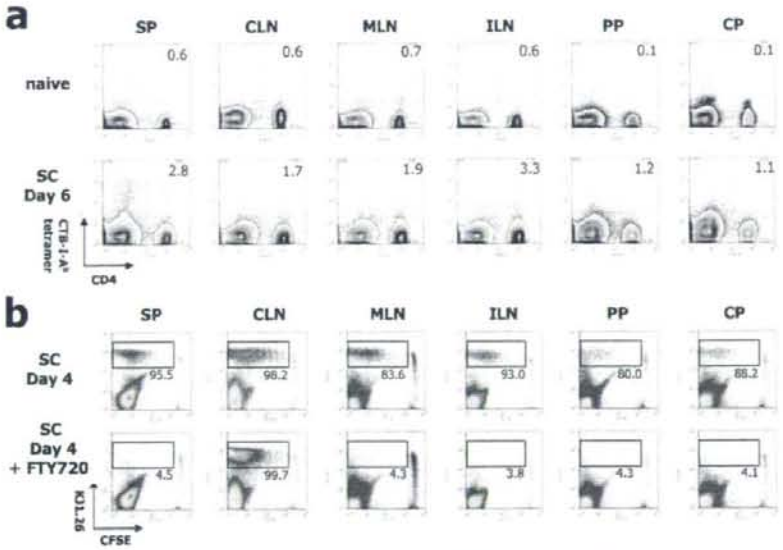

FIGURE 4. Subcutaneous priming alone could induce the increase in Ag-specific CD4$^+$ T cells at mucosal inductive sites. *a*, MNCs isolated from the spleen, CLN, MLN, ILN, PPs, CPs of C57BL/6 mice after s.c. immunization with 1 μg of CT plus 10 μg of TT were stained with CTB-I-A^b tetramer to detect the induction of CTB-specific CD4$^+$ T cells. Numbers represent the percentages of CTB-I-A^b tetramer-positive cells relative to the total number of CD4$^+$ T cells. *b*, Naive BALB/c mice were adoptively transferred with CFSE-labeled OVA-specific TCR transgenic CD4$^+$ T cells of DO11.10 mice and then immunized by s.c. with 1 μg of CT plus 20 μg of OVA with/without FTY 720 treatment. At day 4, the proliferation of OVA-specific TCR transgenic CD4$^+$ T cells was analyzed by detecting the dilution of CFSE dye. The numbers below the boxes indicate the percentages of proliferating populations relative to the total number of OVA-specific TCR transgenic CD4$^+$ T cells.

of functional CXCR4 as well as CCR10 by the Ag-specific IgA ASCs for migration into the colon suggests that they may be of systemic origin and develop into ASCs following s.c. priming.

CPs were the main IgA class switching site after s.c.-IR immunization

To explore the site of class switching into IgA ASCs after s.c.-IR immunization, we checked the molecular markers of IgA class switching recombination (CSR) from the μ-chain to the α-chain. These markers included AID, an essential recombination enzyme for CSR that is strictly expressed by B cells, and Iμ-Cα, the final transcript for IgA production (24). After s.c.-IR immunization, most immune tissues expressed AID, although AID was detected in the spleen, MLN, and CP of mice immunized with IR alone (Fig. 3*a*). In particular, the expression of AID in the CP was much higher after s.c.-IR than after IR alone. The expression of Iμ-Cα was likewise more enhanced in the CP after s.c.-IR than after IR alone. These data suggest that CP might be the major sites of IgA class switching after s.c.-IR immunization.

To confirm that CP was the site of IgA class switching, we used FTY 720 treatment from the day mice received IR boosting following s.c. priming to entrap Ag-specific IgA ASCs at the site of IgA class switching. FTY 720 binds to G protein-coupled sphingosine 1-phosphate type 1 receptors on target cells and inhibits the egress of lymphocytes from lymphoid organs (29). TT-specific IgA ASCs accumulated significantly in the CP but decreased in the lamina propria of the large intestine when treated with FTY 720 (Fig. 3*b*). These results support the premise that systemic priming followed by IR boosting induced IgA isotype class switching in the CP. Collectively, these findings show that CP is the main site of IgA isotype class switching after s.c.-IR immunization, as evidenced by their strong expression of IgA class-switching-related molecules and by the accumulation of Ag-specific IgA ASCs after FTY 720 treatment.

CP might be a major route for Ag delivery to recruit systemically derived cells after s.c.-IR immunization

We initially used CP-null mice to investigate the importance of CP for the induction of IgA ASCs by the s.c.-IR regime. Interestingly, in utero treatment of timed pregnant mice with anti-IL-7Rα mAb completely inhibited both PP and CP formation in the C57BL/6 progeny (data not shown) but only the PP in the BALB/c progeny (16, 30). Thus, we used both PP- and CP-null C57BL/6 and PP-null but CP-intact BALB/c progeny to investigate the role of CP after s.c.-IR. In CP-null C57BL/6 progeny, no IgA or IgG Ab production occurred, whereas the levels of serum IgG and IgA Abs were slightly reduced but still comparable to those in CP-intact wild-type B6 mice (Fig. 3*c*). As expected, no TT-specific IgA and IgG ASCs were observed in the large intestine of CP-null mice, whereas the levels of TT-specific IgG ASCs in the spleen of CP-null mice were similar to those seen in CP-intact wild-type B6 mice. In PP-null but CP-intact BALB/c progeny, high levels of Ag-specific IgA and IgG ASCs were also induced, comparable to those of wild-type BALB/c mice (Fig. 3*d*). These results suggested that PP was not involved in the generation of Ag-specific IgA ASCs in the colon after s.c.-IR together with the exclusion of the unexpected effect after treatment with the anti-IL-7Rα mAb. All together these results demonstrate that CP acts as a critical site for the induction of Ag-specific IgA and IgG ASCs in the large intestine after s.c.-IR and suggest that CP might be the Ag entry site where systemically committed cells infiltrate into the large intestine to initiate IgA class switching.

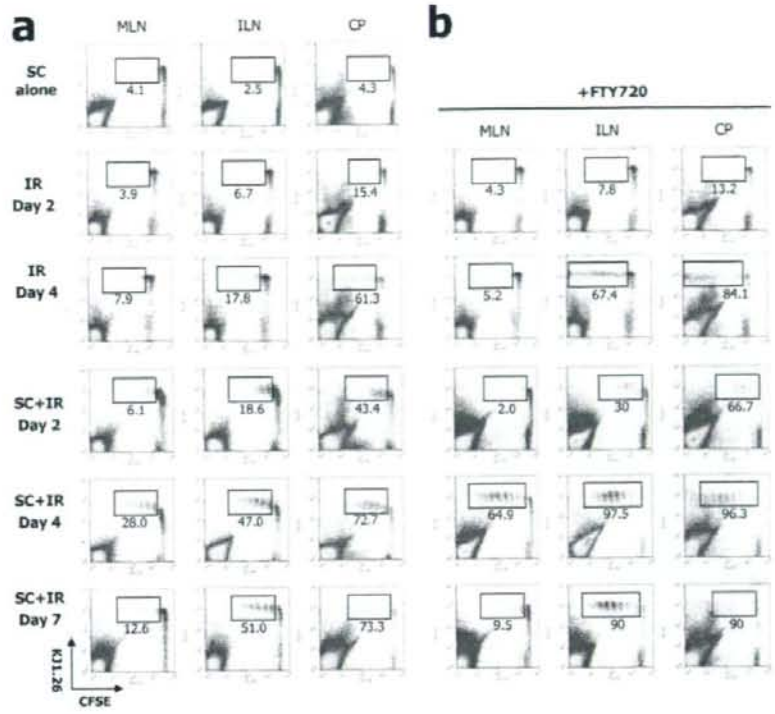

FIGURE 5. s.c. priming could expand Ag-specific $CD4^+$ T cell responses by subsequent IR immunization. *a*, s.c. primed or naive mice were adoptively transferred with CFSE-labeled OVA-specific TCR transgenic $CD4^+$ T cells of DO11.10 mice and then immunized with OVA plus CT by the IR route. *b*, Mice were treated with FTY 720 every other day from the day of IR immunization. At days 2, 4, and 7 after IR immunization, MNCs from MLNs, ILNs, and CPs were stained with KJ1.26-PE specific to DO11.10 TCR. The numbers below the boxes show the percentages of proliferating fractions relative to the total number of OVA-TCR transgenic $CD4^+$ T cells.

s.c. priming could modulate mucosal immune responses by subsequent IR boosting

Our results clearly showed that prior systemic priming could affect the induction of IgA ASCs in the large intestine after IR immunization. We next investigated the effect of s.c. priming on Ag-specific $CD4^+$ T cells in mucosal compartments. At day 6 after s.c. immunization with 1 μg of CT, CTB-specific $CD4^+$ T cells were analyzed in the immune inductive tissues using CTB peptide-I-A^b tetramer staining (Fig. 4*a*). In s.c.-immunized mice, CTB-specific $CD4^+$ T cells were detected in all of the immune tissues. These included draining cutaneous LN (CLN) such as cervical, auxiliary and brachial LN, noncutaneous LN such as MLN and iliac LN (ILN), mucosal inductive sites (PP and CP), as well as spleen. Of interest, CTB-specific $CD4^+$ T cells were most abundant in the ILN, which is known to be a draining LN of colon and genital tract (31) (Fig. 4*a*). Increases in CTB-specific $CD4^+$ T cells in each immune tissue were detected from days 5 to 10 after s.c. immunization (data not shown). However, these $CD4^+$ T cells were not detected in the lamina propria of large intestine, even after IR boosting (data not shown). To definitively confirm this finding by using an alternate detection method, we adoptively transferred mice with CFSE-labeled OVA-specific $CD4^+$ T cells from DO11.10 mice and then immunized them with OVA plus CT via the s.c. route (Fig. 4*b*). DO11.10 $CD4^+$ T cell proliferation was detected in most of the immune tissues and even in the mucosal inductive organs (PP and CP) and the secondary lymphoid organs of the systemic compartment (spleen and LN) at day 4 after s.c. immunization (Fig. 4*b*), confirming the results obtained using CTB-specific $CD4^+$ T cells with CTB peptide-I-A^b tetramer staining (Fig. 4*a*).

To determine whether the presence of Ag-specific $CD4^+$ T cells in unexpected mucosal sites was due to simple dispersion of proliferating Ag-specific $CD4^+$ T cells in the draining LN through the blood circulation, mice were treated with FTY 720 on the day of s.c. immunization to prevent lymphocyte circulation and to encourage accumulation of Ag-specific $CD4^+$ T cells via Ag-bearing DC presentation. Inhibition of lymphocyte egress by FTY 720 treatment clearly showed that the proliferation of Ag-specific $CD4^+$ T cells was restricted to the CLN (Fig. 4*b*). These results suggest that Ag-specific $CD4^+$ T cells generated in the CLN by s.c. priming were disseminated via the blood circulation to nondraining lymphoid tissues and inductive sites in the mucosal compartments.

Next, we examined how s.c. priming affects the responsiveness of naive $CD4^+$ T cells to subsequent IR immunization. At day 14 after s.c. priming with CT plus OVA, CFSE-labeled DO11.10 $CD4^+$ T cells were adoptively transferred into the systemically primed BALB/c recipients; the following day, mice were immunized by IR with and without FTY 720 treatment. In the absence of boosting, no proliferation of DO11.10 $CD4^+$ T cells was noted in s.c.-primed recipient mice transferred with these same cells (Fig. 5*a*). However, after IR boosting, DO11.10 $CD4^+$ T cells began to proliferate at day 2, proliferated briskly at day 4 and continued to proliferate in the ILN and CP until day 7. Interestingly, the proliferation of DO11.10 $CD4^+$ T cells in the MLN appeared at day 4 and prominently decreased at day 7. In the other immune tissues, such as the spleen, CLN, and PP, no Ag-specific proliferation was found (data not shown). Similar data were also obtained after FTY 720 treatment (Fig. 5*b*), demonstrating that the proliferation of Ag-specific $CD4^+$ T cells after s.c.-IR was initiated by Ag-presentation of APC and not via passive dispersion by blood circulation. In contrast, IR immunization in the absence of s.c. priming produced varying results: slight induction of Ag-specific $CD4^+$

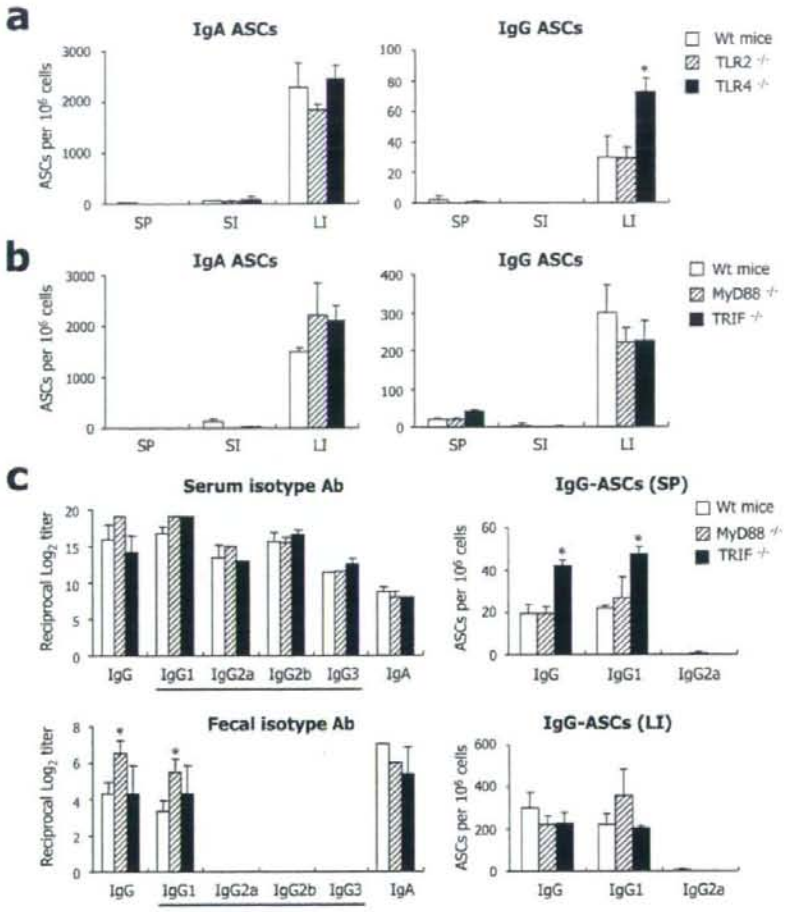

FIGURE 6. Innate immunity may not direct the cross-talk between the large intestine and systemic compartments following s.c.-IR immunization. To examine the involvement of TLR signaling for the induction of Ag-specific ASCs in the large intestine following s.c.-IR immunization, TT-specific IgA and IgG ASCs were measured in TLR2$^{-/-}$ and TLR4$^{-/-}$ mice (*a*) and MyD88$^{-/-}$ and TRIF$^{-/-}$ mice (*b*) at day 7 after s.c.-IR. *c*, The levels of IgG isotype Abs in the serum and fecal extracts, as well as the isotype of IgG ASCs in the spleen and the lamina propria of the large intestine, were assessed in wild-type BALB/c, MyD88$^{-/-}$, and TRIF$^{-/-}$ mice at day 7 after s.c.-IR immunization. *, $p < 0.05$ by ANOVA test.

