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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., $\alpha4\beta7$ 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.

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

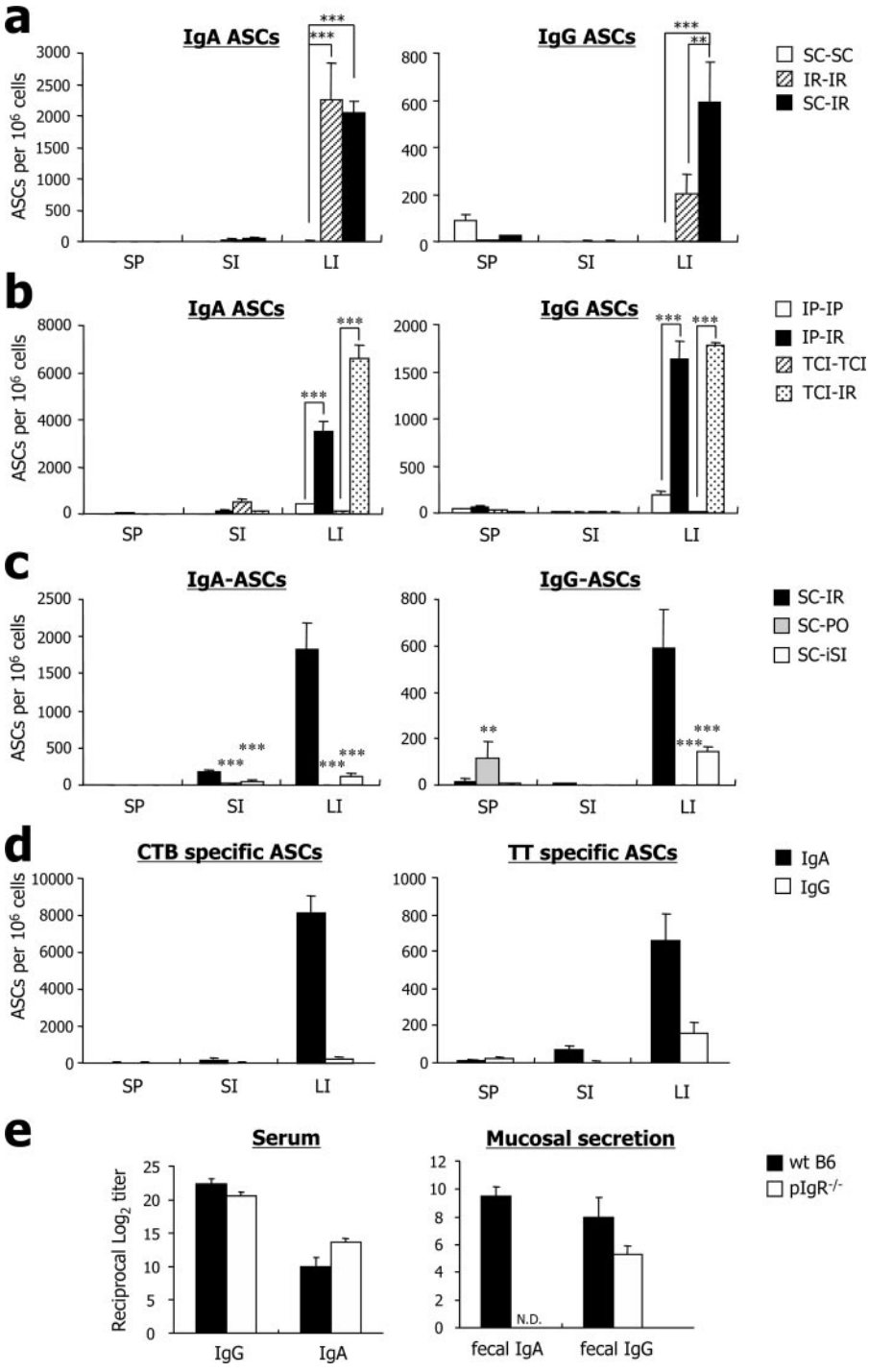

FIGURE 1. High numbers of Ag-specific IgA and IgG ASCs in the mouse large intestine are induced by s.c.-IR immunization. Mice received IR boosting following s.c. (*a*) or i.p. or TCI priming (*b*) with TT plus CT. Seven days after boosting, MNCs isolated from the spleen (SP) and lamina propria of the 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

