Colonic Patches Direct the Cross-Talk Between Systemic Compartments and Large Intestine Independently of Innate Immunity[1]

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Although the mucosal and the systemic immune compartments are structurally and functionally independent, they engage in cross-talk under specific conditions. To investigate this cross-talk, we vaccinated mice with tetanus toxoid together with cholera toxin with s.c. priming followed by intrarectal (IR) boosting. Interestingly, higher numbers of Ag-specific IgA and IgG Ab-secreting cells (ASCs) were detected in the lamina propria of the large intestine of mice vaccinated s.c.-IR. Ag-specific ASCs from the colon migrated to SDF-1α/CXCL12 and mucosae-associated epithelial chemokine/CCL28, suggesting that $CXCR4^+$ and/or $CCR10^+$ IgA ASCs found in the large intestine after s.c.-IR are of systemic origin. In the colonic patches-null mice, IgA ASCs in the large intestine were completely depleted. Furthermore, the accumulation of IgA ASCs in the colonic patches by inhibition of their migration with FTY720 revealed that colonic patches are the IgA class-switching site after s.c.-IR. Most interestingly, s.c.-IR induced numbers of Ag-specific IgA ASCs in the large intestine of $TLR2^{-/-}$, $TLR4^{-/-}$, $MyD88^{-/-}$, and $TRIF^{-/-}$ mice that were comparable with those of wild-type mice. Taken together, our results suggest the possibility that cross-talk could occur between the large intestine and the systemic immune compartments via the colonic patches without the assistance of innate immunity. *The Journal of Immunology,* **2008, 180: 1609–1618.**

The possibility of developing safe and effective mucosal vaccines against a variety of microbial pathogens has aroused great interest because such vaccines would provide two layers of host protection—at mucosal surfaces and in systemic compartments (1). To realize the potential benefits of mucosal vaccines, a number of novel vaccination routes and adjuvants have been proposed but many of these have been challenged (2). Parenteral immunization is generally thought not to induce significant immune responses at mucosal surfaces. For instance, systemic vaccination with vaccinia induces cellular and humoral immune responses in systemic sites but not in mucosal sites. However, vaccination via mucosal routes can elicit both systemic and mucosal immunity (3). Mucosal vaccination has sometimes been used to overcome the barrier to recombinant viral vector immunization caused by preexisting same viral immunity (4). Therefore, an effective mucosal vaccine must be able to overcome preexisting immunity in systemic tissues that can provoke immune tolerance and/or hypersensitivity.

Sensitized T and B cells leave the mucosal inductive sites (e.g., Peyer's patches (PPs)[3] and nasopharynx-associated lymphoid tissue) transit through the thoracic duct and enter the circulation before settling down in selected mucosal tissues where they differentiate into memory/effector or plasma cells (5, 6). Recent studies (7) demonstrate that murine dendritic cells (DCs) isolated from PPs, but not from spleen and peripheral lymph nodes (LNs), increase the expression of mucosal homing receptors (e.g., α4β7 and CCR9). Furthermore, retinoic acid, specifically secreted by mucosal DCs, regulates the imprinting of mucosal DCs including DCs from PPs and mesenteric LNs (MLNs) for gut T (8) and B cell homing (9). Thus, these results may explain how the "common mucosal immune system" is compartmentalized and restricted within mucosal immune tissues.

However, results obtained by our own group and others have revealed the possibility of cross-talk between systemic compartments and some mucosal tissues. For instance, the combined systemic prime/mucosal boost strategy for the induction of both systemic and mucosal immune responses has been used to develop an efficient vaccination regimen (10–12). In addition, intrarectal (IR) immunization following systemic priming results in increases in both systemic and mucosal CTL responses, although systemic immunization alone could induce systemic but not mucosal CTL responses (13). In the murine allergic diarrhea model, systemically primed Ag-specific allergic responses selectively occur in the large but not the small intestine of BALB/c mice (14). Adoptive transfer of Ag-primed splenic $CD4^+$ cells obtained from GFP transgenic donor mice preferentially migrate into the large intestine. In addition, systemically primed Ag-specific $CD4^+$ T cells containing both $CD45RB^{high}$ and $CD45RB^{low}$ populations act as a pathogenic subset, in turn, leading to selective inflammatory responses in the large intestine (15). These results together with those of other studies suggest that a unique and important immunological cross-talk system exists between systemic compartments such as the spleen and the

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[3] Abbreviations used in this paper: PP, Peyer's patch; IR, intrarectal; CT, cholera toxin; TT, tetanus toxoid; ASC, Ab-secreting cells; CP, colonic patch; DC, dendritic cell; MNC, mononuclear cell; LN, lymph node; MLN, mesenteric lymph node; CLN, cutaneous LN; ILN, iliac lymph node; pIgR, polyimmunoglobulin receptor; TCI, transcutaneous; CSR, class switching recombination; AID, activation-induced cytidine deaminase; ILN, iliac LN; MEC, mucosae-associated epithelial chemokine.

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large intestine. We have previously offered several hypotheses to explain the phenomenon as, for example, the differences in the immune environments and microenvironments and/or existence of a specific cross-talk immune pathway. However, the exact underlying mechanism has yet to be elucidated.

To clarify the cross-talk interaction between mucosal and systemic compartments, we used a simple regimen of s.c. immunization followed by an IR boost. Interestingly, this regimen dramatically increased the number of Ag-specific IgA Ab-secreting cells (ASC) expressing functional CXCR4 and CCR10, thought to be systemically derived, in the large intestine. In contrast, two IR immunizations induced IgA ASC expressing CCR10 alone. These responses depended on colonic patches (CP), one of the major inductive sites in the large intestine, and seemed to be independent of innate immunity. Based on these results, we concluded that s.c. priming could modulate immune responses if followed by IR immunization, suggesting that the mucosal compartment, especially the large intestine, long thought to be separate from the systemic immunological compartment, is instead in close communication with it.

Materials and Methods

Mice

C57BL/6 and BALB/c mice were purchased from Charles River Laboratories (Orient). Timed pregnant C57BL/6 mice were purchased from the Daehan Biolink. To generate both PP- and CP-null mice or PP-null but CP-intact mice, pregnant C57BL/6 or BALB/c mice were injected i.v. with 600 μg of anti-IL-7Rα mAb on gestational day 14 (16). The structure of CP is circular and its center forms a protruding configuration giving it the appearance of a dome (17). Naive C57BL/6 mice possess 3 or 4 CP in the large intestine and are completely depleted by in utero treatment with anti-IL-7R mAb (provided by Dr. Masafumi Yamamoto, Nihon University at Matsudo, Chiba, Japan). The polyimmunoglobulin receptor $(pIgR)^{-/-}$ mice (18) was provided by Dr. Masanobu Nanno (Yakult Central Institute for Microbiological Research, Tokyo, Japan) and the OVA epitope (323–339)-specific TCR transgenic (DO11.10) mice was provided by Dr. Kazuhiko Yamamoto (University of Tokyo, Tokyo, Japan). All mice were maintained under pathogen-free conditions in the experimental facility at the International Vaccine Institute (Seoul, Korea), where they received sterilized food and water ad libitum.

Immunization

Mice were injected s.c. into the shoulder region and by i.p. routes with 10 μg of tetanus toxoid (TT) plus 1 μg of cholera toxin (CT; List Biological Laboratories; Campbell, CA) or by the transcutaneous (TCI) route with 100 μg of TT plus 50 μg of CT. For IR immunization, we anesthetized mice with a ketamine-xylazine mixture before administration of a 40-μl vaccine solution containing 10 μg of TT plus 2 μg of CT using disposable polystyrene tubing for oral feeding. For direct injection into the lumen of the small intestine, mouse abdomens were surgically opened, and the same volume and amount of TT plus CT as used in IR immunization was injected above the upper jejunum lumen with a fine needle. TT was provided by Dr. Yasushi Higashi (Biken Foundation, Osaka University, Osaka, Japan).

ELISA and ELISPOT for assessment of Ag-specific ASCs

We used serum and fecal extracts to determine Ag-specific Ab titers by ELISA as described elsewhere (19). Endpoint titers were expressed as the reciprocal $\log_2$ of the last dilution giving an OD at 450 nm of 0.1 greater than background. Mononuclear cells (MNCs) were obtained from the spleen, LN, and intestinal lamina propria as previously described (20). MNCs from the lamina propria of the small and large intestine were dissociated by digestion using a collagenase/DNase I enzyme solution after removal of PPs. Cells were then enriched by a discontinuous density gradient containing 40 and 75% Percoll (Amersham Biosciences). The number of total or Ag-specific ASCs was measured by an ELISPOT assay in accord with an established protocol (21). ASCs were counted with the aid of a stereomicroscope (SZ2-ILST; Olympus, Tokyo, Japan).