T cell proliferation in the ILN but significantly more in the CP at day 4. However, the proliferation intensity in the ILN and CP was less than that in s.c.-primed mice, regardless of FTY 720 treatment. IR immunization induced Ag-specific CD4$^+$ T cell responses in the MLN of s.c.-primed mice but not of naive mice, an interesting finding because MLN has been proposed to be the border between the mucosal compartment and the systemic immune systems (32). These results reveal that s.c. priming could set the stage for a quick and profound response to subsequent IR immunization.

Dramatic increase in IgA ASCs in the large intestine after s.c.-IR immunization was independent of TLR signaling by commensal flora

Recent observations (33, 34) regarding the involvement of TLR signaling on Ab secretion led us to examine the induction of IgA and IgG ASCs after s.c.-IR in TLR-deficient mice. Continuous TLR2 and TLR4 signaling exists in microenvironment of the large intestine where commensal bacteria are extremely abundant. We immunized TLR2$^{-/-}$ and TLR4$^{-/-}$ mice by s.c.-IR with CT plus TT and then determined the numbers of IgA and IgG ASCs. Comparable levels of TT-specific IgA ASCs were elicited in the large intestine of wild-type BALB/c, TLR2$^{-/-}$, and TLR4$^{-/-}$ mice (Fig. 6*a*). Moreover, the numbers of TT-specific IgG ASCs were somewhat increased in the TLR4$^{-/-}$ mice. To further confirm the independence of TLR signaling on s.c.-IR-induced responses, we used MyD88$^{-/-}$ and TRIF $^{-/-}$ mice. In both MyD88$^{-/-}$ and TRIF$^{-/-}$ mice, the generation of TT-specific IgA and IgG ASCs in the large intestine was not impaired (Fig. 6*b*). We checked the levels of IgG isotype Abs in the serum and fecal extracts and of IgG isotype ASCs in the spleen and large intestine because a recent study proposed that TLR signaling in Ab responses depends on Ab isotype (33). The levels of serum IgG, IgG1, IgG2a, IgG2b, IgG3, and IgA Abs from MyD88$^{-/-}$ and TRIF$^{-/-}$ mice after s.c.-IR immunization were quite similar to those of wild-type mice (Fig. 6*c*). However, there were slightly more IgG and IgG1 ASCs in the spleen of TRIF$^{-/-}$ mice. The IgG and IgA Ab levels from fecal extracts were also not impaired in the TLR signaling deficient mice. Instead, MyD88$^{-/-}$ mice but not TRIF$^{-/-}$ mice induced higher levels of IgG Abs in the fecal extract. IgG1 was the major isotype of IgG secreted into the spleen and large intestine, reflecting CT-enhanced dominant Th2-type responses (35). Taken together, these data suggest that the dramatic increase in Ag-specific IgA and IgG ASCs seen in the colon after s.c.-IR immunization occur independent of innate signaling.

Discussion

The mucosal immune tissues have long been considered to be highly compartmentalized and functionally independent from the systemic immune tissues (1, 5). The mucosa-associated lymphoid tissues are populated by T cell, B cell, and accessory cell subpopulations that are phenotypically and functionally distinct from systemic lymphoid tissues. Immune cell recirculation within mucosal tissues is tightly regulated by the common mucosal immune system (1). Once activated at a mucosal site, immune cells are disseminated via the blood circulation and, with the help of distinct chemokines and integrins within the mucosal compartment, migrate to remote mucosal tissues and not to systemic sites (2). However, our group has suggested the possibility of cross-talk between systemic immune tissues and some mucosal tissues such as the large intestine (14, 15). The results presented here also show that systemic priming can accelerate and modulate the immune responses induced by targeting the large intestine but not the small intestine. Interestingly, after IgA isotype class switching in the CPs of the large intestine, the $CXCR4^+$ and/or $CCR10^+$ Ag-specific IgA ASCs induced by s.c.-IR were distinct from the IR-induced $CCR10^+$ IgA ASCs, which acted independently of TLR signaling.

A previous study (26) demonstrated that IgG ASCs tend to traffic to the bone marrow or inflammatory sites irrespective of their site of induction, whereas IgA ASCs arising in mucosal lymphoid tissues migrate into the lamina propria of gastrointestinal, respiratory and urogenital tissues via induction site-specific traffic patterns. The interactions of chemokines and their receptors coordinate the migration and tissue localization of plasma cells. MEC/CCL28 attracts IgA ASCs present in both intestinal and nonintestinal mucosal tissues (28), whereas TECK/CCL25 only attracts a subpopulation of IgA ASCs associated with the small intestine (22). Furthermore, IgA ASCs in the small intestine express CCR9, CCR10, and CXCR4, whereas IgA ASCs in the colon mainly express CCR10 and CXCR4 (36). Reciprocally, the epithelial cells of the small intestine produce TECK and MEC, whereas those of the colon secrete MEC and SDF-1α (36). These findings suggest that IgA ASCs homing into the small intestine are guided by TECK and MEC, whereas those homing into the colon are guided by MEC and SDF-1α. Interestingly, SDF-1α is also constitutively expressed by bone marrow endothelial and stromal cells (37), as well as by dendritic and endothelial cells of skin (38). CXCR4 and its ligand CXCL12 have been shown to be critically involved in the localization of plasma cells within the spleen and LN as well as in their homing to bone marrow. The results presented here reveal that the Ag-specific IgA ASCs induced in the colon by systemic priming followed by IR boosting have different chemokine receptor usages than those induced by IR immunization alone (the latter attracting only MEC). Thus, $CCR10^+$ and/or $CXCR4^+$ Ag-specific IgA ASCs in the large intestine may originate from systemically committed cells and be attracted to colon expressing their cognate ligands. In contrast, IgG ASCs in the large intestine are controlled by a completely different mechanism than the IgA ASCs. The differentiation of IgG plasmablasts is correlated with up-regulated expression of CXCR3; these migrate to the inflamed tissue regardless of induction site (26). CXCR3 ligands including MIG/CXCL9 and 10 kDa IFN-γ-induced protein CXCL10 are widely expressed by the endothelium and other cells in inflamed tissues. It remains to be seen whether CXCR3 is involved in the migration of Ag-specific IgG ASCs into the large intestine or whether there are other mechanisms.

Our results show that IR boosting induces dramatic increases in Ag-specific Ab in the large intestine whereas oral boosting did not do so in the small intestine. We propose several hypotheses to explain this localized phenomenon.

First, differences in the microbial environment may favor the development of colonic inflammation, because the large intestine contains by far the highest number of commensal bacteria; however, our results indicate that the brisk increase in Ag-specific ASCs in the large intestine is independent of innate microflora-activated MyD88 and TRIF signaling.

Second, the mucosal immune environments of the small and large intestine differ. A recent study showed that mice lacking PPs in the small intestine are more susceptible to the onset of OVA-induced allergic diarrhea, suggesting that PPs, as the site where IL-10-producing Treg cells are created, play a role in the regulation of mucosal immunity (30). In addition, the frequency of $CD4^+$ and $\alpha\beta$ T cells as well as expression of LFA-2 and L-selectin is higher in the large intestine than in the small intestine (39). In our study, even direct injection into the lumen of the jejunum via bypass of the stomach could not induce Ag-specific IgA Ab responses. All of these findings suggest that the small intestine, perhaps through the mediation of the MLNs and/or PPs, may play a more significant role in the regulation of mucosal immunity than the large intestine. However, we found no significant differences in the frequencies of $CD4^+CD25^+FoxP3^+$ Treg cells and $CD3^+NK1.1^+$ NKTs in the PPs and CPs of naive and s.c.-IR immunized mice (data not shown). Despite possessing similar numbers of $CD25^+FoxP3^+$ Treg cells, CP may possess less regulatory function than PP. Furthermore, MLNs in addition to PP play a decisive role in the induction of oral tolerance (25, 40). Overall, it is plausible that ILN, the main draining LN of the large intestine (31), have less regulatory function than MLN against exogenous Ags. However, as of yet, we have been unable to verify this hypothesis.

Third, a specific cross-talk immune pathway may exist between the systemic compartments and the large intestine. Indeed, we previously found that systemically primed splenic $CD4^+$ T cells are preferentially recruited into the large intestine (14). Our current data also demonstrate the induction by s.c.-IR immunization of CXCR4- and/or CCR10-expressing IgA ASCs, which are considered to be systemically committed cells, and the modulation of naive $CD4^+$ T cell responses in the s.c. primed mice by subsequent IR boosting. These findings suggest the existence of a unique cross-talk immune pathway between systemic immune compartments and the large intestine. Taken together with the brisk increase in Ag-specific ASCs in the large intestine after s.c.-IR immunization, these findings could partially elucidate the model of cross-talk between systemic tissues and the large intestine. It is even possible that the large intestine has two faces dependent and independent on the common mucosal immune system.

A topic of considerable recent research activity is the question of whether B cell responses are controlled by TLR signaling. Using $MyD88^{-/-}$ mice, Pasare and Medzhitov (33) demonstrated that generation of T cell-dependent Ag-specific Ab responses requires activation of TLRs in B cells. Only Abs of certain isotypes require TLR signaling; the IgM and IgG1 isotypes are largely, but not completely, TLR dependent; IgG2 isotypes are entirely TLR dependent; and IgE and IgA responses are TLR independent. However, using $MyD88^{-/-}TRIF^{-/-}$ mice, which are lacking in TLR signaling, another group showed that robust Ag-specific B cell responses could be elicited after vaccination with adjuvants even in the absence of TLR signals (34). In addition, MyD88-dependent signaling pathways in B cells are essential for generating long-term humoral immunity; however, antiviral Ab responses to a live virus infection were effectively initiated in the absence of MyD88-mediated signaling (41). The large intestine is constantly confronted

with enormous numbers of commensal bacteria and pathogens. The gut epithelium, with its ability to sense commensal microflora, maintains the gut homeostasis and prevents detrimental chronic inflammatory diseases and the initiation of host defense mechanisms against pathogens. Innate signaling has, therefore, been thought key to maintaining homeostasis in the large intestine. Nevertheless, our results reveal that the dramatic increase in Ag-specific IgA and IgG ASCs in the large intestine by s.c.-IR immunization can be obtained in TLR2$^{-/-}$, TLR4$^{-/-}$, as well as in MyD88$^{-/-}$ and TRIF$^{-/-}$ mice, suggesting that TLR activation, a type of innate signaling, is not required for the induction of those responses.

In conclusion, our study provides new evidence showing that the cross-talk between the systemic immune compartments and the large intestine via the CP is independent of innate immunity. The large intestine has unique features that distinguish it from general mucosal tissues, such as the small intestine, and which may render the colon more susceptible to activate T and B cells. Further characterization of the large intestine will be critical to understanding its relationship with systemic compartments and to developing an effective and safe mucosal vaccine with synergic effects with preexisting immunity.

Disclosures

The authors have no financial conflict of interest.

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Sphingosine 1-phosphate–dependent trafficking of peritoneal B cells requires functional NFκB-inducing kinase in stromal cells

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We previously reported that sphingosine 1-phosphate (S1P) regulates peritoneal B-cell trafficking and subsequent intestinal IgA production, but the underlying mechanisms remain obscure. We demonstrate here that nuclear factor κB–inducing kinase (NIK) is involved in the regulation of S1P-mediated trafficking of peritoneal B cells. Although peritoneal B cells from NIK-mutated alymphoplasia *(aly)* mice expressed type 1 S1P receptor ($S1P_1$) at comparable levels and demonstrated normal migration toward S1P, *aly* peritoneal B cells showed decreased sensitivity to FTY720, an $S1P_1$ modulator. NIK-mutated stromal cells showed decreased levels of adhesion molecules (VCAM-1 and ICAM-1) and increased CXCL13 expressions, leading to impaired ability to support S1P-mediated emigration, but not immigration, of peritoneal B cells. Therefore, *aly* peritoneal B cells exhibited normal S1P-mediated peritoneal B-cell trafficking from peritoneum to intestine for IgA production when they were transferred into severe combined immunodeficient or wild-type mice. However, S1P-mediated emigration of wild-type B cells from the *aly* peritoneal cavity was impaired without affecting their immigration from the blood. Further, transfer of wild-type stromal cells into the peritoneum restored S1P-mediated trafficking of *aly* peritoneal B cells. These findings suggest that NIK in stromal cells has a specific role in the regulation of S1P-mediated trafficking of peritoneal B cells. (Blood. 2008;111:4646-4652)

Introduction

The peritoneal cavity contains numerous B cells, especially B1 cells, which play important roles in protective immunity in the peritoneal cavity and in the production of secretory IgA antibody (S-IgA) in the intestine.[1,2] Accumulating evidence has demonstrated that the trafficking of peritoneal B cells is regulated by chemokines (eg, CCL19, CCL21, and CXCL13), cytokines (eg, interleukin [IL]-10), and adhesion molecules (eg, integrins) at various points in the immigration, retention, and emigration of these cells.[3-8]

Sphingosine 1-phosphate (S1P) is another key molecule in the regulation of lymphocyte trafficking.[9,10] Among the 5 types of S1P receptors, type 1 S1P receptor ($S1P_1$) is preferentially expressed on lymphocytes and is required for their emigration from secondary lymphoid organs and the thymus.[11,12] FTY720 is an agonist for S1P receptors, except type 2 S1P receptor ($S1P_2$), and blocks S1P-mediated signaling by inducing internalization of S1P receptors.[12-16] Therefore, treatment with FTY720 decreases the number of circulating lymphocytes in both blood and lymph by inhibiting their emigration from secondary lymphoid organs and the thymus.[12-16]

In addition to its role in the systemic immune compartments, we recently reported that S1P is involved in the trafficking of mucosa-associated immunocompetent cells, including peritoneal B cells, intraepithelial T lymphocytes, and intestinal mast cells.[17-19] In those studies, we showed that peritoneal B1 and B2 cells expressed comparable levels of $S1P_1$ and that FTY720 treatment impaired trafficking of peritoneal B cells into the intestine by enhancing their emigration from the peritoneal cavity and by inhibiting their immigration from blood into the peritoneal cavity. The FTY720-associated disruption of peritoneal B-cell trafficking into the intestine was associated with impaired intestinal immunoglobulin A (IgA) production by peritoneal B cells.[17] These findings provide strong evidence that S1P plays an essential role in the regulation of peritoneal B-cell trafficking into the intestine and subsequent intestinal IgA production.

