Numbers in the dot plots indicate the percentage of cells represented in the quadrant. *, P < 0.05; **, P < 0.01. Five mice per group. Representative of two experiments. Error bars indicate SEM.
Nod2 regulates IEL homeostasis | Jiang et al.

NOD2 genotype (Simms et al., 2008). In this study, our results suggest that IEL dysregulation caused by loss of function of NOD2 may favor the onset of CD. Indeed, multiple studies have described a decreased level of the TCRγδ+ IELs in patients with CD (Fukushima et al., 1991; Lee et al., 1997). This idea is also supported by the results showing that loss of TCRγδ+ IELs or CD8αβ+TCRαβ+ IELs aggravates colitis in the mouse model (Hoffmann et al., 2001; Olivares-Villagómez et al., 2008).

Gut microbiota resides in the intestine and has a beneficial effect ranging from aiding in metabolism to competing with invasive pathogens (Sonnenburg et al., 2006; Honda and Littman, 2012). Increasing evidence from germ-free mice or antibiotic-treated mice reveals that gut microbiota is important for the lymphoid tissue development in the intestine (Abt and Artis, 2009). IELs are a unique T cell population located between IECs and function as a critical component of the epithelial barrier. IELs reduced dramatically in germ-free mice (Guy-Grand et al., 1991; Kawaguchi et al., 1993; Suzuki et al., 2002), suggesting that the defect of IELs may play an important role in the intestinal barrier dysfunction in Nod2−/− mice.

Although the etiology of CD is poorly understood, increasing evidence from genetics, functional studies on innate immunity, and therapeutic trials on patients suggest that CD results from impaired innate immunity and dysfunction of mucosal barrier (Comalada and Peppelenbosch, 2006; Marks et al., 2006). The genetic association of NOD2 with CD established a critical link between innate immunity and the development of the disease, but the underlying mechanisms remain controversial. The CD-associated NOD2 variants show impaired ability to recognize microbial components and lack of activation of NF-κB in monocytes (Hugot et al., 2001). However, the higher NF-κB activity is observed in patients with CD (Podolsky, 2002). Defensin levels are noted to be lower in the Paneth cells of patients with mutant NOD2 (Wehkamp et al., 2005), but recent work has shown that defensin deficiency in CD is independent of the NOD2 genotype (Simms et al., 2008). In this study, our results suggest that IEL dysregulation caused by loss of function of NOD2 may favor the onset of CD. Indeed, multiple studies have described a decreased level of the TCRγδ+ IELs in patients with CD (Fukushima et al., 1991; Lee et al., 1997). This idea is also supported by the results showing that loss of TCRγδ+ IELs or CD8αβ+TCRαβ+ IELs aggravates colitis in the mouse model (Hoffmann et al., 2001; Olivares-Villagómez et al., 2008). Gut microbiota resides in the intestine and has a beneficial effect ranging from aiding in metabolism to competing with invasive pathogens (Sonnenburg et al., 2006; Honda and Littman, 2012). Increasing evidence from germ-free mice or antibiotic-treated mice reveals that gut microbiota is important for the lymphoid tissue development in the intestine (Abt and Artis, 2009). IELs are a unique T cell population located between IECs and function as a critical component of the epithelial barrier. IELs reduced dramatically in germ-free mice (Guy-Grand et al., 1991; Kawaguchi et al., 1993; Suzuki et al., 2002), suggesting...
suggest that IEL loss may contribute to the impaired innate immunity and higher susceptibility to mucosal damage in Nod2−/− mice and patients with CD.

MATERIALS AND METHODS

Mice. Nod2−/−, Rip2−/−, and Myd88−/− mice were described previously (Adachi et al., 1998; Kobayashi et al., 2002, 2005). Nod1−/− mice were provided by Millennium Pharmaceuticals. Il15−/− mice were originally provided by Immunex. CD45.1+ and Rag1−/− mice were purchased from the Jackson Laboratory. All mice were from a C57BL/6 background. The littermates of the mutant mice were used as control. All animal experiments were approved by a local ethics committee (the Ethics Committee of the University of Science and Technology of China; Service Vétérinaire Cantonal, Lausanne, Switzerland).

Cell preparations. Thymocyte and splenocyte suspensions were prepared by grinding the organs through mesh filters. IELs were isolated as previously described (Jiang et al., 2010). In brief, Peyer’s patches were removed, and then the small intestine or colon was opened longitudinally and cut into 1-cm-long pieces. After washing the specimens in PBS containing 100 U/ml penicillin and 100 µg/ml streptomycin twice, the pieces were then stirred at 37°C in prewarmed Dulbecco’s modified Eagle’s medium containing 100 U/ml penicillin, 100 µg/ml streptomycin, and 5% FCS for 30 min.