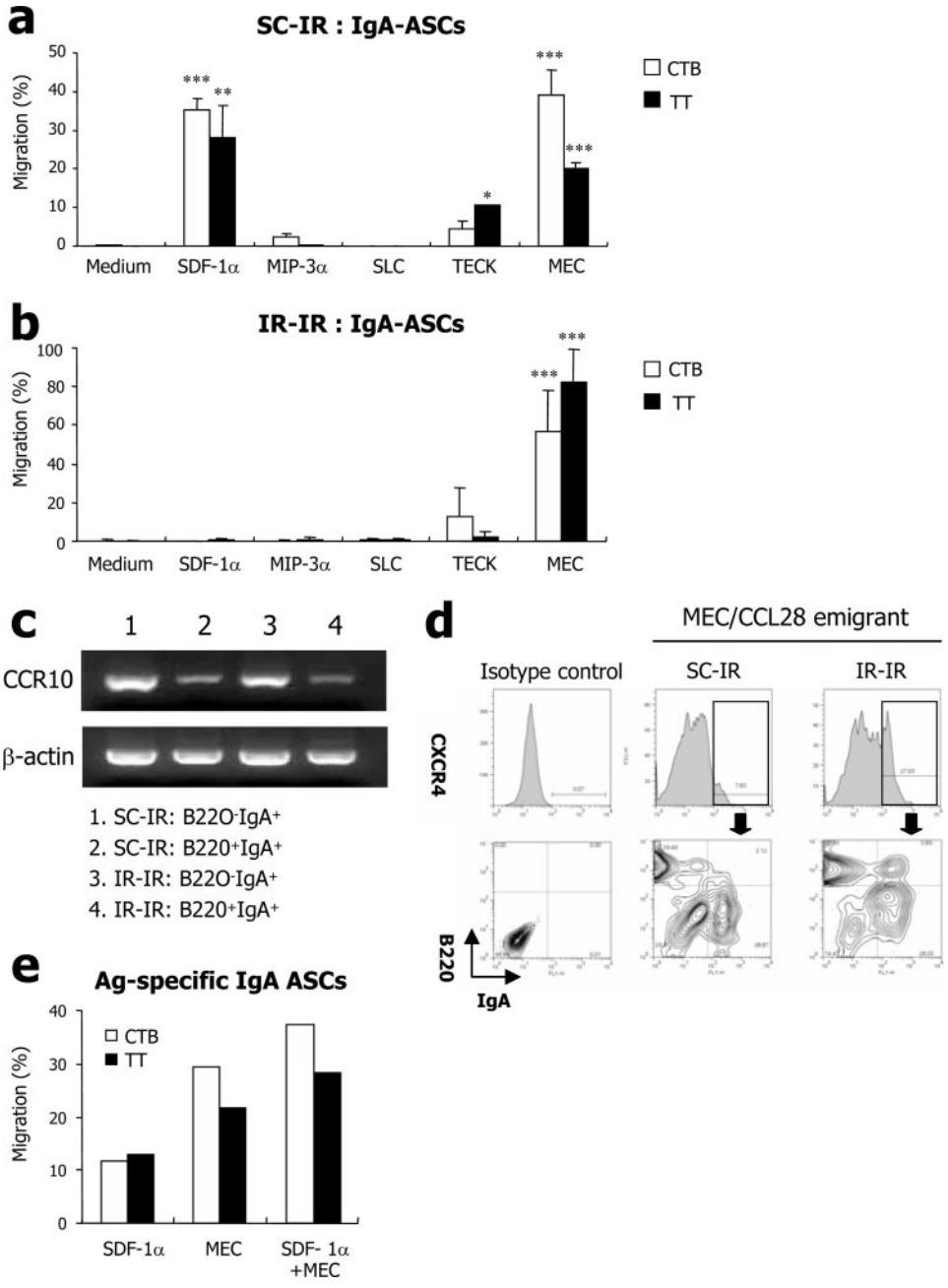

FIGURE 2. SDF-1α and MEC attracted Ag-specific IgA ASCs of the large intestine after s.c.-IR immunization. Each chemokine was added to the lower chamber and MNCs isolated from the lamina propria of the large intestine following s.c.-IR (*a*) or IR-IR immunization (*b*) were applied to the upper chamber 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