Alymphoplasia *(aly)* mice carry a point mutation in nuclear factor κB–inducing kinase (NIK), leading to the inability to bind to IκB kinase α, a molecule essential for nuclear factor κB (NFκB) activation.[20-22] *aly* mice lack lymph nodes and Peyer patches; they have impaired development of the spleen and thymus and showed accumulation of peritoneal B1 cells.[20,23] Because the B1 and B2 cells that originate from the peritoneal cavity and Peyer patches are the primary sources of intestinal IgA,[1,2] the immunologic defects in *aly* mice almost completely ablate intestinal IgA production.[23] A previous study revealed that the impaired peritoneal B-cell trafficking in *aly* mice was, at least in part, due to the defect of signaling pathway coupling with G-proteins, such as chemokine receptors, in lymphocytes.[23] A subsequent study revealed that the impaired function of stromal cells in *aly* mice was also attributable to the defective trafficking of bone marrow–derived naive IgM^+ IgA^- B cells to the intestine.[24]

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Given the defective G-protein signaling pathways in *aly* mice,[23] and the fact that S1P receptors couple to G proteins,[25] our findings led us to hypothesize that the defective peritoneal B-cell trafficking and consequent impaired intestinal IgA production in *aly* mice might be mediated by S1P. We therefore sought here to investigate the interaction between NIK- and S1P-mediated pathways in peritoneal B-cell trafficking and subsequent intestinal IgA production. Our findings provide new evidence that NIK-mediated signaling in stromal cells regulates S1P-mediated trafficking of peritoneal B cells, especially their emigration from the peritoneal cavity.

Methods

Mice, FTY720 treatment, and cell isolation

Female C57BL/6, *aly*, and severe combined immunodeficient (SCID) mice (7-9 weeks) were purchased from Japan Clea (Tokyo, Japan). All mice were maintained in horizontal laminar flow cabinets and provided with sterile food and water ad libitum. For FTY720 treatment, mice were injected intraperitoneally with FTY720 (1 mg/kg; Novartis Pharma, Basel, Switzerland).[17-19] Peritoneal cells were obtained by flushing the peritoneum with 8 mL ice-cold phosphate-buffered saline (PBS).[17] All experiments were approved by the Animal Care and Use Committee of the University of Tokyo and conducted in accordance with its guidelines.

Flow cytometry and cell sorting

A standard protocol was used for flow cytometric analysis and cell sorting.[17-19] Cells were incubated with anti-CD16/32 antibody (Ab; BD Biosciences, San Diego, CA) and then stained with the appropriate fluorescent-conjugated Abs specific for B220, CD11b, ICAM-1, and VCAM-1 (BD Biosciences). Viaprobe (BD Biosciences) was used to discriminate between dead and live cells. Flow cytometric analysis and cell sorting were performed with FACSCalibur (BD Biosciences) and FACSAria (BD Biosciences), respectively.

In vitro migration assay

In vitro migration assays using purified B1 and B2 cells were performed according to a previously established method.[12] Briefly, peritoneal B cells were applied to the upper chambers (pore diameter, 5 μm; Invitrogen, Carlsbad, CA) and 0, 20, 200, or 2000 nM S1P was added to the lower wells. After a 6-hour incubation, the B cells that had migrated into the lower wells were counted with the aid of trypan blue staining.

RT-PCR

To measure mRNA expression for $S1P_1$, quantitative and conventional reverse-transcription–polymerase chain reaction (RT-PCR) using LightCycler (Roche Diagnostics, Mannheim, Germany) were performed.[17-19] Briefly, total RNA was isolated using TRIzol reagent (Invitrogen), and cDNA was synthesized using Powerscript reverse transcriptase (BD Biosciences). The oligonucleotide primers and probes specific for $S1P_1$ (forward primer, TACACTCTGACCAACAAGGA; reverse primer, ATAATGGTCTCTGGGTTGTC; FITC-probe, TGCTGGCAATTCAAGAGGCCCATCATC; LCRed 640-probe, CAGGCATGGAATTTAGCCGCAGCAAATC), glyceraldehyde-3-phosphate dehydrogenase (GAPDH; forward primer, TGAACGGGAAGCTCACTGG; reverse primer, TCCACCACCCTGTTGCTGTA; FITC-probe, CTGAGGACCAGGTTGTCTCCTGCGA; LCRed 640-probe, TTCAACAGCAACTCCCACTCTTCCACC), CCL19 (forward primer, GCCAAGAACAAAGGCAACA; reverse primer, CACACTCACATCGACTCTCTA), CCL21 (forward primer, ACAGACACAGCCCTCAA; reverse primer, CATGAGGTGGCTGCTTT), and CXCL13 (forward primer, GAACAGGCATTTAGTGACAAC; reverse primer, TTTTGGAAGCCTGCGTTTT) were designed and synthesized by Nihon Gene Research Laboratory (Sendai, Japan).[26]

Adoptive cell transfer

For tracing cells in vivo, peritoneal B cells (10^7 cells) were incubated with 0.25 μM 5-(and-6)-carboxyfluorescein diacetate, succinimidyl ester (CFSE; Molecular Probes, Eugene, OR) in the dark for 10 minutes at 37°C and then were washed twice with PBS according to a previously described method.[17,18] Labeled B cells (5×10^6 cells) were transferred into recipient mice intraperitoneally or intravenously; FTY720 was administered intraperitoneally simultaneously. After 12 hours, peritoneal cells were collected for flow cytometric analysis.

For experiments involving stromal cell transfer, stromal cells were isolated from the small intestines of wild-type (WT) mice as previously described.[27] Briefly, cells were isolated from intestinal lamina propria and cultured on 10-cm culture plates in complete RPMI1640 medium. After 1 hour, nonadherent cells were removed by washing with PBS, and remaining adherent cells were cultured overnight in complete RPMI1640 medium. After overnight culture, the plates were washed with PBS, and remaining adherent cells were cultured in complete RPMI1640 medium. After 2 rounds of subculture, confluent cells were used as stromal cells and transferred into the peritoneal cavities of *aly* mice (10^7 cells per mouse). Two weeks after transfer, mice were treated with FTY720 for analysis of peritoneal B-cell trafficking. To analyze Ab production from peritoneal B cells, SCID mice were adoptively transferred with peritoneal B cells (5×10^6 cells per mouse) via the intraperitoneal route and treated with FTY720 every 2 days for 2 weeks. Two weeks after adoptive transfer, fecal extracts were collected for the measurement of total IgA levels by enzyme-linked immunosorbent assay (ELISA).

Measurement of fecal IgA by ELISA

The concentration of fecal IgA was determined by ELISA as previously described.[17] Purified murine IgA Ab (BD PharMingen, San Diego, CA) was used as a standard for the quantification. After blocking of coated anti–mouse Ig Ab (Southern Biotechnology Associates, Birmingham, AL) with 5% bovine serum albumin in PBS, diluted fecal extract was added and incubated in the coated wells for 2 hours at room temperature. Bound Ab was quantified using HRP-conjugated anti–mouse IgA (Southern Biotechnology Associates) and 3,3′5,5′-tetramethylbenzidine (Moss, Pasadena, CA), as previously described.[17]

Statistics

The results were compared using the Student *t* test or Welch *t* test. *P* value of less than .05 was considered statistically significant.

Results

Decreased sensitivity to FTY720 in *aly* mice

To test whether the defective trafficking of peritoneal B cells in *aly* mice was attributable to a dysfunctional S1P-mediated pathway, we compared the effect of FTY720 on peritoneal B cells in *aly* mice with those in WT mice. Consistent with our previous results,[17] a single injection of FTY720 induced rapid reductions in the percentages and absolute cell numbers of peritoneal B1 and B2 cells in WT mice (Figure 1A). In contrast, *aly* mice showed scant reduction in peritoneal B cells after a single injection of FTY720 (Figure 1B). To elucidate whether the FTY720 reactivity of *aly* mice was complete or partial, mice were injected repeatedly with FTY720 and their peritoneal B cells were examined. In agreement with our previous results,[17] treating WT mice with multiple injections of FTY720 did not increase its effect on peritoneal B cells, such that B1- and B2-cell counts and percentages were similar to those of the single-treatment group (Figure 1A). In contrast, repeated, but not single, FTY720 treatment significantly ($P < .05$) reduced the peritoneal B1- and B2-cell populations of *aly* mice (Figure 1B). These findings suggest that the peritoneal B cells

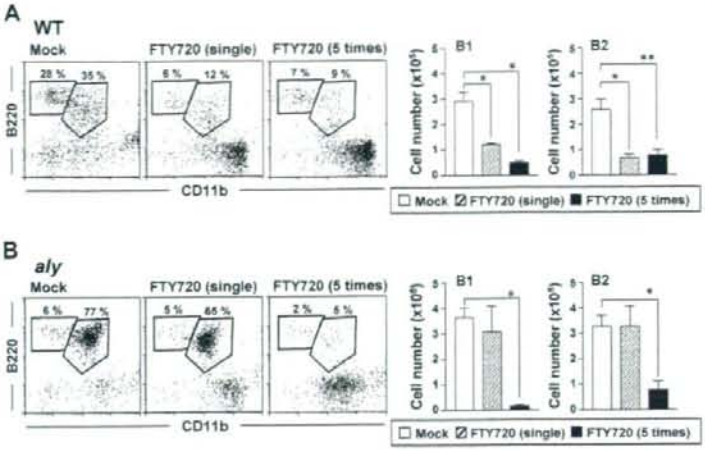

Figure 1. Decreased reactivity of peritoneal B cells to FTY720 in *aly* mice. Cells were isolated from the peritoneal cavities of WT (A) or *aly* (B) mice 12 hours after single or multiple (that is 5) injections of FTY720 (right) or vehicle only (mock; left), and cell populations were analyzed by flow cytometry. The data are representative of at least 4 independent experiments. The numbers of B220$^+$CD11b$^+$ B1 cells and B220$^+$CD11b$^-$ B2 cells were calculated from the total cell number and flow cytometric data. Data are presented as mean plus or minus SEM ($n = 4$). $^*P < .01$; $^{**}P < .05$.

of *aly* mice showed reduced sensitivity but are still reactive to FTY720.

Normal $S1P_1$ expression and migration to S1P in *aly* peritoneal B cells

We hypothesized that the decreased reactivity of *aly* peritoneal B cells to FTY720 was due to their minimal expression of S1P receptors, especially $S1P_1$, whose type was exclusively expressed on peritoneal B cells.[17] To test this hypothesis, we performed quantitative RT-PCR analysis and found that the levels of $S1P_1$ in peritoneal B1 and B2 cells and splenic B cells were comparable between *aly* and WT mice (Figure 2A). Together with a previous report indicating that NIK-mediated signaling is linked to the same G-coupled protein that S1P receptors use,[25] our results suggested that NIK mutation abolished $S1P_1$-mediated signaling. To test whether $S1P_1$-mediated signaling in *aly* peritoneal B cells was functional, we investigated the in vitro migration of peritoneal B cells toward S1P. We found that, like the peritoneal B cells isolated from WT mice, both peritoneal B1 and B2 cells from *aly* mice migrated to the gradient of S1P (Figure 2B). These data indicated that the reduced reactivity to FTY720 in *aly* mice was not due to a defect in $S1P_1$ expression or $S1P_1$-mediated signaling.

To further examine whether *aly* peritoneal B cells show normal reactivity to S1P in vivo, we isolated *aly* peritoneal B cells, labeled them with CFSE, and adoptively transferred them into SCID mice that were treated concurrently with FTY720 to disrupt S1P-mediated signaling. Because our recent study demonstrated that FTY720 inhibited B-cell immigration into the peritoneal cavity and enhanced their emigration from it,[17] we transferred the labeled B cells through 2 different routes, intraperitoneal and intravenous injection. When *aly* peritoneal

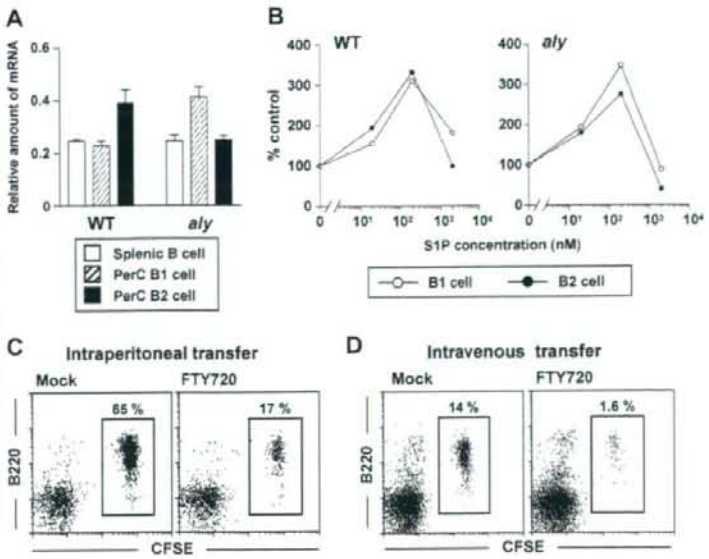

Figure 2. Similar expression of $S1P_1$ and biologic reactivity to S1P among various populations of *aly* peritoneal B cells. (A) Quantitative RT-PCR analysis for $S1P_1$ expression was performed using RNA isolated from sorted splenic B (□), and peritoneal B1 (▨) and B2 (■) cells. The relative quantity of specific mRNA encoding $S1P_1$ was expressed as a ratio to GAPDH. Data are expressed as the mean plus or minus SD of 4 mice. (B) In vitro migration assay was performed with peritoneal B1 (○) and B2 (●) B cells purified from WT (left) and *aly* (right) mice. Peritoneal B cells were added to the upper chamber of a transwell plate in the presence of 0, 20, 200, or 2000 nM S1P in the lower chamber. Six hours later, the number of cells that had migrated into the lower chamber were counted. Data are representative of 3 independent experiments. (C,D) CFSE-labeled *aly* peritoneal B cells were adoptively transferred into SCID mice by the intraperitoneal (C) or intravenous (D) routes: reconstituted SCID mice were treated simultaneously with (right panels) or without (left panels) FTY720. After 12 hours, cells were isolated from the peritoneal cavity for the analysis of CFSE$^+$ B220$^+$ cells. Data are representative of 4 independent experiments.