Chemotaxis assay

To evaluate the expression of chemokine receptors on Ag-specific ASCs, a chemotaxis assay and ELISPOT were combined. MNCs were isolated from the lamina propria of the large intestine and subjected to a chemotaxis assay (22). In brief, 5-μm Transwell inserts (Corning Costar) containing 1×10^6 MNCs were placed in the 24-well plate so as to make contact with 600 μl of the medium alone (basal) or with one of the following chemokines as well: 100 nM of SDF-1α/CXCL12 (R&D Systems), 100 nM of MIP-3α/CCL20 (R&D Systems), 100 nM of SLC/CCL21 (PeproTech), 300 nM of TECK/CCL25 (R&D Systems), 100 nM of CTACK/CCL27 (PeproTech), or 250 nM of mucosae-associated epithelial chemokine/CCL28 (R & D Systems). We did a preliminary experiment to determine the optimal concentration of different chemokines using MNCs from splenocytes and lamina propria of the small intestine. Two hours later, inserts were removed and the population that had migrated to the bottom wells was added into the wells of ELISPOT plates to measure the number of Ag-specific ASCs.

The proliferation of OVA-specific TCR transgenic $CD4^+$ T cells

We isolated MNCs from the spleen and LNs of DO11.10 mice, labeled the $CD4^+KJ1.26^+$ T cells with 10 μM of CFSE (Molecular Probes) for 15 min in RPMI at 37°C and washed the cells several times in PBS. In all 5×10^6 $CD4^+KJ1.26^+$ T cells were transferred into sex-matched naive or s.c.-primed BALB/c mice via the tail vein. One day after adoptive transfer when transferred $CD4^+$ T cells were circulated and distributed throughout the body, naive or s.c.-primed recipient mice were vaccinated IR with a mixture of 200 μg of OVA and 2 μg of CT or s.c. with a mixture of 20 μg of OVA and 1 μg of CT. The CFSE dilution in the MNCs of each tissue was analyzed after staining with anti-KJ1.26-PE (BD Pharmingen) (53-6.7) mAbs specific to DO11.10 clonotypic TCR in accord with the manufacturer's instructions.

CT-I-A^b tetramer staining

To detect CT-specific TCR-expressing $CD4^+$ T cells, CT-I-A^b tetramers were formed by incubation of I-A^b monomers and streptavidin-PE (Molecular Probes) with a molecular ratio of 5:1 for 2 h at 37°C. CT-I-A^b tetramers were incubated with cells for 2.5 h at 37°C in a CO_2 incubator. Then, the cells were stained with anti-CD4-APC. The data were obtained using FACSCalibur (BD Biosciences) with CellQuest (BD Biosciences), and the profiles were analyzed using FlowJo software (Tree Star).

RT-PCR

MNCs isolated from the colon lamina propria were sorted as $B220^-IgA^+$ and $B220^+IgA^+$ cells by FACSAria Cell Sorter (BD Bioscience). Total RNA was extracted from mouse tissues using TRIzol (Invitrogen) according to the manufacturer's instructions. cDNA was synthesized by Superscript II reverse transcriptase (Invitrogen) with oligo(dT) primer (Invitrogen). Activation-induced cytidine deaminase (AID), αCT, Iμ-Cα, and β-actin were amplified as described previously (23, 24). To check CCR10 mRNA expression, murine CCR10-specific primer (forward 5′-AGAGCTCTGTTACAAGGCTGATGTC-3′ and reverse 5′-CAGGTGG TACTTCCTAGATTCCAGC-3′) was used.

FTY 720 treatment

To induce lymphocyte retention in secondary lymphoid organs, we administered mice i.p. with FTY 720 (1 mg/kg body weight; Cayman Chemicals) every other day (25). The effect of FTY 720 treatment was monitored by regular analysis of peripheral blood lymphocyte and tissue lymphocyte counts.

Statistics

Data are expressed as the mean ± SD. Statistical comparison between experimental groups was performed using ANOVA or Student's *t* test.

Results

Dramatic increase in Ag-specific IgA and IgG ASCs in the large intestine after s.c.-IR immunization

As the first step in investigating the relation between systemic and mucosal immune systems, C57BL/6 mice were immunized by the IR route two weeks after s.c. immunization (s.c.-IR) with TT and CT as adjuvant. As expected, mice vaccinated by s.c.-s.c. (Fig. 1*a*) and s.c. or IR (data not shown) did not have significant numbers of Ag-specific ASCs in the small and large intestines; however, Ag-specific IgG ASCs were detected in the spleen of s.c.-immunized mice although in low levels because IgG ASCs migrate to the effective site or bone marrow rather than reside in spleen once they switch class from IgM to IgG (26) (Fig. 1*a*). Interestingly, however, brisk numbers of TT-specific IgA and IgG ASCs were detected in the lamina propria of the large intestine after s.c.-IR at levels similar to those induced by IR-IR immunization (Fig. 1*a*). Dramatic increases in the induction of IgA

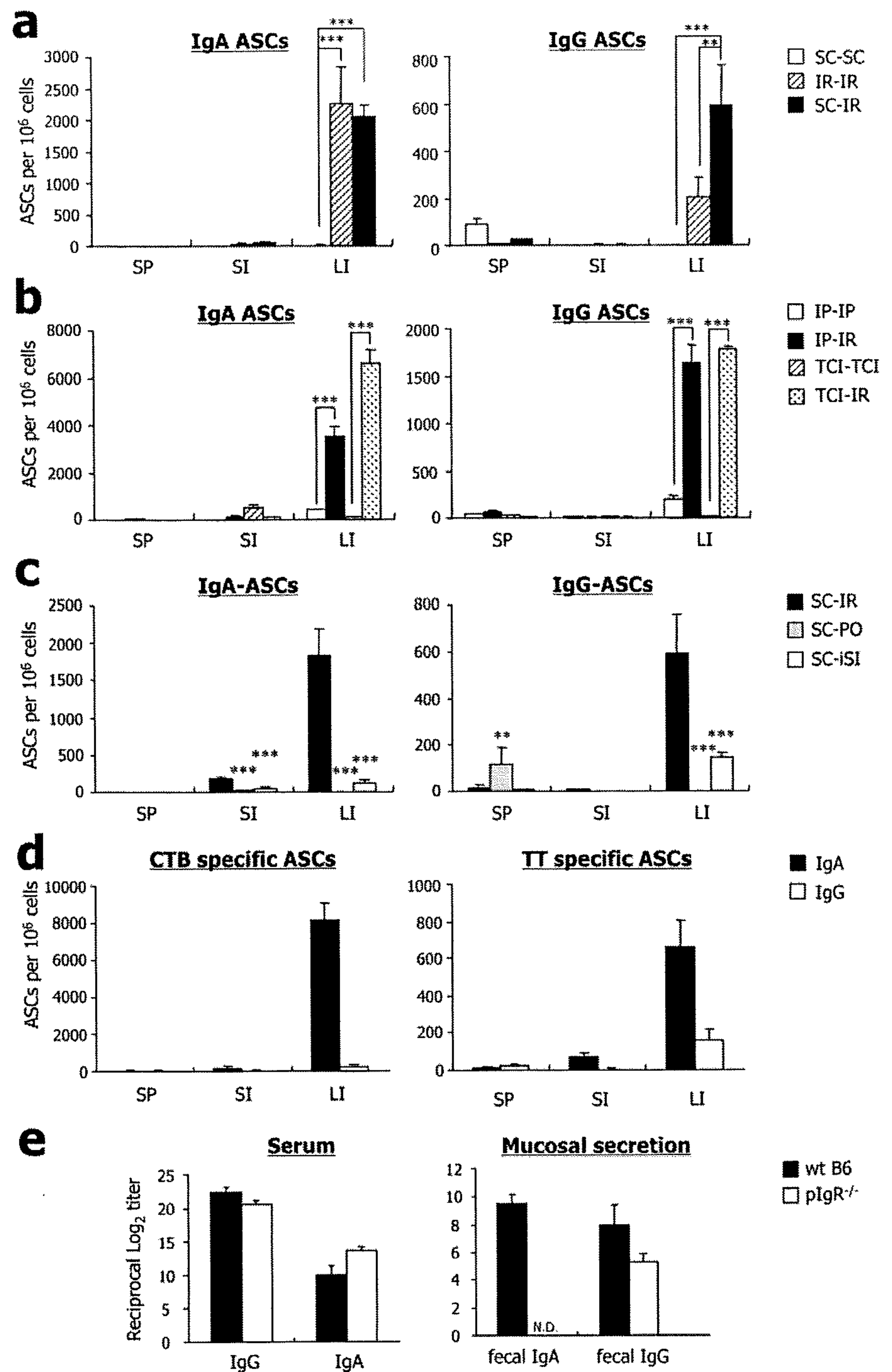

FIGURE 1. High numbers of Ag-specific IgA and IgG ASCs in the mouse large intestine are induced by s.c.-IR immunization. Mice received IR boosting following s.c. (*a*) or i.p. or TCI priming (*b*) with TT plus CT. Seven days after boosting, MNCs isolated from the spleen (SP) and lamina propria of the small (SI) and large intestine (LI) of the immunized mice were prepared. TT-specific ASCs per 10^6 cells were determined by ELISPOT. ***, $p < 0.001$ by ANOVA test (*a*) or Student's *t* test (*b*). *c*, Mice received IR or oral boosting (PO) following s.c. priming with TT plus CT. For another group of s.c. primed mice, the same dose of TT plus CT as used in the IR immunization was injected into the upper jejunum lumen of the small intestine (s.c.-iSI) to exclude the chance of inactivation of protein Ag by the acidic conditions and enzyme digestion of the stomach. **, $p < 0.01$ and ***, $p < 0.001$ by ANOVA test. *d*, Mice received IR boosting 100 days following s.c. priming to check the mucosal boosting effects on the long-term memory phase of systemic immunity. CTB- and TT-specific ASCs per 10^6 cells were determined using ELISPOT. *e*, Wild-type C57BL/6 and $pIgR^{-/-}$ mice receiving s.c.-IR immunization were used to determine the secretion of Ag-specific polymeric secretory IgA Abs into the gut lumen. TT-specific IgG or IgA Ab responses were determined in the sera and fecal extracts by ELISA.