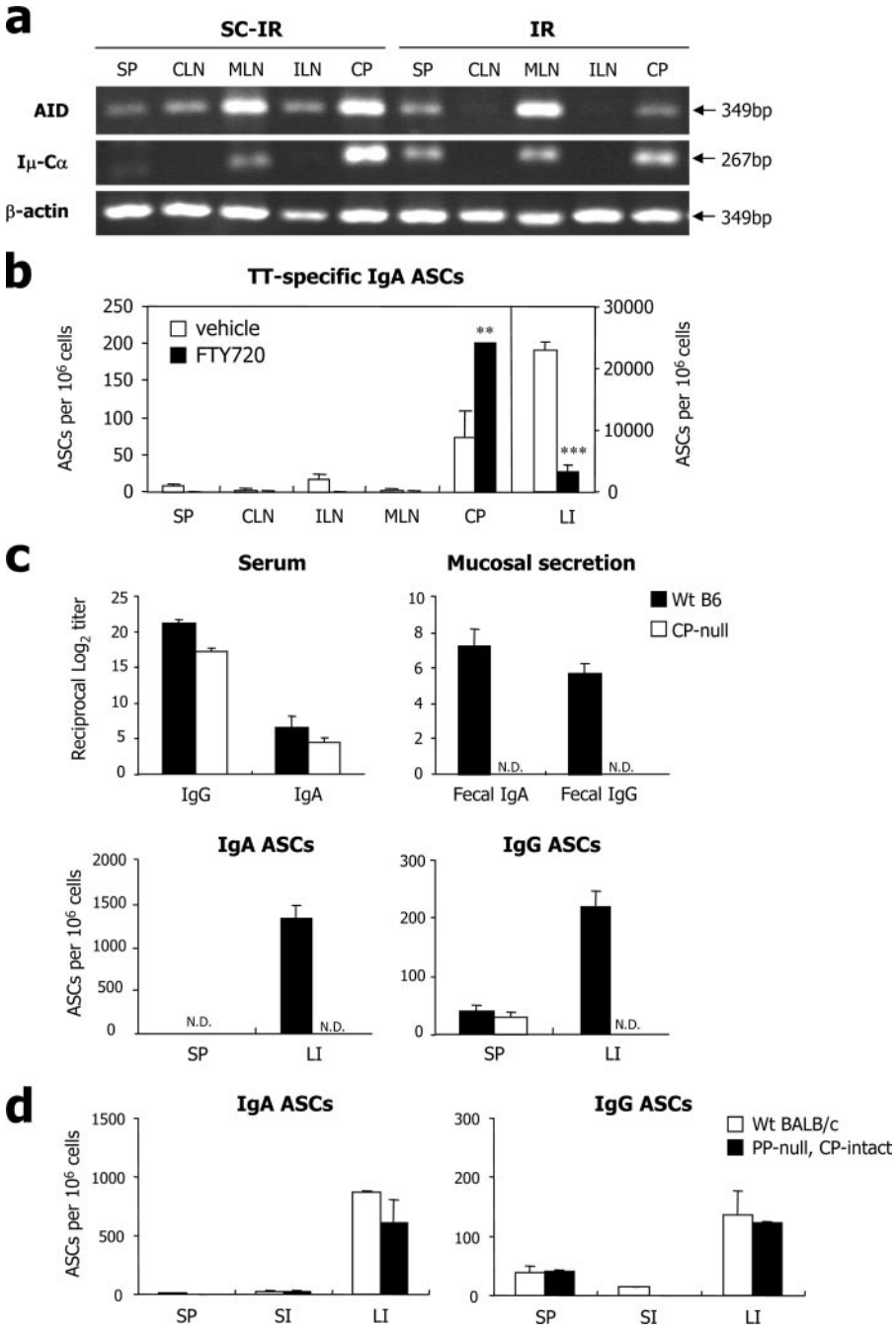

FIGURE 3. CPs are essential for IgA class switching after s.c.-IR immunization. *a*, Expression of a series of IgA isotype CSR-associated mRNA including AID and Iμ-Cα was determined in the organized lymphoid tissues of spleen, CLN, MLN, and ILN, and CP at day 7 after s.c.-IR or IR immunization alone 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