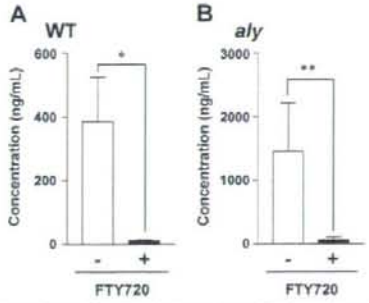

Figure 3. *aly* peritoneal B cells show a comparable dependence on S1P for intestinal IgA production. WT (A) or *aly* (B) peritoneal B cells (5×10^6 cells) were adoptively transferred into SCID mice, which were treated with vehicle only (□) or FTY720 (■) every 2 days. Two weeks after cell transfer, fecal extracts were collected for measurement of IgA levels by ELISA. Data are presented as means plus or minus SEM (n = 4). $^*P < .01$; $^{**}P < .05$.

B cells were adoptively transferred into the peritoneal cavities of SCID mice, FTY720 treatment resulted in a marked reduction in B-cell numbers, suggesting that, as with WT B cells, FTY720 enhanced the emigration of *aly* B cells from the peritoneal cavity (Figure 2C). Further, immigration of *aly* B cells from the blood into the peritoneal cavity was impaired when SCID mice were treated with FTY720 after the intravenous transfer of *aly* peritoneal B cells (Figure 2D). Taken together with the new in vitro and in vivo data, our findings convincingly show that *aly* peritoneal B cells can react to S1P and FTY720. However, the sensitivity to FTY720 is lower in *aly* mice than in WT mice.

A previous study demonstrated that S1P lyase, which degrades S1P to phosphoethanolamine, is abundant in secondary lymphoid organs, thus establishing a S1P gradient with lower concentrations in the secondary lymphoid organs than in the blood and lymph.[28] These findings suggested to us that the lack of secondary lymphoid organs in *aly* mice might contribute to their decreased sensitivity to FTY720 owing to the presence of an impaired S1P gradient. But some evidence obtained in our study disproved this hypothesis. We found that $Id2^{-/-}$ mice, which lacked secondary lymphoid organs due to deficiency of a negative regulator of basic helix-loop-helix transcription factors, showed normal sensitivity to FTY720, and that disruption of the S1P gradient by oral feeding of deoxypyridoxine, an inhibitor of S1P lyase, did not affect the peritoneal B-cell trafficking in WT mice (J.K., unpublished data, January 2007). Taken together with a previous report that lymphoid organs were not required for S1P-mediated trafficking of peripheral lymphocytes,[29] these findings suggest that the impaired reactivity of *aly* mice to FTY720 is not attributable to their defective secondary lymphoid organ structure.

Normal S1P-mediated trafficking of *aly* peritoneal B cells for the intestinal IgA production

Peritoneal B cells are primarily sources of intestinal IgA production,[1,2] and we previously demonstrated that S1P mediates the production of intestinal IgA by peritoneal B cells.[17] Therefore, we next examined whether *aly* peritoneal B cells could produce intestinal IgA in an FTY720-sensitive manner. We addressed this issue by investigating intestinal IgA production in SCID mice adoptively transferred with *aly* peritoneal B cells. Consistent with our previous findings,[17] SCID mice that received WT peritoneal B cells produced considerable amounts of intestinal IgA, and FTY720 treatment inhibited this production (Figure 3A). Similar induction of intestinal IgA production occurred when SCID mice were reconstituted with *aly* peritoneal B cells (Figure 3B), and, as seen after transfer of WT peritoneal B cells, FTY720 abolished intestinal IgA production in the mice that received *aly* peritoneal B cells (Figure 3B). These data indicate that *aly* B cells migrate normally into the intestine and subsequently produce intestinal IgA in an FTY720-sensitive manner in SCID mice. In light of these data, NIK in B cells seems to be redundant in the S1P-mediated trafficking of peritoneal B cells and subsequent intestinal IgA production. Therefore, the impaired sensitivity of *aly* mice to FTY720 is not due to defective S1P-mediated signaling in B cells.

NIK-mediated pathway in non-B cells is essential for the sensitivity of S1P-mediated peritoneal B-cell emigration but not immigration

In light of our current findings that *aly* peritoneal B cells react to FTY720 and S1P, we hypothesized that their decreased sensitivity to FTY720 was due to the NIK mutation in the non-B cells. We therefore examined the FTY720 reactivity of WT peritoneal B cells adoptively transferred into SCID and *aly* mice. Regardless of the injection route, WT peritoneal B cells showed normal reactivity to FTY720 and thus were decreased after treatment with FTY720 when they were adoptively transferred into SCID mice (Figure 4A,B). In contrast, WT peritoneal B cells transferred intraperitoneally into *aly* mice lacked reactivity to FTY720. Indeed, the numbers

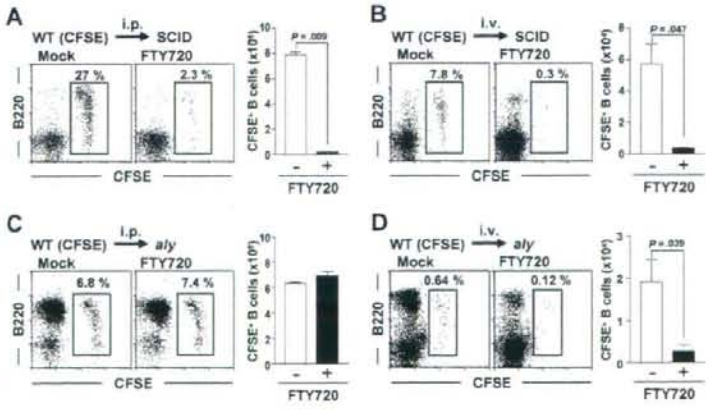

Figure 4. NIK-mediated signaling in non-B cells controls S1P-mediated peritoneal B-cell emigration of peritoneal B cells, but not their immigration. (A,B) Peritoneal B cells were isolated from WT mice, labeled with CFSE, and adoptively transferred via the intraperitoneal (i.p.) (A) or intravenous (i.v.) (B) routes into SCID mice. (C,D) Similarly, CFSE-labeled peritoneal WT B cells were transferred into *aly* mice from which peritoneal cells were removed 8 hours before transfer. The reconstituted mice were treated simultaneously with (right panels) or without (left panels) FTY720. After 12 hours, cells were isolated from the peritoneal cavity for the analysis of $CFSE^+$ $B220^+$ cells. Flow cytometric data are representative of 3 independent experiments and are presented as means plus or minus SEM (n = 3).

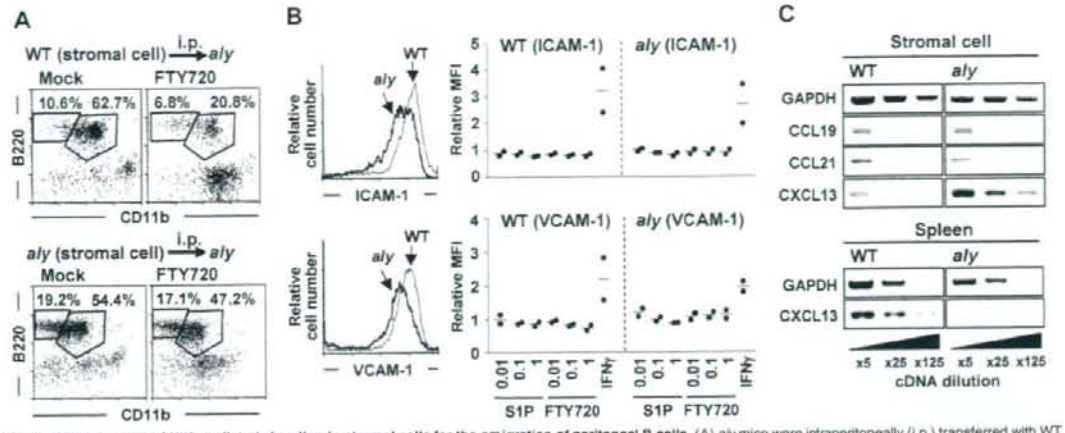

Figure 5. Requirement of NIK-mediated signaling in stromal cells for the emigration of peritoneal B cells. (A) *aly* mice were intraperitoneally (i.p.) transferred with WT (top panels) or *aly* (bottom panels) stromal cells. Two weeks after cell transfer, mice were treated with FTY720 for the analysis of peritoneal B-cell populations. Flow cytometric data are representative of 3 independent experiments and are presented as means plus or minus SEM (n = 3). (B) Expression of ICAM-1 (top panels) and VCAM-1 (bottom panels) on WT (thin lines) and *aly* (thick lines) stromal cells was determined by flow cytometry (left). Twenty-four hours after treatment of stromal cells with various concentrations of S1P, FTY720, or IFNγ (50 units/mL), expression of ICAM-1 and VCAM-1 was determined by flow cytometry. Relative mean fluorescence intensity (MFI) was expressed as a ratio to MFI of untreated cells. Data are representative of 2 independent experiments, and bars indicate mean values. (C) Expression of chemokines (CCL19, CCL21, and CXCL13) in stromal and spleen cells was examined by RT-PCR. Data are representative of 3 independent experiments.

of peritoneal B cells were similar with or without FTY720 treatment (Figure 4C).

We next addressed whether NIK mutation affected B-cell immigration from the blood into the peritoneal cavity. In this experiment, we removed the peritoneal cells from recipient *aly* mice 8 hours before adoptive transfer, because the *aly* peritoneal B cells were too numerous to allow detection of intravenously transferred B cells. Twenty-four hours after depletion of the peritoneal B cells, the peritoneal cavities of *aly* mice contained more B2 cells than untreated *aly* peritoneal B cells (data not shown), demonstrating that peritoneal B cells were removed and that many cells were derived from the blood. Removing peritoneal cells from recipient mice before adoptive transfer enabled us to detect intravenously injected WT peritoneal B cells in *aly* mice (Figure 4D). Unlike the case with intraperitoneally transferred WT B cells (Figure 4C), FTY720 prevented the immigration of intravenously transferred WT peritoneal B cells from the blood into the peritoneal cavity (Figure 4D). The number of adoptively transferred B cells was decreased consistently and significantly ($P < .05$) in the peritoneal cavities of FTY720-treated *aly* mice (Figure 4D). These findings suggest that NIK-mediated signaling in non-B cells participates in the regulation of S1P-mediated emigration of B cells from the peritoneal cavity but not in their immigration from the blood.

Requirement of NIK-mediated pathway in stromal cells for S1P-mediated emigration of peritoneal B cells

Because both *aly* and WT B cells showed normal emigration from the peritoneal cavities of SCID mice (Figures 2C and 4A, respectively), T cells likely do not play a role in this pathway. We therefore speculated that NIK-mediated signaling in stromal cells was involved in the emigration of peritoneal B cells. To test this hypothesis, we transferred WT stromal cells into the peritoneal cavities of *aly* mice treated with FTY720 and noted a subsequent reduction in the number of peritoneal B cells (Figure 5A top panels). In contrast, FTY720 had no discernible effect when *aly* stromal cells were transferred into *aly* mice (Figure 5A bottom panels). These data suggest that NIK-mediated signaling in stromal cells participates in the regulation of S1P-mediated peritoneal B-cell emigration.

To investigate the mechanisms of peritoneal B-cell trafficking mediated by NIK in stromal cells, we compared the expression of vascular cell adhesion molecule (VCAM)-1 and intercellular adhesion molecule (ICAM)-1, adhesion molecules regulating peritoneal B-cell trafficking, between WT and *aly* stromal cells. The expression of these adhesion molecules was lower on *aly* stromal cells than on WT stromal cells (Figure 5B). A previous report that S1P regulated the expression of VCAM-1 on endothelial cells[30] led us to hypothesize that S1P affects the expression of VCAM-1 and ICAM-1 on stromal cells. However, unlike endothelial cells, WT and *aly* stromal cells showed negligible expression of all types of S1P receptors (data not shown). Therefore, treatment of stromal cells with S1P or FTY720 influenced neither VCAM-1 nor ICAM-1 expression, although IFNγ increased the expression of both of these molecules in both WT and *aly* stromal cells (Figure 5B).

We then examined the expression of chemokines that were reported to be involved in peritoneal B-cell trafficking (CCL19, CCL21, and CXCL13).[3,4] Our results showed that expression of CCL19 and CCL21 was comparable between WT and *aly* stromal cells (Figure 5C). In contrast, CXCL13 expression was increased in *aly* stromal cells compared with WT stromal cells (Figure 5C), although CXCL13 expression in the *aly* spleen was lower than in the WT spleen (Figure 5C), as previously reported.[23] These findings collectively indicate that, in S1P-mediated peritoneal B-cell trafficking, S1P directly affects peritoneal B cells, not stromal cells, but stromal cells are involved in the S1P-mediated pathway through the expression of adhesion molecules and chemokines. Decreased expression of VCAM-1 and ICAM-1 on stromal cells, or unbalanced CXCL13 expression between the peritoneal cavity and other sites (eg, spleen) in *aly* mice (or both mechanisms), may explain the impaired S1P-mediated trafficking of peritoneal B cells in *aly* mice.