and IgG ASCs in the large intestine against the B subunit of CT (CT-B) used as an adjuvant were also noted (data not shown). In addition, these highly enhanced responses were obtained regardless of mouse strain (data not shown). Ag-specific IgA Ab was also greatly increased in vaginal and nasal wash samples and in saliva and fecal extracts after s.c.-IR vaccination with TT plus CT (data not shown). These results strongly suggest that s.c.-IR combine effects not only in the colon but also in other mucosal compartments. To determine whether similar responses could be obtained by other routes of systemic priming combined with IR boosting, mice were boosted with IR immunization following i.p. or TCI immunization (i.p.-IR, TCI-IR) (Fig. 1*b*). The i.p.-IR and TCI-IR regimens induced large numbers of Ag-specific IgA and IgG ASCs in the large intestine, suggesting systemic priming was not limited to the s.c. route. However, when s.c.-primed animals were boosted orally (s.c.-PO), few TT-specific IgA and IgG ASCs were induced in the gut, demonstrating that the large but not the small intestine is the mucosal site interconnected with the systemic immune system (Fig. 1*c*). To confirm definitively the low responsiveness of small intestine-targeting immunization following systemic priming by bypassing the digestion and acidic conditions of the stomach, s.c.-primed mice were injected in the upper jejunum lumen of the small intestine (s.c.-iSI) with the same dose of TT plus CT used for IR immunization. This direct injection into the lumen of the small intestine induced a small amount of Ag-specific IgA and IgG ASCs in the small intestine and induced few Ag-specific IgA and IgG ASCs in the large

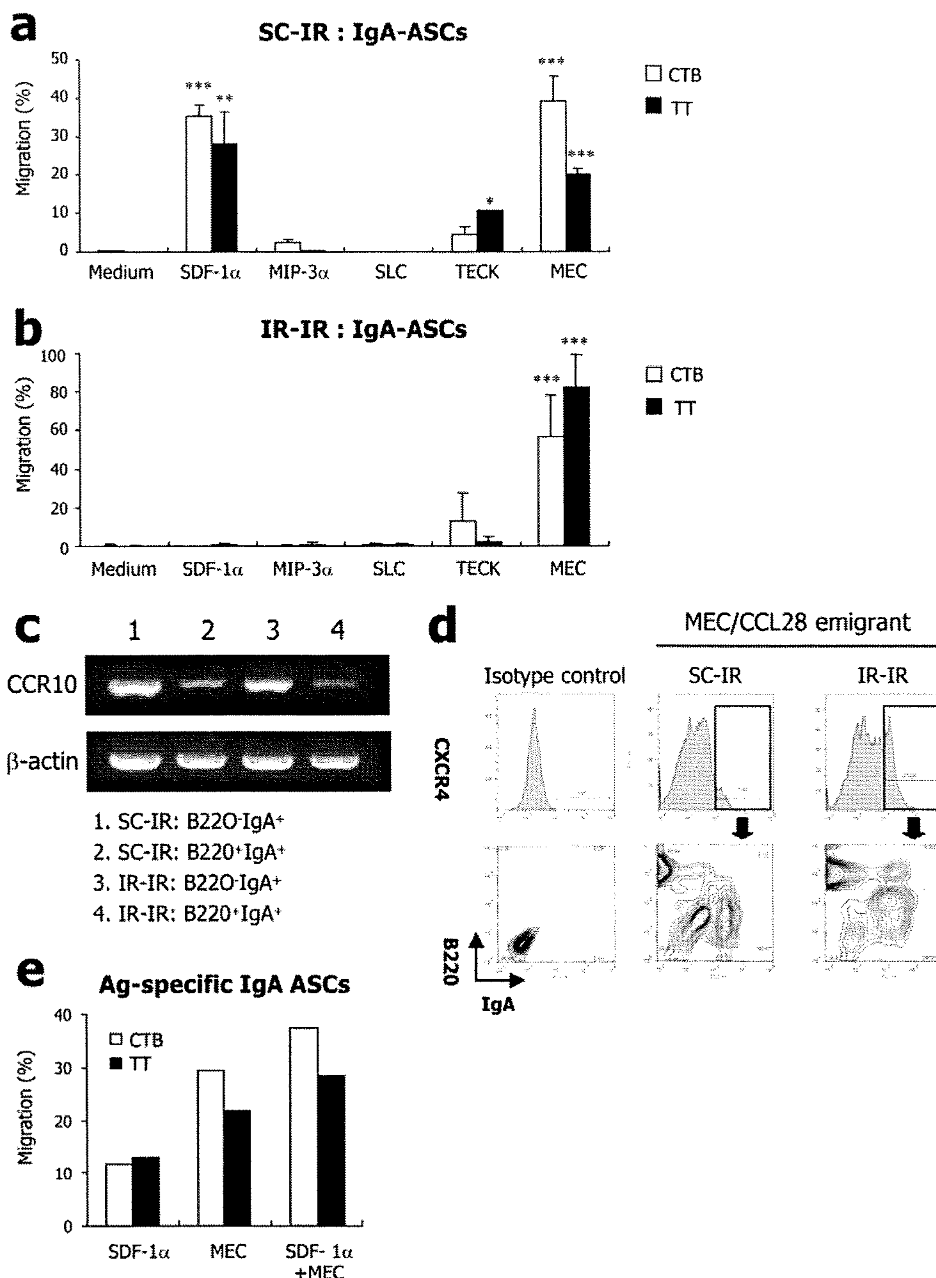

FIGURE 2. SDF-1α and MEC attracted Ag-specific IgA ASCs of the large intestine after s.c.-IR immunization. Each chemokine was added to the lower chamber and MNCs isolated from the lamina propria of the large intestine following s.c.-IR (*a*) or IR-IR immunization (*b*) were applied to the upper chamber well. CTB-specific and TT-specific IgA ASCs were determined using an ELISPOT assay to analyze the cells that had migrated into the lower chamber. The data represent the percentage of Ag-specific IgA ASCs that migrated into each chemokine relative to the total Ag-specific IgA ASCs. *, $p < 0.05$, **, $p < 0.01$, ***, $p < 0.001$ by ANOVA test. *c*, MNCs isolated from the colon lamina propria at day 7 after s.c.-IR or IR-IR were sorted as two population of $B220^-IgA^+$ and $B220^+IgA^+$ cells. CCR10 mRNA expression was determined by RT-PCR using CCR10-specific primer. β-actin was used as a control. *d*, MNCs from colon lamina propria after s.c.-IR or IR-IR were used for chemotaxis assay with a Transwell system. Cells that migrated to MEC/CCL28 were stained with anti-IgA, anti-B220, and anti-CXCR4 Ab to evaluate the CXCR4 expression on $CCR10^+$ IgA ASCs. *e*, MNCs isolated from colon lamina propria after s.c.-IR were applied to chemotaxis assay by a mixture of SDF-α and MEC and then CTB-specific and TT-specific IgA ASCs were determined using an ELISPOT assay to analyze the cells that had migrated into the lower chamber.

intestine, but not enough to be statistically significant (Fig. 1*c*). To assess the stress of surgery, we did sham surgery with PBS in s.c.-IR immunized mice. As expected, there were no significant changes in the numbers of Ag-specific IgA and IgG ASCs in the large intestine after sham surgery when compared with s.c.-IR mice that did not have surgery (data not shown).