Figure 9. IEL loss contributes to the high susceptibility to TNBS-induced colitis in Nod2−/− mice. (A) Sorted splenic CD8+ T cells or CD8+ IELs from wild-type mice were transferred into Nod2−/− mice to reconstitute IELs, and 3 d later, the numbers of total IELs or indicated IEL subsets were counted and the reconstitution levels were normalized to the corresponding numbers of total IELs or indicated IEL subsets in Nod2+/+ mice. *, P < 0.05; **, P < 0.01. Three mice per group. (B–D) Sorted splenic CD8+ T cells or CD8+ IELs from wild-type mice were transferred into Nod2−/− mice, and 3 d later, the mice were treated with TNBS, and the survival rate (B), H&E staining of colon sections (C), and colitis scores (D) are shown. *, P < 0.05. 13–17 mice per group. One representative experiment out of two is shown. Error bars indicate SEM.

the important role of gut microbiota in the maintenance of IELs. In this study, we demonstrate that gut microbiota-derived products, probably MDP, can signal NOD2 to maintain IELs. Thus, our data provide a new mechanism through which gut microbiota controls the homeostasis of IELs. However, it is unclear which bacterial species are associated with the homeostasis of IELs observed in this study. It will be important to define the commensal bacterial species that elicit this effect.

NOD2 has an important role in host defense against the invasive intracellular pathogen, including L. monocytogenes (Kobayashi et al., 2005); however, little is known whether NOD2 can recognize commensal bacteria. In this study, we demonstrate that Nod2 maintains IELs via recognition of gut microbiota, suggesting the important role of Nod2 in sensing of commensal bacteria. Interestingly, the expression of Nod2 in the intestine is dependent on the presence of gut microbiota (Petnicki-Ocwieja et al., 2009). In summary, our results demonstrate that NOD2 signaling by sensing of gut microbiota is important for maintaining the homeostasis of IELs and suggest that IEL loss may contribute to the impaired innate immunity and higher susceptibility to mucosal damage in Nod2−/− mice and patients with CD.
Figure 10. MDP supplementation reduces the susceptibility of gut microbiota–depleted mice to TNBS–induced colitis. (A and B) Mice were treated with antibiotics (Ab) or MDP or iEDAP (InvivoGen) drinking water at a fixed concentration as previously reported (Reikvam et al., 2011). Mice were supplemented with neomycin sulfate (Sigma-Aldrich), and 1 g/liter metronidazole (Sigma-Aldrich), 500 mg/liter cefuroxime (Sigma-Aldrich), 500 mg/liter vancomycin (Sigma-Aldrich), and 1 g/liter metronidazole (Sigma-Aldrich) in drinking water at a fixed concentration of 1 µM from the age of 2 wk for 4 wk to reconstitute MDP or iEDAP in the intestine.

Colitis model. Induction of TNBS (Sigma-Aldrich) colitis was performed as described previously (Scheiffele and Fuss, 2002). Severity of colitis was assessed using a semiquantitative scoring system (Scheiffele and Fuss, 2002).

Immunofluorescence. Frozen sections of tissues were stained with FITC–CD8α or FITC–TCRβ antibodies (eBioscience) and then mounted using ProLong Gold reagent with DAPI (Invitrogen). Confocal images were obtained using an LSM 700 microscope (Carl Zeiss), and for image analysis we used the LSM software (Carl Zeiss).

ELISA. Tissues were homogenized, and then the lysates were assayed for mouse IL–15 (eBioscience) according to the manufacturer’s instructions.

Adoptive transfer. For prevention of chemical-induced colitis, CD8+ IELs or splenic T cells were isolated from wild-type mice and sorted and then were intravenously transferred into Nod2−/− mice (200 µl PBS/107 cells). 3 d later, the mice were used to analyze IELs or induce colitis using TNBS or DSS.

For BM chimera experiments, Nod2−/− or Nod2+/+ BM cells were intravenously transferred into Rag2−/− mice, or CD45.1+ WT BM cells were intravenously transferred into lethally irradiated (12 Gy given 1 d before adoptive transfer) Nod2−/− or Nod2+/+ mice, or Nod2−/− and Nod2+/+ BM cells were intravenously transferred into lethally irradiated CD45.1+ WT mice. After 8 wk, IELs were analyzed by flow cytometry.

In vivo treatment with IL–15. Mice were injected intraperitoneally with PBS or PBS containing human IL–15 (5 µg/mouse; R&D Systems) once daily for 2 wk. IELs were harvested 24 h after the last injection of IL–15.

Real-time PCR. The Il–15 and Nod2 primers were obtained from SA Biosciences. Real-time PCR using SYBR was performed on a LightCycler (Roche) as previously described (Jiang et al., 2010).

Hematoxylin and eosin (H&E) staining. For histology, colon tissue was fixed in 10% neutral-buffered formalin and embedded in paraffin. 5-µm sections were affixed to slides, deparaffinized, and stained with H&E. Morphological changes in the stained sections were examined under light microscopy (BX53; Olympus).

Statistical analysis. Data are expressed as mean ± SEM. Differences were analyzed by Student’s t test (with 95% confidence interval). P-values <0.05 were considered significant.

The authors have no conflicting financial interests.
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