Discussion

Lymphocyte trafficking into and from lymph nodes and nonlymphoid organs is regulated through several bioactive molecules (eg, chemokines and adhesion molecules).[9] We previously demonstrated that a lipid mediator, S1P, regulated mucosa-associated lymphocyte trafficking of peritoneal B cells, intraepithelial T lymphocytes, and mast cells into the intestine.[17-19] To this end, our recent study[17] showed that S1P plays important roles in both the immigration and emigration of B cells into and from the peritoneal cavity. Our current study extends this observation by showing that NIK-mutated *aly* mice were less sensitive (~5 times) to FTY720 than WT mice (Figure 1). We found that NIK-mediated signaling in stromal cells was involved in the emigration, but not immigration, step of S1P-mediated trafficking of peritoneal B cells (Figure 4). Although our results showed that the specific involvement of NIK-mediated signaling in stromal cells in the emigration of peritoneal B cells is a critical and major factor determining less sensitivity (~5 times) of *aly* mice to FTY720, it is simply possible that elevated numbers of peritoneal B cells in *aly* mice (~10 times) may at least partly provide additional explanation for the lower sensitivity of *aly* mice to FTY720.

Our current study also revealed that *aly* peritoneal B cells are functionally normal and therefore show normal $S1P_1$ expression and reactivity to S1P (Figure 2). In addition, *aly* peritoneal B cells show normal expression of CXCR5, a receptor for CXCL13.[23] In contrast, functional defects of *aly* stromal cells led to impaired S1P-mediated peritoneal B-cell trafficking in *aly* mice, although stromal cells did not express any types of S1P receptors (Figure 5 and data not shown). Regarding this issue, we found 2 possible major defects in stromal cell expression of adhesion molecules (ICAM-1 and VCAM-1) and chemokine, CXCL13. First, in agreement with previous findings that the expression of VCAM-1 and ICAM-1 was positively regulated by NFκB/NIK pathway,[31,32] *aly* stromal cells showed reduced expression of VCAM-1 and ICAM-1, thereby weakening the attachment of peritoneal B cells to stromal cells in their trafficking pathway (Figure 5B). Second, although CXCL13 expression was decreased in the spleens of *aly* mice compared with WT control (Figure 5C), which is in agreement with a previous work,[23] *aly* stromal cells showed increased CXCL13 expression compared with WT stromal cells (Figure 5C). Underlying mechanisms of the opposite effect of NIK mutation on CXCL13 expression between stromal and spleen cells remain enigmatic and represent challenges for future studies. But our current findings indicate that NIK-mediated signalings are involved in both positive and negative regulation of CXCL13 expression and which is used depends on cell types. This idea is supported by a previous report that the regulation of inflammatory cytokine–mediated CXCL13 expression was different among cells types (eg, bone marrow stromal cells and osteoblasts).[33] Taken together with these facts that S1P and CXCL13 mutually regulate marginal B-cell trafficking and the S1P function is dominant to CXCL13,[34] it seems that, under normal conditions, the disruption of $S1P_1$-mediated signaling by FTY720 treatment may allow peritoneal B cells to react to the CXCL13 gradient between the peritoneal cavity and other sites (eg, spleen), leading to B-cell emigration from the peritoneal cavity through the interaction with stromal adhesion molecules (eg, ICAM-1 and VCAM-1). Our results indicated that the functional defects of stromal cells in this pathway caused decreased sensitivity of *aly* mice to FTY720 and thus replacement of *aly* stromal cells with WT rescued normal S1P-mediated emigration of peritoneal B cells.

The molecular mechanisms of peritoneal B-cell trafficking for intestinal IgA production remain enigmatic.[1,2] In this regard, the enhanced expression of β_7 integrin induced by peritoneal environment plays a role in establishing the commitment of peritoneal B cells to home back to the peritoneal cavity as well as migrate to the intestine.[8] In addition, gut-associated dendritic cells (eg, Peyer patches and mesenteric lymph nodes) can allow B cells to migrate into the intestine by inducing the expression of $\alpha_4\beta_7$ integrin and CCR9 through retinoic acid.[35] However, the molecules involved in the peritoneum-dependent gut tropism of peritoneal B cells remain unknown. In a previous study,[24] NIK-dependent stromal cell activation was required for the direct migration of bone marrow–derived B cells to the intestinal lamina propria but not for the migration of B cells primed in the Peyer patches. Our current results similarly suggest that the peritoneum-mediated trafficking of B cells into the intestine involves NIK-dependent stromal cells. The present study therefore shows that both NIK-dependent signaling in stromal cells and S1P were required for B-cell trafficking from the peritoneal cavity to the intestine for intestinal IgA production, especially in the step of B-cell emigration from the peritoneal cavity (Figure 4). Therefore, molecular interaction among S1P, NIK in stromal cells, and unknown gut-imprinting molecules likely uniquely coordinates the trafficking of B cells from the peritoneum into the intestine for subsequent intestinal IgA production.

Considering all previous and current findings together, we suggest that the destiny of peritoneal B cells is controlled by a pleonastic regulatory network comprising S1P, chemokines, and integrins. In this pathway, NIK-mediated signaling in stromal cells regulates the S1P-mediated emigration of B cells from the peritoneum to the intestine for subsequent production of intestinal IgA.

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Authorship

Contribution: J.K. and M.G. designed and performed research, analyzed data, and wrote the paper; Y.K., M.H., and I.I. performed research and analyzed data; H.K. designed research and wrote the paper.

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Regulation of humoral and cellular gut immunity by lamina propria dendritic cells expressing Toll-like receptor 5

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The intestinal cell types responsible for defense against pathogenic organisms remain incompletely characterized. Here we identify a subset of CD11c^{hi}CD11b^{hi} lamina propria dendritic cells (LPDCs) that expressed Toll-like receptor 5 (TLR5) in the small intestine. When stimulated by the TLR5 ligand flagellin, TLR5$^+$ LPDCs induced the differentiation of naive B cells into immunoglobulin A–producing plasma cells by a mechanism independent of gut-associated lymphoid tissue. In addition, by a mechanism dependent on TLR5 stimulation, these LPDCs promoted the differentiation of antigen-specific interleukin 17–producing T helper cells and type 1 T helper cells. Unlike spleen DCs, the LPDCs specifically produced retinoic acid, which, in a dose-dependent way, supported the generation and retention of immunoglobulin A–producing cells in the lamina propria and positively regulated the differentiation interleukin 17–producing T helper cells. Our findings demonstrate unique properties of LPDCs and the importance of TLR5 for adaptive immunity in the intestine.

The gastrointestinal tract is constantly exposed to food proteins and commensal bacteria. Although the intestinal immune system has evolved mechanisms to maintain immunological tolerance to food and commensal organisms, it also recognizes invasive pathogens and properly induces protective immune responses to eliminate them. Dendritic cells (DCs) are thought to be critical in the 'decision' of whether to mount tolerant or protective immune responses[1]. Many subsets of DCs have been identified in the intestine[2]. In the Peyer's patches and mesenteric lymph nodes, conventional DCs consist of CD11c^{hi}CD11b$^+$CD8α^-, CD11c^{hi}CD11b$^-$CD8α^+ and CD11c^{hi}CD11b$^-$CD8α^- subsets[2]. In addition, there are CD11c^{int} plasmacytoid DCs in these sites[3,4]. Peyer's patch DCs produce interleukin 10 (IL-10) rather than IL-12, polarize naive T cells toward T helper type 2 (T_H2) or regulatory phenotypes[5] and induce the differentiation of plasma cells positive for immunoglobulin A (IgA)[6,7].

In contrast, lamina propria DCs (LPDCs) are less well studied. Although DCs are dominant antigen-presenting cells in the small intestine, colonic DCs are concentrated mainly in isolated lymphoid follicles, few of which are present in the lamina propria[8]. However, studies have shown that LPDCs of the small intestine and DCs in mesenteric lymph nodes that express CD103 have regulatory functions[9,10]. CD103$^+$ LPDCs migrate from the lamina propria to the mesenteric lymph nodes in a CCR7-dependent way[11–13] and promote the generation of Foxp3$^+$ regulatory T cells by means of retinoic acid[14]. Subsequent studies, however, have shown that CD11b$^+$F4/80$^+$CD11c$^-$ macrophages in the lamina propria are more potent inducers of regulatory T cells than are LPDCs and that CD11b$^+$ LPDCs generate T cells producing IL-17 *in vitro*[15]. These findings collectively suggest that LPDCs induce both 'tolerogenic' regulatory T cells and 'inflammatory' IL-17–producing T helper cells (T_H-17 cells). However, it remains unclear what kind of stimulation triggers the LPDC-induced generation of T_H-17 cells.

The Toll-like receptor (TLR) family, which is key for innate immunity, consists of 13 mammalian members[16]. TLRs are 'preferentially' expressed in 'professional' antigen-presenting cells such as DCs and macrophages and recognize specific components of

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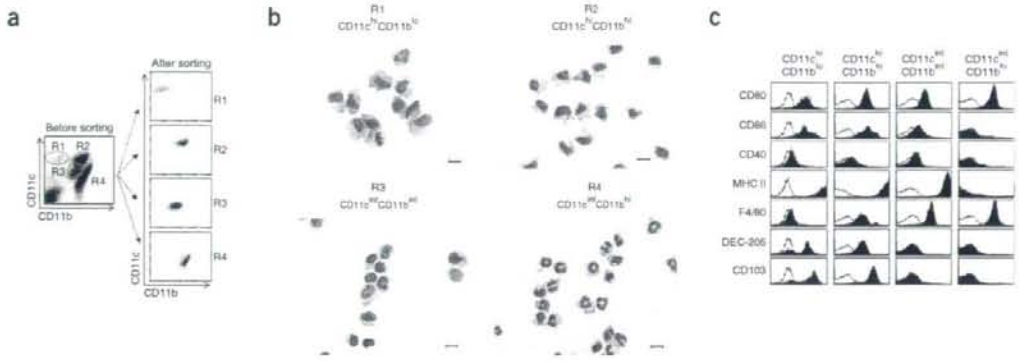

Figure 1 Four subsets of CD11c$^+$ LPCs in the small intestine. (**a**) Flow cytometry of intestinal low-density LPCs stained for CD11b and CD11c, before and after sorting. (**b**) May-Grunwald-Giemsa staining of four leukocyte subsets (gated in **a**) from the lamina propria. Scale bars, 10 μm. (**c**) Surface expression of CD80, CD86, CD40, major histocompatibility complex class II (MHC II), F4/80, DEC-205 and CD103 (filled histograms) on the four leukocyte subsets gated in **a**. Open histograms, isotype control. Data are representative of at least three independent experiments.

microorganisms to induce innate immune responses[16]. Each TLR activates specific signaling pathways that elicit biological responses to microorganisms, as well as DC maturation and cytokine production that shape adaptive immune responses[16]. Although the function of TLRs has been examined extensively in intestinal epithelial cells[17], the function of TLRs in lamina propria antigen-presenting cells has not been fully elucidated. Intestinal CD11c$^+$ lamina propria cells (LPCs) have high expression of TLR5 (A002297) and induce inflammatory responses when stimulated with the TLR5 ligand bacterial flagellin[18]. Unlike conventional DCs, such as those in the spleen (SPDCs), CD11c$^+$ LPCs do not express TLR4, which recognizes the Gram-negative bacterial component lipopolysaccharide (LPS)[18]. Nevertheless, *Tlr5*$^{-/-}$ mice show resistance to oral *Salmonella typhimurium* infection, as this facultative intracellular flagellated bacteria seems to use TLR5 and CD11c$^+$ LPCs as 'carriers' for systemic infection[18].

Mouse CD11c$^+$ LPCs consist of four subsets distinguished by differential expression patterns of CD11c and CD11b. Here we have identified a subset of CD11c^{hi}CD11b^{hi} LPDCs as TLR5-expressing cells. In response to flagellin, these LPDCs induced the differentiation of naive B cells into IgA$^+$ (A001174) plasma cells by a mechanism independent of gut-associated lymphoid tissue (GALT) and triggered the differentiation of antigen-specific T_H-17 and T_H1 cells. In a dose-dependent way, retinoic acid produced by LPDCs supported the generation and retention of IgA-producing cells in the lamina propria and positively regulated T_H-17 cell differentiation.

RESULTS

High TLR5 expression on CD11c^{hi}CD11b^{hi} LPDCs

CD11c$^+$ DCs constituted 10–15% of leukocytes in the small intestinal lamina propria and consisted of at least two subsets (CD11c^{hi}CD11b^{lo} (R1) and CD11c^{hi}CD11b^{hi} (R2))[12] (**Fig. 1a,b**), each of which had a DEC-205$^+$ major histocompatibility complex class II–high CD80$^+$CD86$^+$CD103$^+$ surface phenotype (**Fig. 1c**). In addition, CD11c^{hi}CD11b^{hi} cells had moderate expression of F4/80, which indicated a macrophage-like character. The remaining CD11c$^+$ subsets consisted of CD11c^{int}CD11b^{int} cells (R3), which are F4/80$^+$DEC-205$^-$ major histocompatibility complex class II$^+$ phagocytic macrophages[15,19], and CD11c^{int}CD11b^{hi} cells (R4), which are eosinophils with uniquely shaped nuclei and eosinophilic granules[12] (**Fig. 1b,c**). Of these four subsets from the lamina propria of C57BL/6 mice, only CD11c^{hi}CD11b^{hi} LPDCs expressed *Tlr5* mRNA (**Fig. 2a**). Consistent with the expression of functional TLR5, CD11c^{hi}CD11b^{hi} LPDCs produced proinflammatory cytokines such as IL-6, IL-12p40 and IL-12p70, but not IL-23 or IL-10, in response to flagellin (**Fig. 2b**). In contrast, LPDCs (R1) did not produce such cytokines in response to either flagellin or LPS (**Supplementary Fig. 1** online). Thus, CD11c^{hi}CD11b^{hi} LPDCs are responsible for TLR5-mediated innate immune responses.