To evaluate whether IR boosting affects the memory phase of immunity after systemic priming and induces a dramatic increase in Ag-specific IgA ASCs in the large intestine, mice received IR boosting 100 days after s.c. priming. Brisk numbers of CTB- and TT-specific IgA and IgG ASCs in the lamina propria of the large intestine were induced by IR boosting of the memory phase of systemic immunization (Fig. 1*d*). To further determine whether the IgA ASCs in the large intestine after s.c.-IR could secrete the dimeric form of IgA Abs associated with the secretory component into the gut lumen, IgA Ab responses after s.c.-IR were analyzed in $pIgR^{-/-}$ mice lacking this IgA secretion pathway (18). No IgA Abs were found in the fecal extracts of these mice, showing that Ag-specific IgA ASCs in the large intestine after s.c.-IR are able to secrete polymeric IgA into the lumen of the colon (Fig. 1*e*). As expected, no impairment in the Ag-specific IgG Ab responses was noted in the fecal extract of $pIgR^{-/-}$ mice, because the secretion of IgG Abs was regulated by the neonatal Fc receptor but not by pIgR (27). All of these data suggest that IR boosting during the effector and memory phases of systemic priming could induce a dramatic increase in Ag-specific IgA and IgG ASCs, which secrete soluble Abs into the lumen in the lamina propria of the large intestine.

CXCR4- and CCR10-expressing IgA ASCs were detected in the large intestine after s.c.-IR immunization

Because IR-IR immunization can induce numerous Ag-specific IgA ASCs in the colon (Fig. 1*a*), we wondered whether Ag-specific IgA ASCs induced by s.c.-IR differed from those induced by IR-IR. If so, IgA ASCs induced by s.c.-IR might originate from or

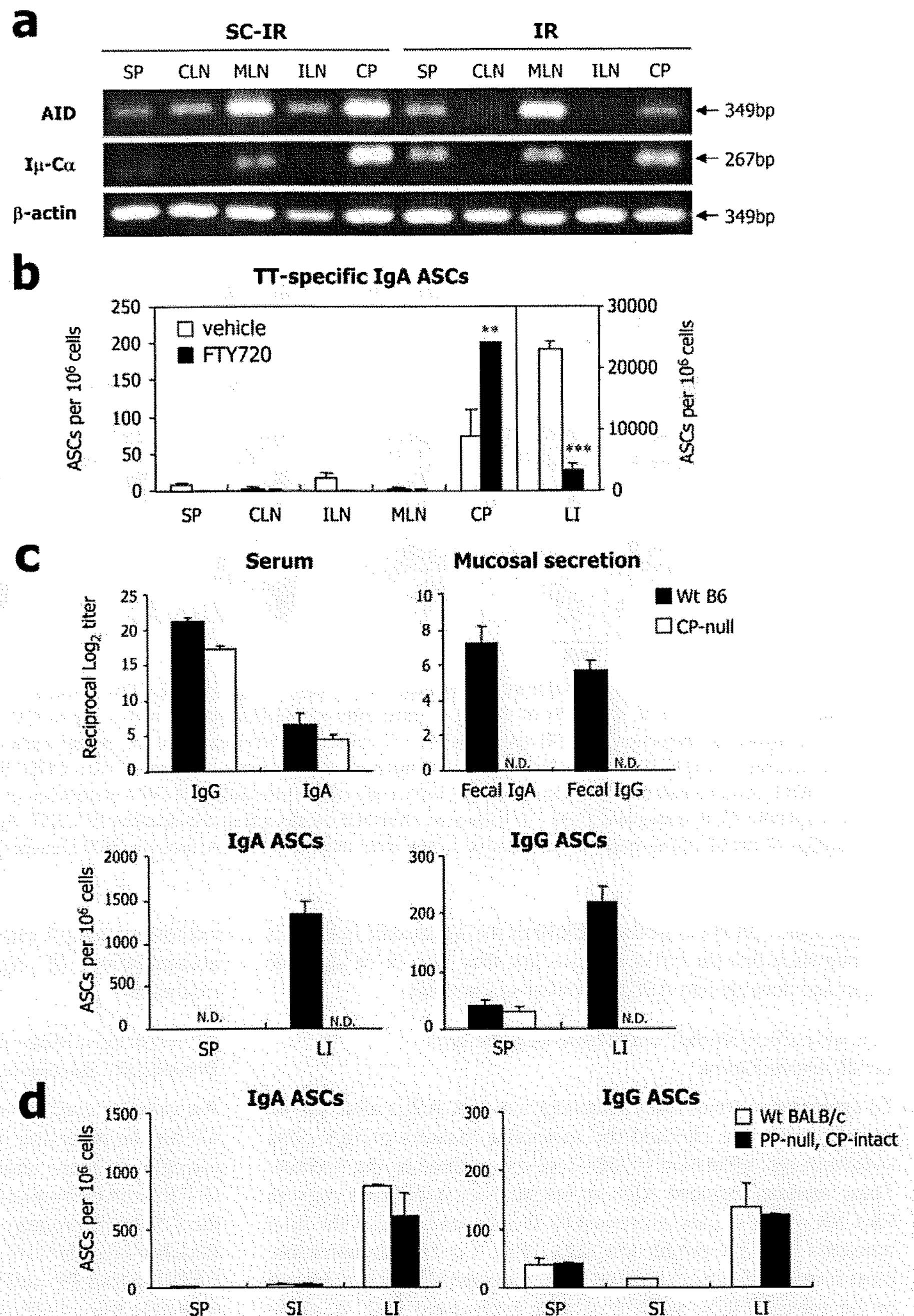

FIGURE 3. CPs are essential for IgA class switching after s.c.-IR immunization. *a*, Expression of a series of IgA isotype CSR-associated mRNA including AID and Iμ-Cα was determined in the organized lymphoid tissues of spleen, CLN, MLN, and ILN, and CP at day 7 after s.c.-IR or IR immunization alone using RT-PCR. β-actin was used as a cDNA control. *b*, Mice were treated with FTY 720 every other day beginning 1 day before IR immunization. The numbers of TT-specific IgA ASCs in the spleen, CLN, ILN, MLN, CP, and lamina propria of the large intestine were measured at day 7 after s.c.-IR immunization. **, $p < 0.01$, ***, $p < 0.001$ compared with the vehicle group (Student's *t* test). *c*, TT-specific Ab responses in the serum and fecal extract, and TT-specific IgA and IgG ASCs in the MNCs isolated from spleen and large intestine were evaluated in the CP-null C57BL/6 progeny treated with anti-IL-7Rα mAb in utero at day 7 following s.c.-IR immunization. N.D.; Not detected. *d*, TT-specific IgA and IgG ASCs were evaluated in the PP-null and CP-intact BALB/c progeny treated with anti-IL-7Rα mAb in utero following s.c.-IR.

be affected by systemic derived cells. We evaluated the migratory characteristics of Ag-specific IgA ASCs induced by s.c.-IR or IR-IR immunization by testing the chemokine responsiveness of these cells using a Transwell chemotaxis assay. As expected on the basis of earlier findings that MEC expression predominates in the colon (28), we found that Ag-specific IgA ASCs elicited by an IR-IR regime migrated principally toward MEC/CCL28 (~70–80%) and, to a much lesser degree, toward TECK/CCL25 (5–10%) (Fig. 2*b*). Surprisingly, similar numbers of Ag-specific IgA ASCs (30–40%) induced by an s.c.-IR regime migrated toward SDF-1α/CXCL12 and toward MEC (Fig. 2*a*). Similar results were obtained from both TT-specific and CTB-specific IgA ASCs. In addition, we checked expression levels of CCR10 mRNA on polyclonal B cells such as $B220^-IgA^+$ and $B220^+IgA^+$ cells after s.c.-IR or IR-IR immunization (Fig. 2*c*). CCR10 mRNA was highly expressed on IgA^+ cells in the colon LP after s.c.-IR or IR-IR. The level of CCR10 mRNA was higher in the $B220^-IgA^+$ cells than in $B220^+IgA^+$ cells. To investigate whether Ag specific IgA ASCs induced by s.c.-IR will coexpress CXCR4 and CCR10, we stained colon MNCs with anti-IgA, anti-B220, or anti-CXCR4 Ab following migration toward MEC. The $CCR10^+$ IgA ASCs after both s.c.-IR and IR-IR immunization partially coexpressed CXCR4 (Fig. 2*d*). Furthermore, we performed the chemotaxis assay to check whether the number of IgA ASCs that migrate toward SDF-1α plus MEC will be additive or not. Of interest, more Ag-specific IgA ASCs migrated toward the mixture of SDF-α and MEC than did each chemokine (Fig. 2*e*). These results also suggest that Ag-specific IgA ASCs partially coexpressed CCR10 and CXCR4. In contrast, Ag-specific IgG ASCs elicited by s.c.-IR or IR-IR immunization did not migrate toward any chemokine we tested (data not shown), suggesting that they do not use these chemokine-chemokine receptor interactions for migration into the colon. The use