CD11c^{hi}CD11b^{hi} LPDCs induce IgA production

To determine the function of CD11c^{hi}CD11b^{hi} LPDCs in adaptive immunity, we examined IgA synthesis in the small intestine. IgA is the

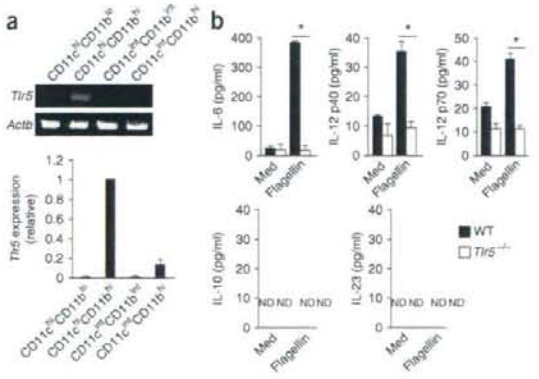

Figure 2 CD11c^{hi}CD11b^{hi} LPDCs specifically express TLR5. (**a**) RT-PCR (top) and quantitative real-time PCR (bottom) of *Tlr5* expression in the four leukocyte lamina propria subsets. *Actb* encodes β-actin (top, loading control). Expression (bottom) is relative to that of *Actb*. Data are representative of three independent experiments. (**b**) Cytokine production by CD11c^{hi}CD11b^{hi} LPDCs from wild-type (WT) and *Tlr5*$^{-/-}$ mice in response to medium alone (Med) or flagellin (1 μg/ml). ND, not detected. *, $P < 0.05$ (unpaired Student's *t*-test). Data represent the mean and s.d. of three independent experiments.

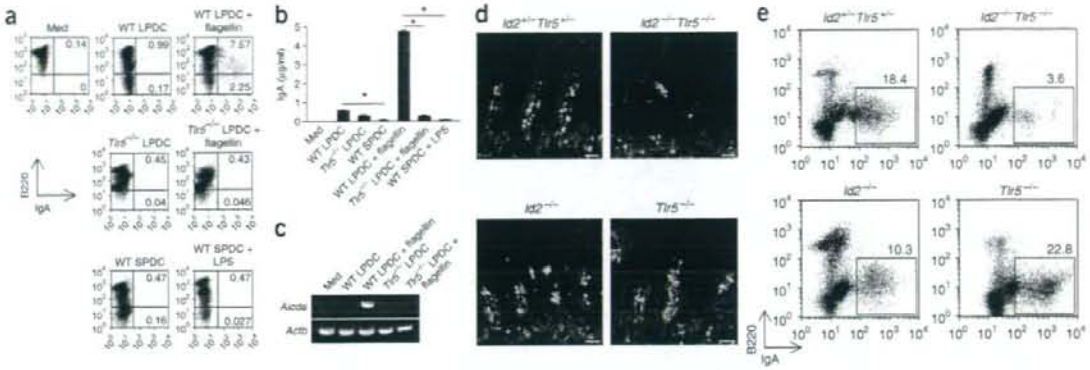

Figure 3 CD11c^{hi}CD11b^{hi} LPDCs induce IgA$^+$ plasma cell differentiation. (**a**,**b**) Flow cytometry (**a**) and ELISA (**b**) of peritoneal IgM$^+$IgD$^+$ cells cultured for 5 d in various conditions (above plots (**a**) and below horizontal axis (**b**)). (**a**) Cells stained for B220 and IgA (isotype controls, **Supplementary Fig. 2**). Numbers in quadrants indicate percent B220$^+$IgA$^+$ cells (top right) or B220$^-$IgA$^+$ cells (bottom right). Data are representative of three independent experiments. (**b**) Concentration of IgA in coculture supernatants. *, $P < 0.05$ (unpaired Student's *t*-test). Data represent the mean and s.d. of three independent experiments. (**c**) Expression of *Aicda* mRNA (encoding activation-induced cytidine deaminase) in IgM$^+$IgD$^+$ cells cultured together with wild-type or *Tlr5*$^{-/-}$ CD11c^{hi}CD11b^{hi} LPDCs with or without flagellin. Data are representative of three independent experiments. (**d**) Immunohistochemistry of IgA$^+$ cells (green) in the small intestine ($n = 4$ mice per group). Scale bars, 50 μm. Data are representative of three independent experiments. (**e**) Flow cytometry of LPCs stained for B220 and IgA. Numbers above outlined areas indicate percent B220$^-$IgA$^+$ cells. Data are representative of three independent experiments.

most abundant immunoglobulin in the gut[20]. Intestinal IgA$^+$ plasma cells are generated mainly in GALT, including Peyer's patches, isolated lymphoid follicles and mesenteric lymph nodes, by a mechanism dependent on antigen, T cells and the formation of germinal centers[21–23]. Differentiated IgA$^+$ cells are 'imprinted' by GALT DC–derived retinoic acid for gut homing through the selective expression of gut-homing receptors, including integrin $\alpha_4\beta_7$ and CCR9 (ref. 7). However, reports have shown that IgA$^+$ cell development does not necessarily require T cell help and the formation of germinal centers[21,24] and that GALT DC–derived retinoic acid can potently act in synergy with cytokines produced by DCs and/or other cells to generate T cell–independent IgA$^+$ cells[7]. Furthermore, it seems that some IgM$^+$ B cells, especially peritoneal B1 cells, migrate directly to the gut lamina propria by a mechanism dependent on sphingosine 1-phosphate[25,26] and differentiate into IgA$^+$ plasma cells in the lamina propria with the help of stroma cells[24]. Commensal bacteria induce natural secretory IgA, and this process is mediated by DCs loaded with commensal bacteria[6,27]. Nevertheless, although published work has suggested the involvement of DCs in gut IgA production[28,29], it is unknown what subset of DCs is responsible for this event and how this is achieved. We thus examined whether CD11c^{hi}CD11b^{hi} LPDCs are involved in the generation of IgA$^+$ cells; we used SPDCs (TLR5$^-$TLR4$^+$) for comparison[18]. Flagellin-stimulated CD11c^{hi}CD11b^{hi} LPDCs but not LPS-stimulated SPDCs efficiently induced the differentiation of B220$^-$ IgA$^+$ plasma cells in the absence of T cells in a TLR5-dependent way (**Fig. 3a,b** and **Supplementary Fig. 2** online). Expression of

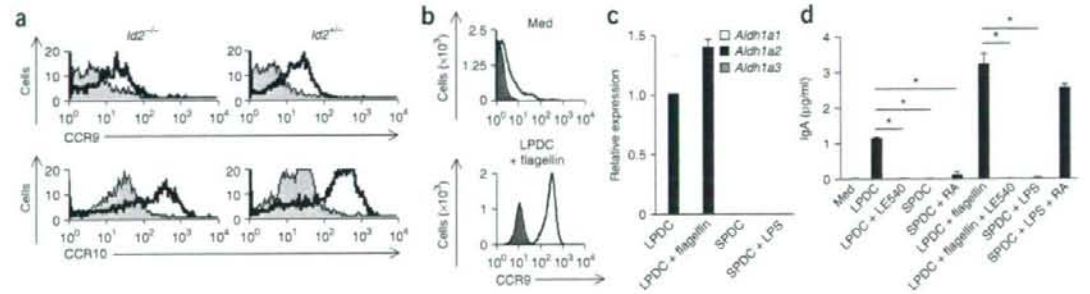

Figure 4 Function of retinoic acid released by CD11c^{hi}CD11b^{hi} LPDCs in IgA synthesis. (**a**) Flow cytometry of LPCs stained for B220, IgA, CCR9 or CCR10 (open histograms), gated on B220$^-$IgA$^+$ cells. Filled histograms, isotype control. Data are representative of three independent experiments. (**b**) Flow cytometry of peritoneal B220$^+$ cells cultured for 5 d with or without flagellin-stimulated CD11c^{hi}CD11b^{hi} LPDCs. Data for CCR9 (open histograms) were acquired after gating on B220$^+$ cells (top) or B220$^-$IgA$^+$ cells (bottom). Filled histograms, isotype control. Data are representative of three independent experiments. (**c**) Quantitative real-time PCR of mRNA encoding retinal dehydrogenase isozymes (key) in CD11c^{hi}CD11b^{hi} LPDCs and SPDCs left unstimulated or stimulated with LPS or flagellin (horizontal axis). Data are representative of three independent experiments (mean and s.d.). (**d**) ELISA of IgA in supernatants of peritoneal B220$^+$ cells cultured for 5 d in various conditions (horizontal axis) with or without LE540 (1 μM) or retinoic acid (RA; 1 nM). *, $P < 0.05$ (unpaired Student's *t*-test). Data represent the mean and s.d. of three independent experiments.

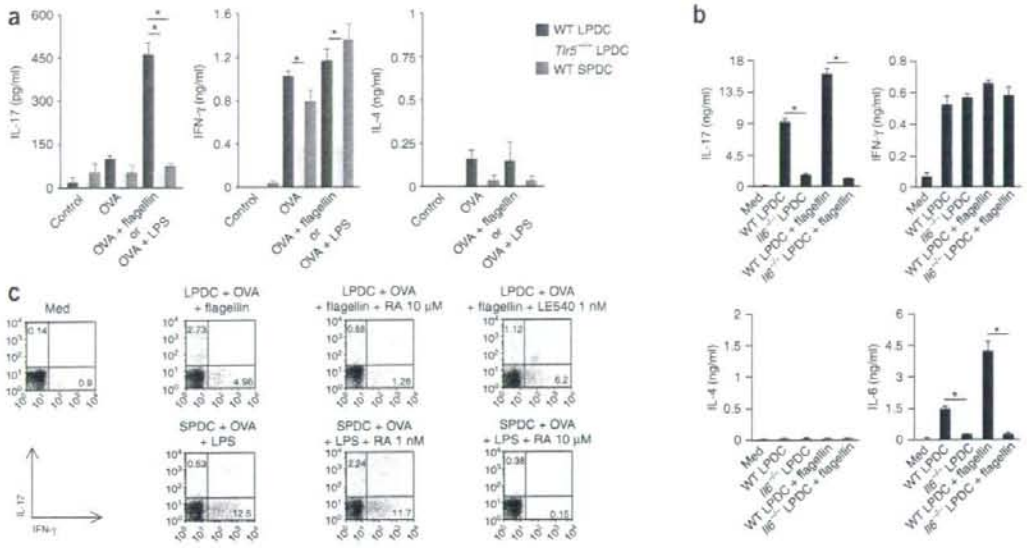

Figure 5 TLR5-dependent T_H-17 cell differentiation by $CD11c^{hi}CD11b^{hi}$ LPDCs. (**a**) ELISA of IFN-γ, IL-17 and IL-4 in culture supernatants. $CD11c^{hi}CD11b^{hi}$ LPDCs or SPDCs cultured for 12 h with OVA protein (100 μg/ml) in the presence or absence of flagellin (1 μg/ml) or LPS (1 μg/ml) were injected on days 0 and 14 into the peritoneal cavities of naive *Tlr5*$^{-/-}$ mice (wild-type $CD11c^{hi}CD11b^{hi}$ LPDCs) or *Tlr4*$^{-/-}$ mice (wild-type SPDCs) at a dose of 5×10^4 antigen-loaded cells per mouse; control mice were treated with PBS. At 1 week after the final immunization, splenocytes were collected and were cultured for 4 d with OVA protein (10 μg/ml) or with OVA peptide (amino acids 323–339; 10 μg/ml; **Supplementary Fig. 6**). *, $P < 0.05$ (unpaired Student's *t*-test). Data represent the mean and s.d. of three independent experiments. (**b**) ELISA of cytokines in supernatants of OT-II transgenic $CD4^+$ T cells cultured for 4 d together with wild-type or *Il6*$^{-/-}$ $CD11c^{hi}CD11b^{hi}$ LPDCs (conditions, horizontal axes). *, $P < 0.05$ (unpaired Student's *t*-test). Data represent the mean and s.d. of three independent experiments. (**c**) Flow cytometry of OT-II transgenic $CD4^+$ T cells cultured for 4 d in various conditions (above plots) and stained intracellularly for IL-17 and IFN-γ (isotype controls, **Supplementary Fig. 7a**). Numbers in quadrants indicate percent $IL\text{-}17^+IFN\text{-}\gamma^-$ cells (top left) or $IL\text{-}17^-IFN\text{-}\gamma^+$ cells (bottom right). Data are representative of three independent experiments.

mRNA encoding activation-induced cytidine deaminase[30], an enzyme essential for class-switch recombination, was upregulated in naive B cells cultured together with flagellin-stimulated $CD11c^{hi}CD11b^{hi}$ LPDCs (**Fig. 3c**).

Although the results presented above demonstrated that $CD11c^{hi}CD11b^{hi}$ LPDCs were able to induce T cell–independent differentiation of IgA^+ cells *in vitro*, we also examined the *in vivo* function of TLR5 in IgA synthesis by using GALT-deficient mice that intrinsically lack secondary lymphoid organs but have LPDCs. Mice lacking the transcription factors Id2 or RORγt, as well as bone marrow–reconstituted mice lacking lymphotoxin-α or both lymphotoxin-α and tumor necrosis factor, do not develop GALT, yet they retain intestinal IgA production[21,31]. Indeed, we detected many IgA^+ cells in the lamina propria of *Id2*$^{-/-}$ mice, which confirmed that gut IgA can be generated without GALT (**Fig. 3d,e**). Furthermore, we found no defects in the *in vitro* differentiation of IgA^+ plasma cells induced by $CD11c^{hi}CD11b^{hi}$ LPDCs from peritoneal B cells isolated from *Id2*$^{-/-}$ mice (**Supplementary Fig. 3** online). Although *Tlr5*$^{-/-}$ mice did not have fewer IgA^+ B cells, *Id2*$^{-/-}$*Tlr5*$^{-/-}$ mice had far fewer IgA^+ cells in the lamina propria (**Fig. 3d,e**). Thus, TLR5 signaling in $CD11c^{hi}CD11b^{hi}$ LPDCs is critical for GALT-independent IgA synthesis *in vivo*.

Retinoic acid in LPDC-induced IgA synthesis

We next examined the expression of gut-homing receptors on lamina propria IgA^+ cells in *Id2*$^{-/-}$ mice. Unexpectedly, $B220^-IgA^+$ plasma cells in the lamina propria of *Id2*$^{-/-}$ mice had high expression of CCR9, despite the lack of GALT in these mice (**Fig. 4a**). These cells also expressed CCR10, another chemokine receptor important for gut tropism[32]. As we did not detect high CCR9 expression on either peritoneal or splenic unstimulated $B220^+$ cells from wild-type or *Id2*$^{-/-}$ mice (**Fig. 4b** and data not shown), CCR9 might be induced on B cells only after their migration to the lamina propria in *Id2*$^{-/-}$ mice. In contrast, coculture with flagellin-treated $CD11c^{hi}CD11b^{hi}$ LPDCs induced CCR9 expression on peritoneal $B220^-IgA^+$ cells (**Fig. 4b**). We therefore determined whether $CD11c^{hi}CD11b^{hi}$ LPDCs synthesize retinoic acid, a mediator able to induce CCR9 expression. Retinal is converted into retinoic acid by retinal dehydrogenase enzymes. Although we detected no mRNA molecules encoding retinal dehydrogenase isoforms in SPDCs, $CD11c^{hi}CD11b^{hi}$ LPDCs specifically expressed *Aldh1a2* mRNA, which encodes retinal dehydrogenase 2 (**Fig. 4c**). To determine if the $CD11c^{hi}CD11b^{hi}$ LPDC–mediated development of IgA^+ cells was controlled by retinoic acid, we added the retinoic acid receptor inhibitor LE540 during the *in vitro* coculture of B cells and $CD11c^{hi}CD11b^{hi}$ LPDCs. LE540 abrogated IgA production by B cells cultured together with flagellin-activated $CD11c^{hi}CD11b^{hi}$ LPDCs (**Fig. 4d**). Moreover, supplementation of LPS-activated SPDCs with retinoic acid increased IgA concentrations to an extent similar to that induced by flagellin-activated $CD11c^{hi}CD11b^{hi}$ LPDCs. Thus, the characteristic ability to synthesize retinoic acid grants $CD11c^{hi}CD11b^{hi}$ LPDCs the ability to generate T cell–independent IgA^+ cells.

LPDC-induced T_H-17 cell differentiation

We next assessed the ability of $CD11c^{hi}CD11b^{hi}$ LPDCs to induce antigen-specific T helper cell differentiation of ovalubumin

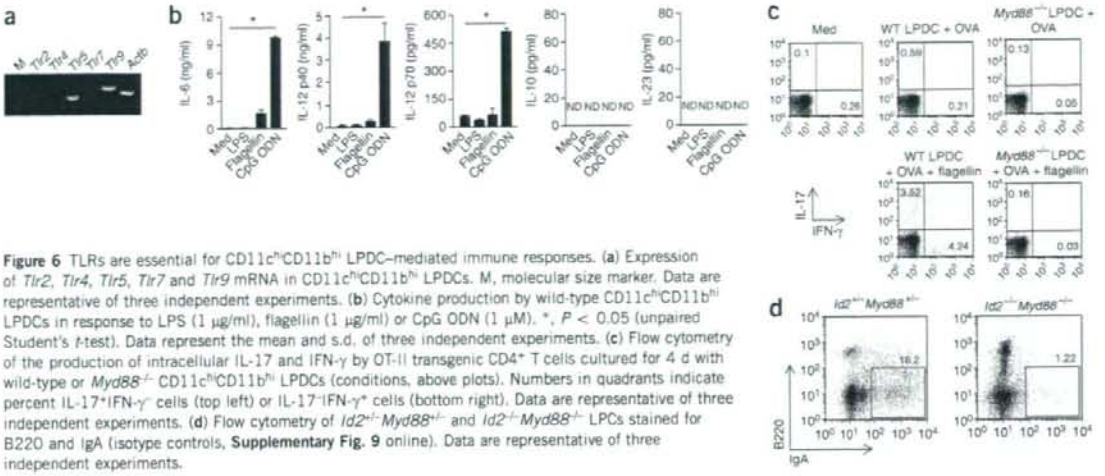

Figure 6 TLRs are essential for $CD11c^{hi}CD11b^{hi}$ LPDC–mediated immune responses. (**a**) Expression of *Tlr2*, *Tlr4*, *Tlr5*, *Tlr7* and *Tlr9* mRNA in $CD11c^{hi}CD11b^{hi}$ LPDCs. M, molecular size marker. Data are representative of three independent experiments. (**b**) Cytokine production by wild-type $CD11c^{hi}CD11b^{hi}$ LPDCs in response to LPS (1 μg/ml), flagellin (1 μg/ml) or CpG ODN (1 μM). *, $P < 0.05$ (unpaired Student's *t*-test). Data represent the mean and s.d. of three independent experiments. (**c**) Flow cytometry of the production of intracellular IL-17 and IFN-γ by OT-II transgenic $CD4^+$ T cells cultured for 4 d with wild-type or *Myd88*$^{-/-}$ $CD11c^{hi}CD11b^{hi}$ LPDCs (conditions, above plots). Numbers in quadrants indicate percent $IL\text{-}17^+IFN\text{-}\gamma^-$ cells (top left) or $IL\text{-}17^-IFN\text{-}\gamma^+$ cells (bottom right). Data are representative of three independent experiments. (**d**) Flow cytometry of *Id2*$^{+/-}$*Myd88*$^{+/-}$ and *Id2*$^{-/-}$*Myd88*$^{-/-}$ LPCs stained for B220 and IgA (isotype controls, **Supplementary Fig. 9** online). Data are representative of three independent experiments.

(OVA)-specific OT-II-transgenic $CD4^+$ T cells. Although we detected only interferon-γ (IFN-γ)-producing cells in cocultures of OT-II T cells and LPS-stimulated SPDCs, we detected both IL-17- and IFN-γ-producing cells in cocultures of OT-II T cells and $CD11c^{hi}CD11b^{hi}$ LPDCs; the numbers of IL-17- and IFN-γ-producing OT-II cells were further increased by flagellin stimulation of LPDCs[33–36] (**Supplementary Fig. 4a,b** online). In support of the idea that $TLR5^+$ $CD11c^{hi}CD11b^{hi}$ LPDCs induce T_H-17 differentiation, naive $CD4^+$ T cells cultured together with wild-type $CD11c^{hi}CD11b^{hi}$ LPDCs had higher expression of RORγt and IL-21, but those cultured together with *Tlr5*$^{-/-}$ $CD11c^{hi}CD11b^{hi}$ LPDCs did not (**Supplementary Fig. 4c,d**). In contrast, other LPDC subsets (R1, R3 and R4) induced neither IL-17 nor IFN-γ production in response to flagellin (**Supplementary Fig. 5** online).

Next we examined *in vivo* the T helper cell responses of mice immunized with antigen-loaded DCs. We detected antigen-specific IFN-γ production after injection of both SPDCs and $CD11c^{hi}CD11b^{hi}$ LPDCs, and this production was augmented by stimulation of TLR5 and TLR4 (**Fig. 5a** and **Supplementary Fig. 6** online). In addition, large amounts of IL-17 were produced by splenocytes from mice injected with flagellin-stimulated $CD11c^{hi}CD11b^{hi}$ LPDCs but not those injected with LPS-stimulated SPDCs. Those responses were impaired when mice were injected with *Tlr5*$^{-/-}$ $CD11c^{hi}CD11b^{hi}$ LPDCs. As IL-6 is an essential cytokine for T_H-17 cell differentiation, and as $CD11c^{hi}CD11b^{hi}$ LPDCs produced IL-6 in response to TLR5 stimulation (**Fig. 2b**), we then examined the involvement of IL-6 in $CD11c^{hi}CD11b^{hi}$ LPDCs–induced T_H-17 cell differentiation[37]. Despite normal induction of IFN-γ, IL-17 production induced by flagellin-stimulated *Il6*$^{-/-}$ $CD11c^{hi}CD11b^{hi}$ LPDCs was significantly lower than that elicited by flagellin-stimulated wild-type $CD11c^{hi}CD11b^{hi}$ LPDCs (**Fig. 5b**).

A series of studies has shown that retinoic acid negatively regulates T_H-17 cell differentiation[38–40]. In agreement with those results, supplementation of cocultures of T cells and $CD11c^{hi}CD11b^{hi}$ LPDCs with 10 μM retinoic acid effectively inhibited *in vitro* T_H-17 cell differentiation; retinoic acid supplementation also suppressed T_H1 cell differentiation (**Fig. 5c** and **Supplementary Fig. 7** online). However, we suspected that this concentration of retinoic acid may have been too high, as plasma retinoic acid concentrations are usually on the order of 10 nM and retinoic acid efficiently enhances the expression of gut-homing receptors on $CD8^+$ T cells even at a concentration of 0.1 nM (ref. 41). Notably, the retinoic acid inhibitor LE540 inhibited the differentiation of T_H-17 cells but not T_H1 cells, which suggested that retinoic acid from $CD11c^{hi}CD11b^{hi}$ LPDCs is actually necessary for T_H-17 cell differentiation. In line with that observation, LPS-stimulated SPDCs induced T_H-17 cell differentiation to the same extent as flagellin-stimulated $CD11c^{hi}CD11b^{hi}$ LPDCs when cultured together with 1 nM retinoic acid, and 10 μM retinoic acid abolished T_H1 cell differentiation induced by LPS-stimulated SPDCs (**Fig. 5c** and **Supplementary Fig. 7**). Thus, retinoic acid at a low concentration acts as a positive regulator of T_H-17 cell differentiation, and the effect of retinoic acid on T_H-17 cell differentiation depends on its concentration.

$CD11c^{hi}CD11b^{hi}$ LPDCs induce antigen-specific T_H-17 cells and T_H1 cells, but it is not clear whether these adaptive immune responses are protective against bacterial infection. T_H-17 cells constitute approximately 2% of the total $CD4^+$ T cell population in the small intestinal lamina propria of C57BL/6 mice without infection, and the number of T_H-17 cells did not change during the acute phase of oral *S. typhimurium* infection (**Supplementary Fig. 8a** online). Mice immunized with OVA-loaded $CD11c^{hi}CD11b^{hi}$ LPDCs had greater proportions of T_H-17 and T_H1 cells in the lamina propria (**Supplementary Fig. 8b**). Challenge of the immunized mice with oral OVA further increased the proportions of lamina propria T_H-17 and T_H1 cells. Similarly, immunization with *S. typhimurium* flagellin–loaded wild-type LPDCs resulted in a significant increase in the proportion of lamina propria T_H-17 cells after oral challenge with *S. typhimurium*, ($P < 0.05$; **Supplementary Fig. 8c**) and resulted in partial protection against lethal challenge with *S. typhimurium* (**Supplementary Fig. 8d**), but similar immunization with *Tlr5*$^{-/-}$ LPDCs did not. Thus, $CD11c^{hi}CD11b^{hi}$ LPDC–mediated immunization contributed to host defense against *S. typhimurium*.

TLR signals in LPDC-mediated inflammation

Although we demonstrated the importance of TLR5 in the activation of adaptive immunity by $CD11c^{hi}CD11b^{hi}$ LPDCs, *Tlr5*$^{-/-}$ $CD11c^{hi}CD11b^{hi}$ LPDCs nevertheless induced small amounts of IL-17- and IFN-γ-producing cells (**Supplementary Fig. 4a**). In

addition, we detected residual B220$^-$IgA$^+$ plasma cells in the lamina propria of *Id2*$^{-/-}$*Tlr5*$^{-/-}$ mice (**Fig. 3e**). Thus, other TLRs may contribute to such responses. Accordingly, CD11c^{hi}CD11b^{hi} LPDCs expressed TLR9 as well as TLR5 and produced proinflammatory cytokines in response to the TLR9 ligand CpG DNA (**Fig. 6a,b**). Notably, unlike wild-type CD11c^{hi}CD11b^{hi} LPDCs, *Myd88*$^{-/-}$ CD11c^{hi}CD11b^{hi} LPDCs failed to induce the *in vitro* differentiation of T_H-17 and T_H1 cells (**Fig. 6c**). Furthermore, B220$^-$IgA$^+$ cells were almost completely absent from the lamina propria of *Id2*$^{-/-}$*Myd88*$^{-/-}$ mice (**Fig. 6d**). These data collectively suggest that TLR signals in general are critical for CD11c^{hi}CD11b^{hi} LPDC–mediated activation of acquired immunity.

DISCUSSION

In this work we have demonstrated the unique characteristics of CD11c^{hi}CD11b^{hi} TLR5-expressing LPDCs. It is noteworthy that TLR5 activation by flagellin triggered CD11c^{hi}CD11b^{hi} LPDC–mediated adaptive immune responses. Studies have shown that adjuvant effects are associated with the induction of protective immunity in the intestine. Injection of the ligand for the receptor tyrosine kinase Flt3, which expands DC populations in the intestine, enhances both tolerance and immunity to orally administered antigens[42,43]. Relative to mice fed antigen alone, those receiving Flt3 ligand and antigen show greater susceptibility to the induction of oral tolerance[42]. However, such oral tolerance is abrogated and immune responses are induced when mice are fed the same antigen with an adjuvant such as IL-1 or cholera toxin[43]. Such findings indicate that DC activation is a crucial parameter determining whether tolerance or protective immunity is induced in the intestine. In physiological conditions, antigens such as food proteins may be presented by quiescent CD11c^{hi}CD11b^{hi} LPDCs in the absence of inflammation, leading to tolerance. However, when inflammatory stimuli such as flagellin are present, CD11c^{hi}CD11b^{hi} LPDCs will undergo maturation, release inflammatory cytokines and initiate protective acquired immunity.