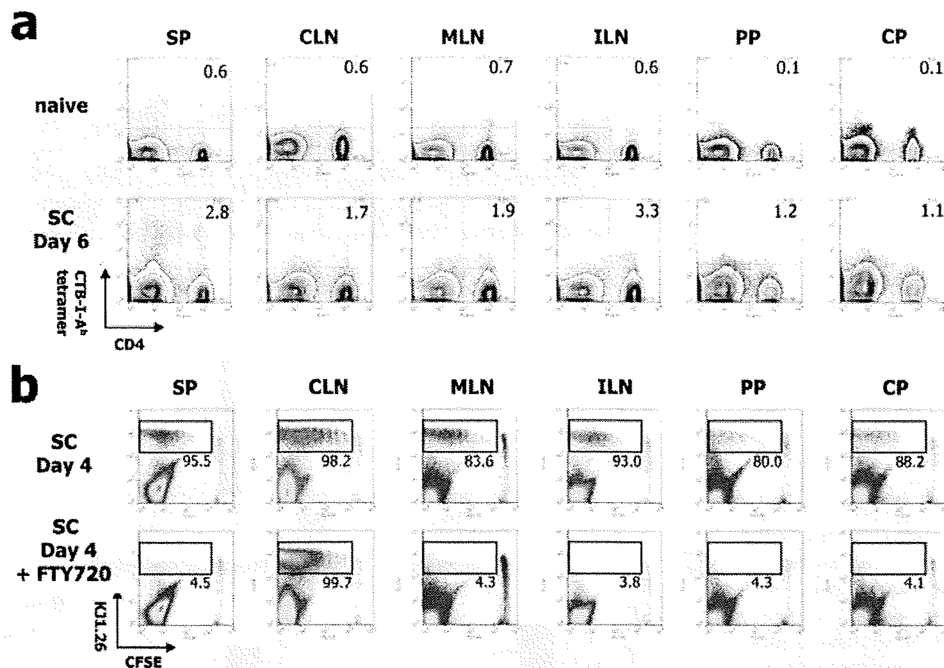

FIGURE 4. Subcutaneous priming alone could induce the increase in Ag-specific CD4$^+$ T cells at mucosal inductive sites. *a*, MNCs isolated from the spleen, CLN, MLN, ILN, PPs, CPs of C57BL/6 mice after s.c. immunization with 1 μg of CT plus 10 μg of TT were stained with CTB-I-A^b tetramer to detect 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.

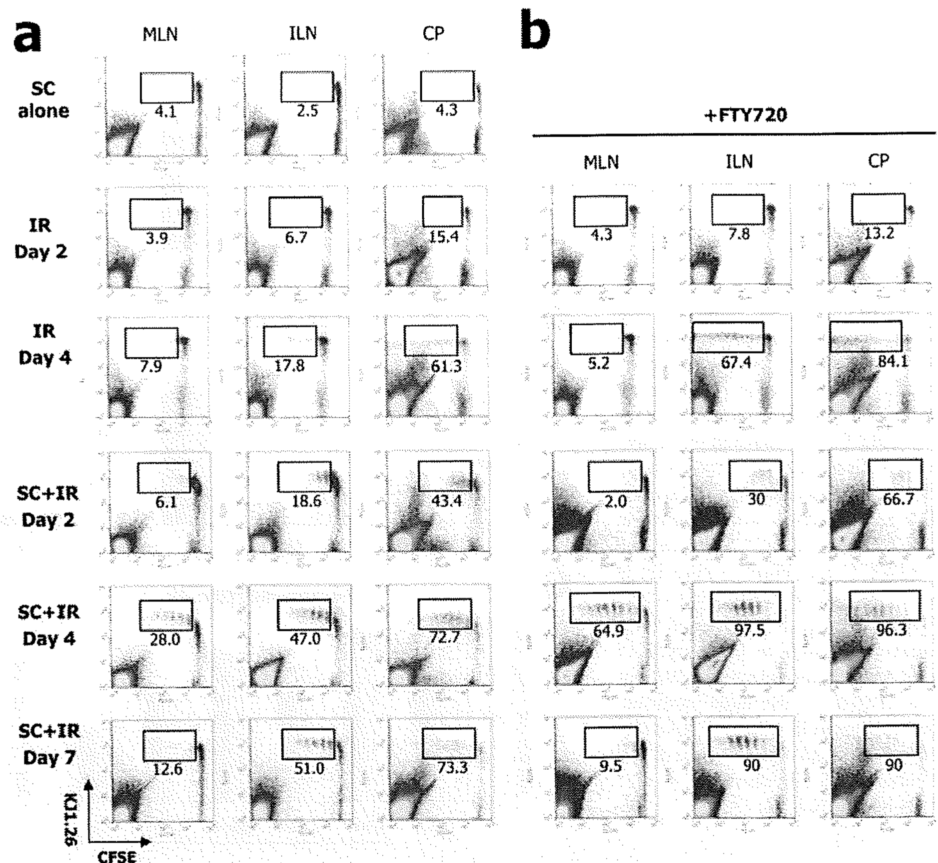

FIGURE 5. s.c. priming could expand Ag-specific CD4$^+$ T cell responses by subsequent IR immunization. *a*, s.c. primed or naive mice were adoptively transferred with CFSE-labeled OVA-specific TCR transgenic CD4$^+$ T cells of DO11.10 mice and then immunized with OVA plus CT by the IR route. *b*, Mice 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$^+$

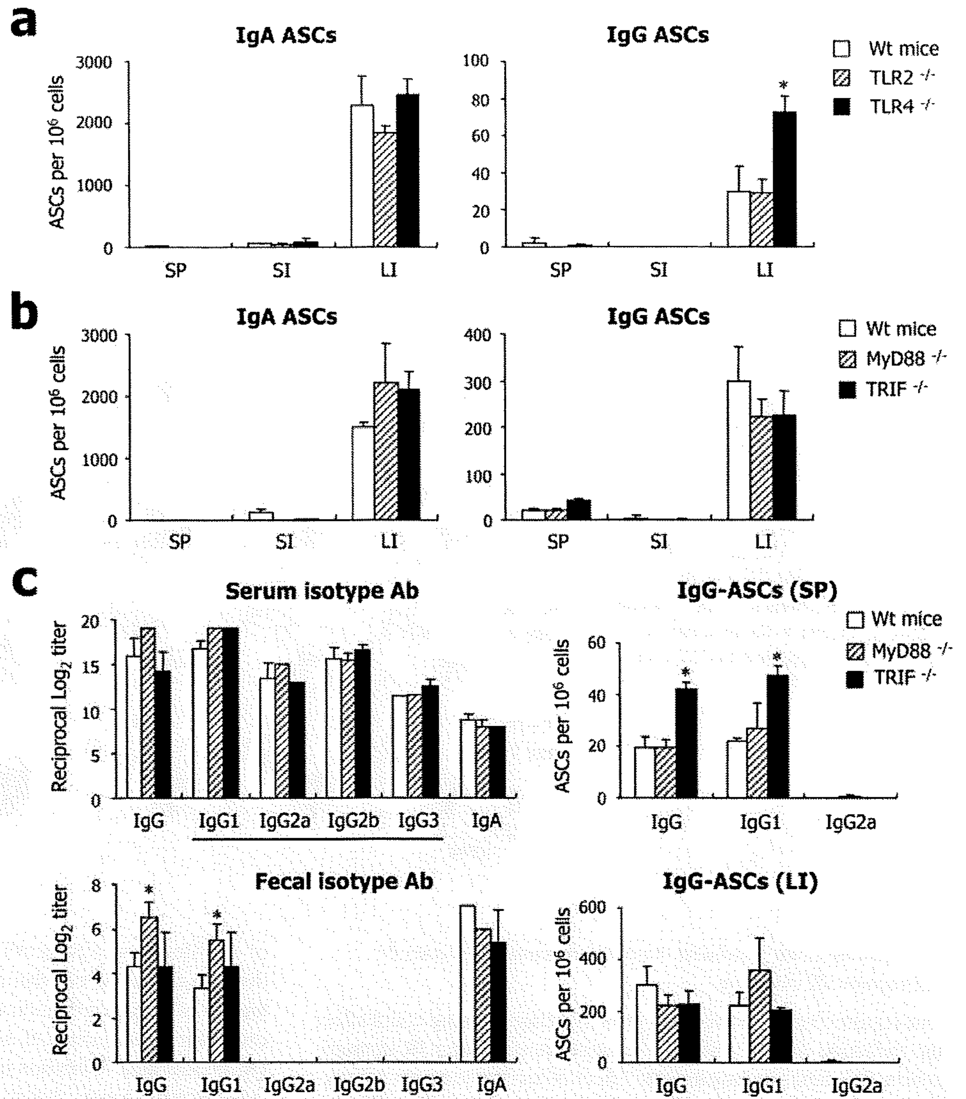

FIGURE 6. Innate immunity may not direct the cross-talk between the large intestine and systemic compartments following s.c.-IR immunization. To examine the involvement of TLR signaling for the induction of Ag-specific ASCs in the large intestine following s.c.-IR immunization, TT-specific IgA and IgG 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)