Commensal bacteria are present at a high density in the intestinal lumen (up to 1×10^{12} bacteria per gram of lumenal contents). Most commensal organisms reside outside the layer of mucus that covers the intestinal epithelial cells. Some bacteria penetrate the enterocyte epithelial layer but are rapidly killed by macrophages[44]. However, some commensal bacteria are ingested by DCs, where they survive for several days[6]. Moreover, intraepithelial DCs send protrusions into the lumen of the small intestine in a CX3CR1-dependent way and directly sample lumenal commensal bacteria[45,46]. Commensal bacteria–loaded DCs mediate the induction of natural secretary IgA[27], and germ-free mice have a profound deficiency in IgA production in the intestinal mucosa[44]. Thus, the presence of intestinal microbiota influences IgA production in the intestine. As the induction of B220$^-$IgA$^+$ plasma cells was impaired in *Id2*$^{-/-}$*Tlr5*$^{-/-}$ mice and was almost completely abrogated in *Id2*$^{-/-}$*Myd88*$^{-/-}$ mice, GALT-independent IgA production seems to be mediated by TLR stimulation. These results indicate that TLRs represent a 'missing link' between commensal bacteria and IgA synthesis in the lamina propria.

The ability to synthesize retinoic acid enables CD11c^{hi}CD11b^{hi} LPDCs to modulate various immune response parameters. CD11c^{hi}CD11b^{hi} LPDCs induced IgA$^+$ cell differentiation without T cell help in a retinoic acid–dependent way. In the process, CD11c^{hi}CD11b^{hi} LPDCs also promoted the upregulation of CCR9 expression on B cells. Notably, IgA$^+$ plasma cells had high expression CCR9 in the lamina propria of GALT-deficient *Id2*$^{-/-}$ mice. These results were unexpected, because GALT DCs are believed to 'imprint' gut tropism on lymphocytes. The ability of CD11c^{hi}CD11b^{hi} LPDCs to induce CCR9 on differentiated IgA$^+$ plasma cells may promote retention in the lamina propria, as the CCR9 ligand CCL25 is abundantly secreted by the crypt epithelium[21]. Although previous studies have shown that retinoic acid negatively regulates T_H-17 cell differentiation, here we have shown that the effect of retinoic acid on T helper cell differentiation depended strictly on its concentration. It is difficult to determine the local concentrations of retinoic acid secreted by CD11c^{hi}CD11b^{hi} LPDCs. However, studies intensively examining the concentration of retinoic acid secreted by GALT DCs in the work of T cell 'imprinting' have shown that 1 nM of retinoic acid is the optimum concentration for the induction of gut-homing receptors on T cells[41]. Notably, high concentrations of retinoic acid inhibited the differentiation of both T_H1 and T_H-17 cells, which suggested that the inhibitory effect of high concentrations of retinoic acid is not specific for T_H-17 polarization. Such observations indicate that the effect of retinoic acid on T_H-17 cell differentiation should be considered more cautiously. In any case, like the CD11c^{hi}CD11b^{hi} LPDC–induced differentiation of IgA$^+$ plasma cells, T_H-17 cell differentiation required retinoic acid. Unlike other conventional DCs, LPDCs can induce the differentiation of antigen-specific T_H-17 cells as well as T_H1 cells in response to TLR stimulation. The ability to produce retinoic acid may support this unique function of CD11c^{hi}CD11b^{hi} LPDCs.

We conclude that CD11c^{hi}CD11b^{hi} LPDCs may work against bacterial infection by inducing 'local' IgA secretion and 'systemic' T helper cell responses through TLR stimulation. As IL-17 can influence cytokine production by a wide range of cell types and can induce the activation and migration of neutrophils[47], CD11c^{hi}CD11b^{hi} LPDCs and T_H-17 cells may modulate the pathogenesis of intestinal bowel diseases such as Crohn's disease. In addition, the ability of CD11c^{hi}CD11b^{hi} LPDCs to induce the differentiation of T_H1 and IgA$^+$ cells suggests that CD11c^{hi}CD11b^{hi} LPDCs might be useful targets of mucosal vaccination.

METHODS

Mice. *Tlr4*$^{-/-}$ (C57BL/6) mice, *Tlr5*$^{-/-}$ mice (C57BL/6), *Id2*$^{-/-}$ mice and *Myd88*$^{-/-}$ mice have been described[18,48]. *Il6*$^{-/-}$ mice (C57BL/6) and OT-II-transgenic mice (C57BL/6) were provided by M. Kopf[49] and W.R. Heath[50], respectively. All animal experiments were done with the approval of the Animal Research Committee of the Research Institute for Microbial Diseases at Osaka University.

Reagents. LPS, flagellin and CpG oligodeoxynucleotides (ODN 1668) were purified as described[18]. *S. typhimurium* flagellin was from Invivogen. All-*trans* retinoic acid (Sigma) was dissolved in dimethyl sulfoxide, was stored at –80 °C with light interception and was added to cultures at a final concentration of 1 nM. LE540 (Wako) was dissolved in dimethyl sulfoxide and was added to cultures at a final concentration of 1 μM.

Cells. Segments of the small intestine were treated for 30 min at 37 °C with PBS containing 10% (vol/vol) FCS, HEPES (20 mM), pH 7.4, penicillin (100 U/ml), streptomycin (100 μg/ml), sodium pyruvate (1 mM), EDTA (10 mM) and polymyxin B (10 μg/ml; Calbiochem) for removal of epithelial cells, then were washed extensively with PBS. Segments of the small intestine and spleen were digested for 45–90 min with continuous stirring at 37 °C with collagenase D (400 Mandl units/ml; Roche) and DNase I (10 μg/ml; Roche) in RPMI 1640 medium plus 10% (vol/vol) FCS. EDTA was added (final concentration, 10 mM) and cell suspensions were incubated for an additional 5 min at 37 °C. Cells were spun through a 17.5% (wt/vol) solution of Accudenz (Accurate Chemical & Scientific) for enrichment for DCs. The cells obtained were incubated with fluorescein isothiocyanate–conjugated antibody to CD11b (anti-CD11b; M170; 557396) and phycoerythrin-conjugated anti-CD11c (HL3; 557401; both from BD Pharmingen) after blockade of Fc receptors. DC subsets were sorted on the basis of their expression of CD11c and CD11b with a FACSVantage SE or FACSAria (BD Biosciences). The purity of the sorted DCs

was routinely over 95%. For morphological studies, cytospin preparations from purified DC subsets were stained with May-Grunwald-Giemsa solution. For analysis of leukocytes, cells were subjected to density-gradient centrifugation in 40% to 75% (vol/vol) Percoll (approximately density, 1.058 g/ml and 1.093 g/ml, respectively) after enzyme treatment. Cells collected from the interface were washed and were used as lamina propria leukocytes in assays. Naive $CD4^+$ T cells from the spleens of OT-II transgenic mice and $B220^+$ cells from the peritoneal cavities of C57BL/6 mice were purified by magnetic sorting with mouse anti-CD4 beads and mouse anti-B220 beads, respectively. Peritoneal cells from C57BL/6 mice were incubated with fluorescein isothiocyanate–conjugated conjugated anti-IgD (11-26c.2a; 553439; BD Pharmingen) and phycoerythrin-indotricarbocyanine–conjugated anti-IgM (R6-60.2; 553409; BD Pharmingen) after blockade of Fc receptors. Naive B cells were sorted on the basis of their expression of IgD and IgM with a FACSVantage SEM or FACSAria (BD Biosciences). The purity of the sorted cells was routinely over 95%.

***In vitro* T cell differentiation.** OT-II transgenic $CD4^+$ T cells (1×10^6) were cultured with $CD11c^{hi}CD11b^{hi}$ LPDCs or SPDCs (1×10^5) in the presence of OVA protein (100 μg/ml), unsupplemented or supplemented with flagellin (1μg/ml) or LPS (1 μg/ml), respectively. After 4 d, cells were restimulated for 4 h with phorbol 12-myristate 13-acetate (50 ng/ml; Sigma) and ionomycin (500 ng/ml; Calbiochem) in the presence of GolgiStop (BD Pharmingen), then cells producing IL-17 and IFN-γ were analyzed by flow cytometry.

Immunization. $CD11c^{hi}CD11b^{hi}$ LPDCs or SPDCs were cultured for 12 h with OVA protein (100 μg/ml) in the presence or absence of flagellin (1 μg/ml) or LPS (1 μg/ml). Antigen-loading cells (5×10^4 per mouse) were injected on days 0 and 14 into the peritoneal cavities of naive *Tlr5*$^{-/-}$ mice ($CD11c^{hi}CD11b^{hi}$ LPDCs) or *Tlr4*$^{-/-}$ mice (SPDCs); control mice were treated with PBS. At 1 week after the final immunization, splenocytes were collected and were cultured for 4 d with OVA protein (10 μg/ml). The concentration of IFN-γ, IL-17 and IL-4 in the culture supernatants was measured by enzyme-linked immunosorbent assay (ELISA).

***In vitro* IgA^+ plasma cell differentiation.** Peritoneal IgM^+IgD^+ cells or $B220^+$ cells (1×10^6) were cultured in medium supplemented with B cell–activating factor (50 ng/ml) together with $CD11c^{hi}CD11b^{hi}$ LPDCs or SPDCs (1×10^5 to 5×10^5) in the presence or absence of flagellin (1 μg/ml) or LPS (1 μg/ml), respectively. After 5 d, cells were analyzed by flow cytometry and the concentration of IgA in culture supernatants was measured by ELISA.

Flow cytometry. Before staining, Fc receptors were blocked for 15 min at 4 °C. Low-density LPCs were stained with the following biotinylated monoclonal antibodies: anti-CD11b (M1/70; 557395), anti-CD11c (HL3; 553800), anti-CD40 (3/23; 553789), anti-CD80 (16-10A1; 553767), anti-CD86 (GL1; 553690), anti-I-A/I-E (2G9; 553622) and anti-CD103 (M290; 557493; all from BD Pharmingen); anti-F4/80 (A3-1; MF48015; Caltag Laboratories); and anti-DEC-205 (NLDC-145; CL89145PE; Cedarlane Laboratories). The surfaces of cocultured T cells were stained with fluorescein isothiocyanate–labeled anti-CD4 (L3T4; 553055; BD Pharmingen). Then, cells were fixed and made permeable with Cytofix/Cytoperm (BD Pharmingen) and were stained intracellularly with phycoerythrin-labeled anti-IL-17 (TCC11-18H10.1; 559502) and allophycocyanin-labeled anti-IFN-γ (XMG1.2; 554413; both from BD Pharmingen). The surfaces of cocultured B cells or lamina propria leukocytes were stained with phycoerythrin-labeled anti-B220 (RA3-6B2; 553090; BD Pharmingen). Then, cells were fixed and made permeable with Cytofix/Cytoperm and were stained intracellularly with fluorescein isothiocyanate–labeled anti-IgA (C10-3; 559354) or were incubated with biotin-conjugated IgA (C10-1; 556978) and then stained intracellulary with allophycocyanin-labeled streptavidin (all from BD Pharmingen). CCR9 expression on lamina propria leukocytes and cocultured B cells was assessed with rat anti–mouse CCR9 (242503; FAB2160A; R&D Systems). CCR10 expression on lamina propria leukocytes and cocultured B cells was assessed with rat anti–mouse CCR10 (248918; FAB2815A; R&D Systems). Samples were acquired on a FACSCalibur with CELLQuest software (BD Biosciences) and data were analyzed with FlowJo software (TreeStar).

RT-PCR and quantitative real-time PCR. RNA (1 μg) was reverse-transcribed with Superscript2 (Invitrogen) according to the manufacturer's instructions with random hexamers as primers. Primer pairs specific for *Tlr2*, *Tlr4*, *Tlr5*, *Tlr7*, *Tlr9*, *Aicda*[30] or *Actb* (**Supplementary Table 1** online) and Taq polymerase[18] (Takara Shuzo) were used for PCR of 25 cycles at 97 °C for 30 s, 57 °C for 30 s and 72 °C for 30 s; products were separated by agarose gel electrophoresis. A 7700 Sequence Detector (Applied Biosystems) was used for quantitative real-time PCR of cDNA amplified as described above with 2× PCR Master Mix (Applied Biosystems) and primers for 18S rRNA (as an internal control; Applied Biosystems) or primers specific for *Tlr5*, *Rorc*, *Aldh1a1*, *Aldh1a2* or *Aldh1a3* (Applied Biosystems), in a final volume of 25 μl. After incubation at 95 °C for 10 min, products were amplified by 35 cycles of 95 °C for 15 s, 60 °C for 60 s and 50 °C for 120 s.

Measurement of cytokines in supernatants. The concentrations of IFN-γ, IL-17, IL-4, IL-6, IL-10 and IL-12p40 were measured with the Bio-plex system (Bio-Rad) according to the manufacturer's instructions. The concentrations of IL-21, IL-23 and IgA were determined by ELISA (R&D Systems, eBioscience and Southern Biotech, respectively).

Immunohistochemical analysis. For analysis of the number of IgA^+ cells in the small intestinal lamina propria, fluorescein isothiocyanate–conjugated anti–mouse IgA (C10-3; 559354; BD Pharmingen) was applied overnight at 4 °C to sections cut from frozen tissue. Immunohistochemical staining was analyzed with a Radiance 2100 Bio-Rad confocal laser microscope (Bio-Rad).

Bacterial infection. *S. enterica* serovar *typhimurium* has been described[18]. *S. typhimurium* was grown in Luria-Bertani medium without shaking at 37 °C. The concentration of bacteria was determined on the basis of the absorbance at 600 nm. Bacteria were injected orally into mice.

Statistical analysis. Statistical significance was evaluated with an unpaired two-tailed Student's *t*-test in all experiments except **Supplementary Figure 8d**. A *P* value of less than 0.05 was considered significant. Kaplan-Meier plots and log-rank tests were used to assess the survival differences of control and mutant mice after bacterial infection (**Supplementary Fig. 8d**).

Accession codes. UCSD-Nature Signaling Gateway (http://www.signaling-gateway.org/): A002297 and A001174.

Note: Supplementary information is available on the Nature Immunology website.

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AUTHOR CONTRIBUTIONS

K.F. and S.U. did most of the experiments; S.U., K.J.I. and M.H.I. designed all the experiments; B.-G.Y. helped with the immunohistochemical analysis; Y.-J.I. and M.N. helped to isolate cells; S.S., T.T. and M.Y. provided advice for the experiments; Y.Y. provided *Id2*$^{-/-}$ mice; H.K. and M.M. provided advice for the experiments and manuscript; S.U. and S.A. prepared the manuscript; and S.A. directed the research.

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