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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

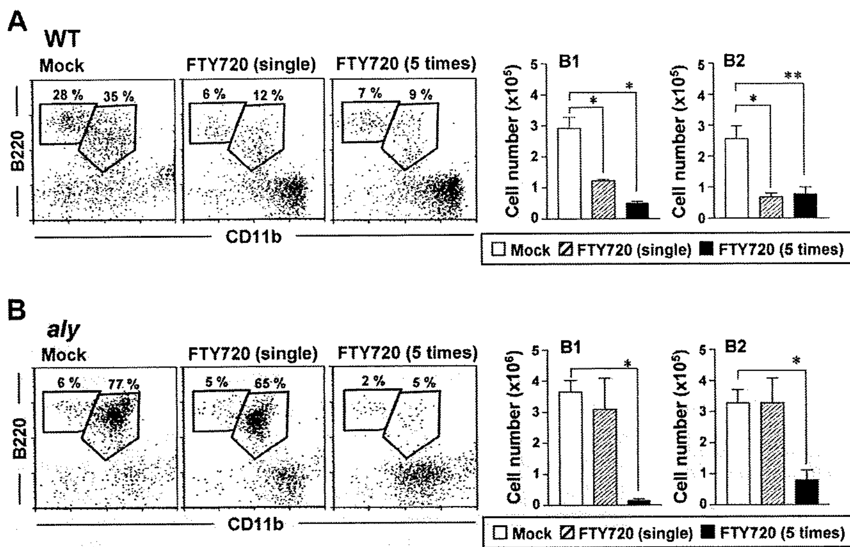

Figure 1. Decreased reactivity of peritoneal B cells to FTY720 in *aly* mice. Cells were isolated from the peritoneal cavities of WT (A) or *aly* (B) mice 12 hours after single or multiple (that is 5) injections of FTY720 (right) or vehicle only (mock; left), and cell populations were analyzed by flow 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

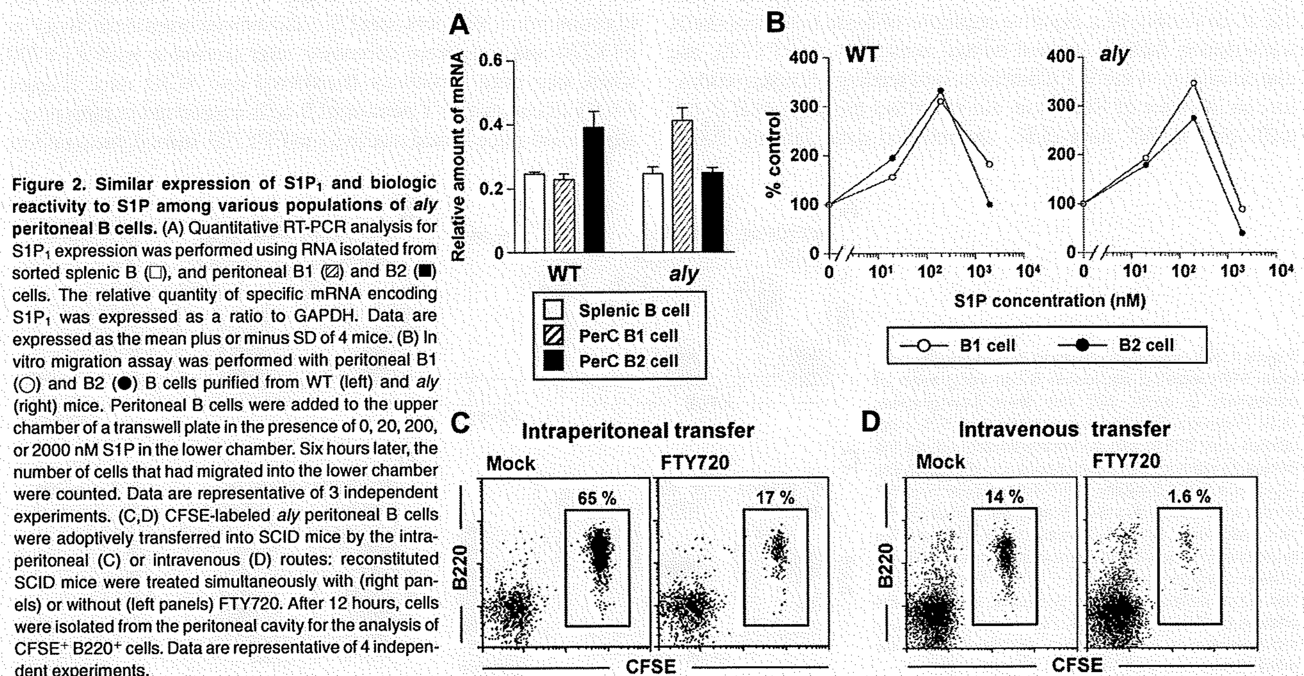

Figure 2. Similar expression of S1P$_1$ and biologic reactivity to S1P among various populations of *aly* peritoneal B cells. (A) Quantitative RT-PCR analysis for S1P$_1$ expression was performed using RNA isolated from sorted splenic B (□), and peritoneal B1 (▨) and B2 (■) cells. The relative quantity 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.

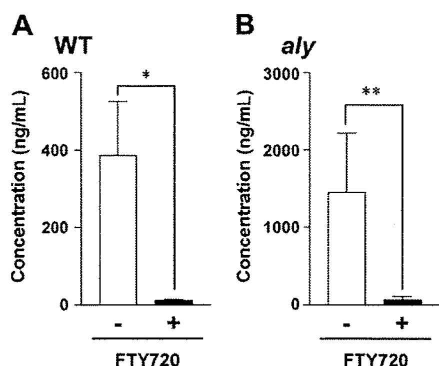

Figure 3. *aly* peritoneal B cells show a comparable dependence on S1P for intestinal IgA production. WT (A) or *aly* (B) peritoneal B cells (5×10^6 cells) were adoptively transferred into SCID mice, which were treated with vehicle only (□) or FTY720 (■) every 2 days. Two weeks after cell transfer, fecal 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

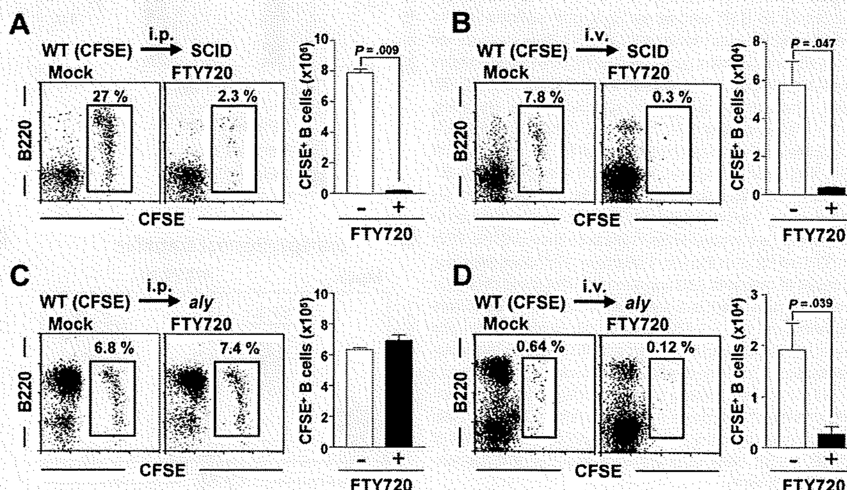

Figure 4. NIK-mediated signaling in non-B cells controls S1P-mediated peritoneal B-cell emigration of peritoneal B cells, but not their immigration. (A,B) Peritoneal B cells were isolated from WT mice, labeled with CFSE, and adoptively transferred via the intraperitoneal (i.p.) (A) or intravenous (i.v.) (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).

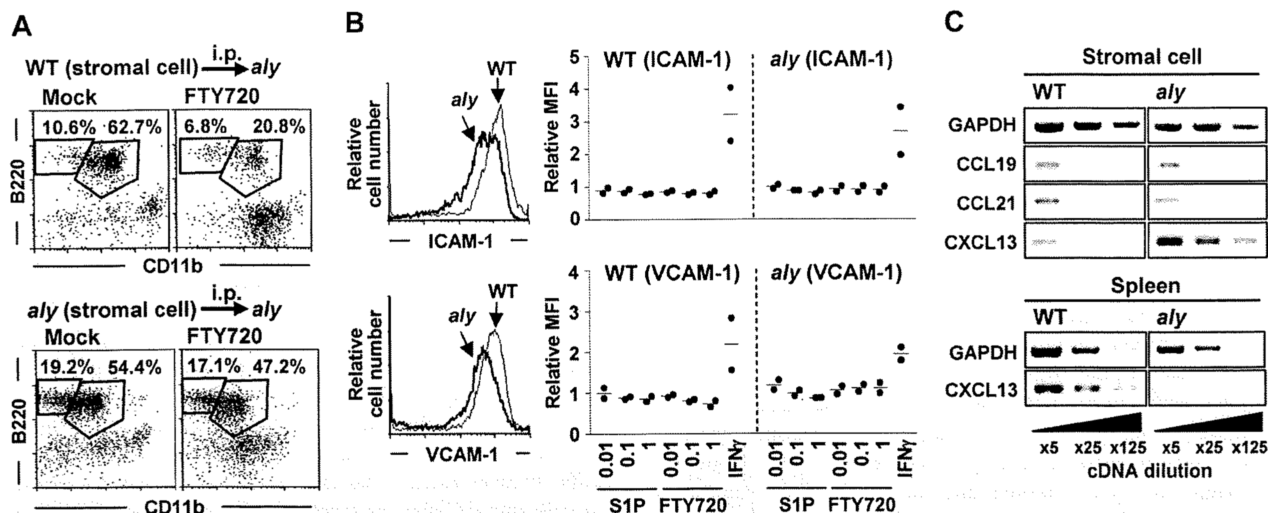

Figure 5. Requirement of NIK-mediated signaling in stromal cells for the emigration of peritoneal B cells. (A) *aly* mice were intraperitoneally (i.p.) transferred with WT (top panels) or *aly* (bottom panels) stromal cells. Two weeks after cell transfer, mice were treated with FTY720 for the analysis 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.

Conflict-of-interest disclosure: The authors declare no competing financial interests.

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Cell

Pattern Recognition Receptors and Inflammation

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Infection of cells by microorganisms activates the inflammatory response. The initial sensing of infection is mediated by innate pattern recognition receptors (PRRs), which include Toll-like receptors, RIG-I-like receptors, NOD-like receptors, and C-type lectin receptors. The intracellular signaling cascades triggered by these PRRs lead to transcriptional expression of inflammatory mediators that coordinate the elimination of pathogens and infected cells. However, aberrant activation of this system leads to immunodeficiency, septic shock, or induction of autoimmunity. In this Review, we discuss the role of PRRs, their signaling pathways, and how they control inflammatory responses.

Introduction

Inflammation is a protective response by the body to ensure removal of detrimental stimuli, as well as a healing process for repairing damaged tissue (Medzhitov, 2008). Inflammation is caused by various factors such as microbial infection, tissue injury, and cardiac infarction. Classically, inflammation is characterized by five symptoms: redness, swelling, heat, pain, and loss of tissue function. These macroscopic symptoms reflect increased permeability of the vascular endothelium allowing leakage of serum components and extravasation of immune cells. The inflammatory response is then rapidly terminated and damaged tissues are repaired. However, overproduction of cytokines (a cytokine storm) by immune cells to overwhelm pathogens can be fatal. A cytokine storm can also be caused by noninfectious diseases such as graft-versus-host disease (GVHD). Inflammatory responses are also critical for the pathogenesis of autoimmune diseases.

The innate immune system is the major contributor to acute inflammation induced by microbial infection or tissue damage (Akira et al., 2006; Beutler et al., 2006). Furthermore, innate immunity is also important for the activation of acquired immunity. Although innate immune cells including macrophages and dendritic cells (DCs) play important roles, nonprofessional cells such as epithelial cells, endothelial cells, and fibroblasts also contribute to innate immunity. Germline-encoded pattern recognition receptors (PRRs) are responsible for sensing the presence of microorganisms. They do this by recognizing structures conserved among microbial species, which are called pathogen-associated molecular patterns (PAMPs). Recent evidence indicates that PRRs are also responsible for recognizing endogenous molecules released from damaged cells, termed damage-associated molecular patterns (DAMPs). Currently, four different classes of PRR families have been identified. These families include transmembrane proteins such as the Toll-like receptors (TLRs) and C-type lectin receptors (CLRs), as well as cytoplasmic proteins such as the Retinoic acid-inducible gene (RIG)-I-like receptors (RLRs) and NOD-like receptors (NLRs). These PRRs are expressed not only in macrophages and DCs but also in various nonprofessional immune cells. With the exception of some NLRs, the sensing of PAMPs or DAMPs by PRRs upregulates the transcription of genes involved in inflammatory responses. These genes encode proinflammatory cytokines, type I interferons (IFNs), chemokines and antimicrobial proteins, proteins involved in the modulation of PRR signaling, and many uncharacterized proteins. The expression patterns of the inducible genes differ among activated PRRs.

The inflammatory response is orchestrated by proinflammatory cytokines such as tumor necrosis factor (TNF), interleukin (IL)-1, and IL-6. These cytokines are pleiotropic proteins that regulate the cell death of inflammatory tissues, modify vascular endothelial permeability, recruit blood cells to inflamed tissues, and induce the production of acute-phase proteins. Although TNF and IL-6 are mainly regulated at the transcriptional and translational levels, the production of IL-1β is regulated by a two-step mechanism. The first step is the expression of an IL-1β zymogen, pro-IL-1β, which is regulated by the synthesis of its mRNA in a TLR signal-dependent manner. However, IL-1β maturation requires cleavage of pro-IL-1β by a protease, caspase-1, which is activated independently of TLR signaling. The complex that activates caspase-1, called the inflammasome, is composed of NLRs, ASC, and caspase-1 (see Review by K. Schroder and J. Tschopp on page 821 of this issue). Type I IFNs, including multiple forms of IFN-α and single forms of IFN-β, IFN-ω, etc., are also involved in the modulation of inflammation (Honda et al., 2006). Type I IFNs play central roles in antiviral responses by inducing apoptotic cell death in virally infected cells, rendering cells resistant to virus infection, activating acquired immunity, and stimulating hematopoietic stem cell turnover and proliferation. Secreted type I IFNs alert the surrounding cells via type I IFN receptors by triggering a signaling cascade that leads to the phosphorylation and nuclear translocation of IFN-stimulated gene factor 3 (ISGF3), a complex composed of Signal Transducers and Activators of Transcription 1 (STAT1), STAT2, and IFN-regulatory factor (IRF) 9. ISGF3

Table 1. PRRs and Their Ligands

PRRs	Localization	Ligand	Origin of the Ligand
TLR			
TLR1	Plasma membrane	Triacyl lipoprotein	Bacteria
TLR2	Plasma membrane	Lipoprotein	Bacteria, viruses, parasites, self
TLR3	Endolysosome	dsRNA	Virus
TLR4	Plasma membrane	LPS	Bacteria, viruses, self
TLR5	Plasma membrane	Flagellin	Bacteria
TLR6	Plasma membrane	Diacyl lipoprotein	Bacteria, viruses
TLR7 (human TLR8)	Endolysosome	ssRNA	Virus, bacteria, self
TLR9	Endolysosome	CpG-DNA	Virus, bacteria, protozoa, self
TLR10	Endolysosome	Unknown	Unknown
TLR11	Plasma membrane	Profilin-like molecule	Protozoa
RLR			
RIG-I	Cytoplasm	Short dsRNA, 5'triphosphate dsRNA	RNA viruses, DNA virus
MDA5	Cytoplasm	Long dsRNA	RNA viruses (Picornaviridae)
LGP2	Cytoplasm	Unknown	RNA viruses
NLR			
NOD1	Cytoplasm	iE-DAP	Bacteria
NOD2	Cytoplasm	MDP	Bacteria
CLR			
Dectin-1	Plasma membrane	β-Glucan	Fungi
Dectin-2	Plasma membrane	β-Glucan	Fungi
MINCLE	Plasma membrane	SAP130	Self, fungi

induces expression of IFN-inducible antiviral genes such as protein kinase R (PKR) and 2′5′-oligoadenylate synthase (OAS) among others. PKR suppresses the proliferation of virus-infected cells and 2′5′-OAS activates RNase L, which cleaves viral nucleotides in order to inhibit virus replication.

In this Review, we discuss how distinct PRRs (with particular emphasis on TLRs and RLRs) sense the presence of pathogens and cellular insults and the mechanisms by which these PRR signals elicit inflammation.

TLRs and Their Ligands

The TLR family is one of the best-characterized PRR families and is responsible for sensing invading pathogens outside of the cell and in intracellular endosomes and lysosomes (Akira et al., 2006). TLRs are characterized by N-terminal leucine-rich repeats (LRRs) and a transmembrane region followed by a cytoplasmic Toll/IL-1R homology (TIR) domain. Ten TLRs have been identified in humans and 12 in mice. Different TLRs recognize the different molecular patterns of microorganisms and self-components (Table 1).

TLR2 senses various components from bacteria, mycoplasma, fungi, and viruses. These components include the lipoproteins of bacteria and mycoplasma. TLR2 recognizes its ligands by forming a heterodimer with either TLR1 or TLR6 (Figure 1). The resulting TLR1/TLR2 and TLR6/TLR2 complexes recognize distinct ligands (triacyl and diacyl lipoproteins, respectively). The crystal structures of the extracellular domains of TLR2, TLR1, and TLR6 revealed that they form M-shaped structures and that their cognate ligands interact with internal pockets formed by the TLR1/TLR2 or TLR6/TLR2 heterodimers (Jin et al., 2007). Stimulation with TLR2 ligands, such as triacyl and diacyl lipoproteins, induces the production of various proinflammatory cytokines (but not type I IFNs) in macrophages and DCs. However, another report showed that TLR2 in inflammatory monocytes induced type I IFNs in response to viral infection, suggesting that the cellular responses to TLR2 ligands differ depending on the cell types involved (Barbalat et al., 2009). TLR10 is related to TLR1 and TLR6 based on sequence similarity. TLR10 seems to be functional in humans, although mouse TLR10 is disrupted by insertion of an endogenous retrovirus. The ligand for TLR10 has not been identified.

TLR4 recognizes lipopolysaccharide (LPS) together with myeloid differentiation factor 2 (MD2) on the cell surface. LPS is a component derived from the outer membrane of Gram-negative bacteria and is known to be a cause of septic shock. The crystal structure of a complex comprising TLR4, MD2, and LPS revealed that two complexes of TLR4-MD2-LPS interact symmetrically to form a TLR4 homodimer (Park et al., 2009). TLR4 is also involved in the recognition of viruses by binding to viral envelope proteins. In addition, TLR4 modulates the pathogenesis of H5N1 avian influenza virus infection by recognizing a DAMP rather than the virus itself (Imai et al., 2008). Acute lung injury caused by avian influenza virus infection produces endogenous oxidized phospholipids, which stimulate TLR4. Mice lacking TLR4 were found to be resistant to avian flu-induced lethality.

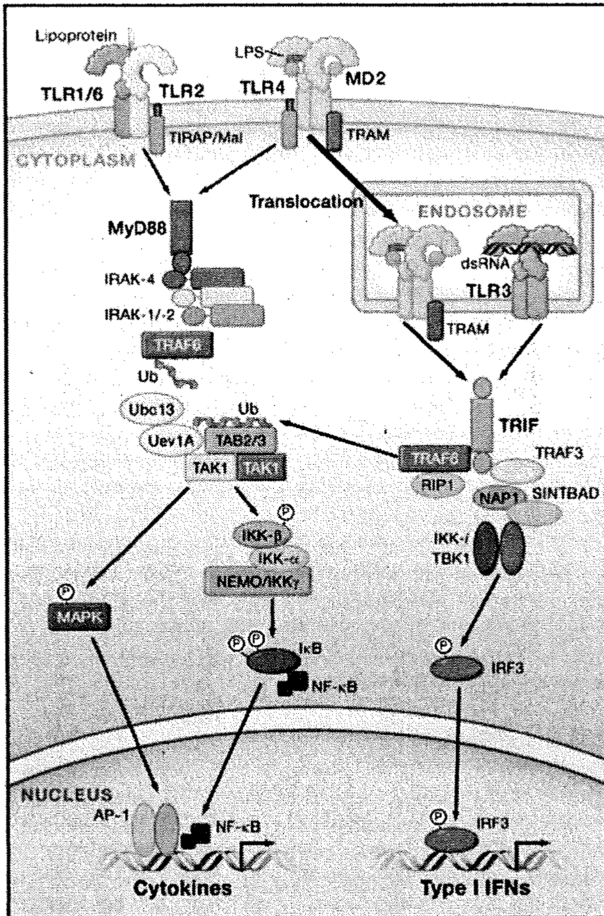

Figure 1. TLR2, TLR3, and TLR4 Signaling Pathways

Lipoproteins and LPS are recognized on the cell surface by a heterodimer of TLR1/6 and TLR2, and by 2 sets of TLR4/MD2 complexes, respectively. Ligand stimulation recruits MyD88 and TIRAP to the TLR, and a complex of IRAKs and TRAF6 is subsequently formed. TRAF6 acts as an E3 ubiquitin ligase and catalyzes formation of a K63-linked polyubiquitin chain on TRAF6 itself and generation of an unconjugated polyubiquitin chain with an E2 ubiquitin ligase complex of Ubc13 and Uev1A. Ubiquitination activates a complex of TAK1, TAB1, and TAB2/3 resulting in the phosphorylation of NEMO and the activation of an IKK complex. Phosphorylated IκB is degraded, and the freed NF-κB translocates to the nucleus where it drives expression of cytokine genes. Simultaneously, TAK1 activates MAP kinase cascades leading to the activation of AP-1, which is also critical for the induction of cytokine genes. LPS induces translocation of TLR4 to the endosome together with TRAM. TLR3 is present in the endosome and recognizes dsRNA. TLR3 and TLR4 activate TRIF-dependent signaling, which activates NF-κB and IRF3 resulting in the induction of proinflammatory cytokine genes and type I IFNs. TRAF6 and RIP1 activate NF-κB, whereas TRAF3 is responsible for phosphorylation of IRF3 by TBK1/IKK-*i*. NAP1 and SINTBAD are required for the activation of TBK1/IKK-*i*. Phosphorylated IRF3 translocates into the nucleus to induce expression of type I IFN genes.

TLR5 is highly expressed by DCs of the lamina propria (LPDCs) in the small intestine, where it recognizes flagellin from flagellated bacteria. In response to flagellin, LPDCs induce B cells to differentiate into IgA-producing plasma cells and trigger the differentiation of naive T cells into antigen-specific Th17 and Th1 cells (Uematsu et al., 2008). TLR11, which is present in mice but not in humans, shows close homology to TLR5. TLR11 recognizes uropathogenic bacteria and a profilin-like molecule derived from the intracellular protozoan *Toxoplasma gondii* (Yarovinsky et al., 2005).

A set of TLRs, comprising TLR3, TLR7, TLR8, and TLR9, recognize nucleic acids derived from viruses and bacteria, as well as endogenous nucleic acids in pathogenic contexts (Akira et al., 2006). Activation of these TLRs leads to the production of type I IFNs in addition to proinflammatory cytokines. TLR3 detects viral double-stranded (ds) RNA in the endolysosome. TLR3 is involved in the recognition of polyinosinic polycytidylic acid (poly I:C), a synthetic dsRNA analog. Although inoculation of mice with poly I:C induces the production of cytokines as well as type I IFNs in mice, TLR3 is essential for the production of cytokines such as IL-12p40, but not type I IFNs in sera (Kato et al., 2006). The crystal structure of TLR3 bound to dsRNA revealed that dsRNA binds to the N-terminal and C-terminal portions of TLR3 LRRs, and this ligand binding dimerizes two TLR3 molecules (Choe et al., 2005; Liu et al., 2008). Mouse TLR7 and human TLR7/8 recognize single-stranded (ss) RNAs from RNA viruses, as well as small purine analog compounds (imidazoquinolines). TLR7 also detects RNAs from bacteria such as Group B Streptococcus in endolysosomes in conventional DCs (cDCs) (Mancuso et al., 2009). TLR9 senses unmethylated DNA with CpG motifs derived from bacteria and viruses. Although the CpG motif was thought to be essential for TLR9 stimulation, the DNA sugar backbone of 2′ deoxyribose also mediates TLR9 recognition (Haas et al., 2008). In addition to DNA, TLR9 also recognizes hemozoin, a crystalline metabolite of hemoglobin produced by the malaria parasite (Coban et al., 2005). TLR9 directly binds to hemozoin, and a crude extract of the malaria parasite elicits parasite-antigen-specific immune responses via TLR9 (Coban et al., 2010). However, another report shows that TLR9 recognizes malaria parasite DNA contained in purified hemozoin, and that hemozoin only transports malaria parasite DNA to the endosome, where TLR9 is present (Parroche et al., 2007). Further studies will clarify the role of TLR9 in the recognition of malaria parasite components. TLR7 and TLR9, but not TLR3, are highly expressed in plasmacytoid DCs (pDCs), a cell type that produces large amounts of type I IFNs in response to virus infection.

Accumulating evidence underscores the importance of the localization of TLRs in the cell for their recognition by ligand (Barton and Kagan, 2009). Given that self-nucleotides are potent TLR ligands and may facilitate autoimmunity, TLRs that recognize self-nucleotides are compartmentalized to avoid unwanted activation. Although TLR1, TLR2, TLR4, TLR5, and TLR6 are present on the plasma membrane, TLR3, TLR7, and TLR9 are mainly present on the endoplasmic reticulum (ER) membrane. It has been proposed that self-nucleic acids are degraded by extracellular or endosomal DNases prior to recognition by TLRs. Nucleic acid-sensing TLRs are recruited from the ER to endolysosomes following stimulation by their ligands (Figure 2). The mechanism by which nucleotide-recognizing TLRs are recruited from the ER to the endolysosome compartment remains to be clarified. However, a forward genetics screen in mice