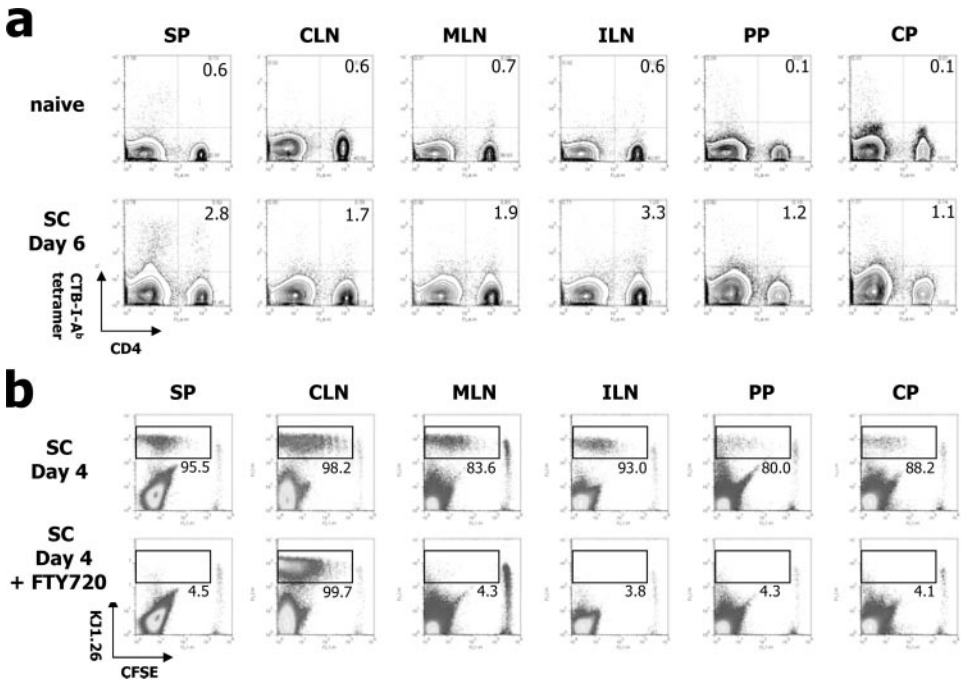

FIGURE 4. Subcutaneous priming alone could induce the increase in Ag-specific $CD4^+$ T cells at mucosal inductive sites. *a*, MNCs isolated from the spleen, CLN, MLN, ILN, PPs, CPs of C57BL/6 mice after s.c. immunization with 1 μg of CT plus 10 μg of TT were stained with CTB-I-A^b tetramer to detect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

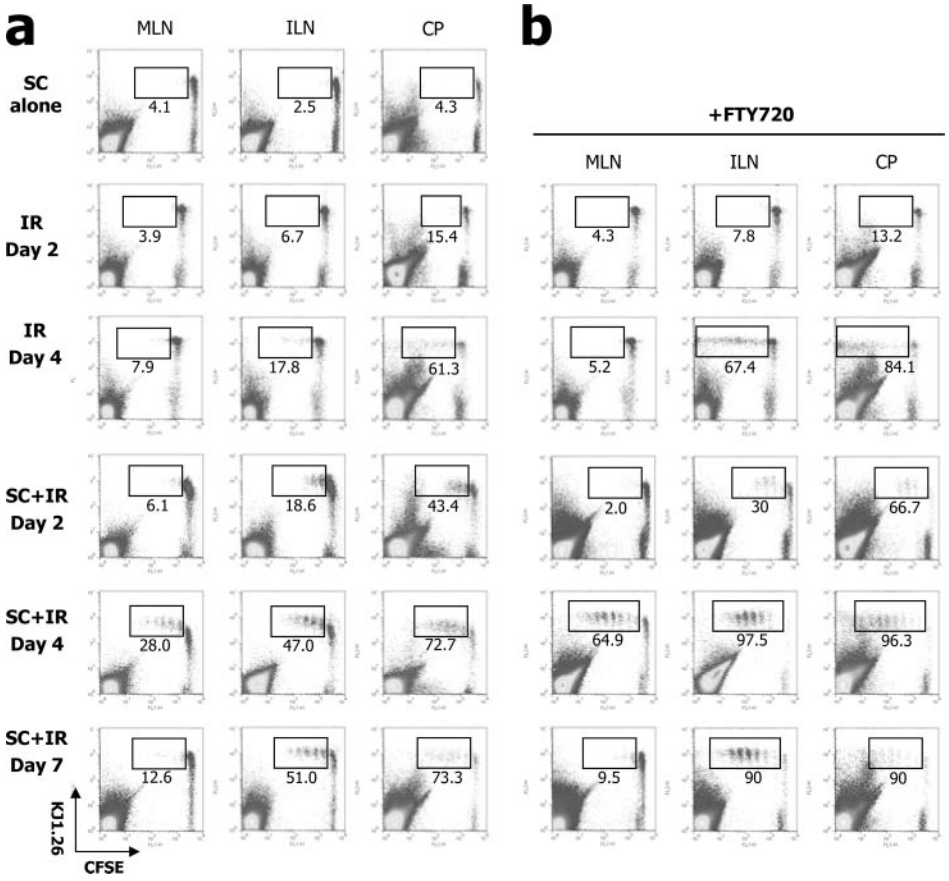

FIGURE 5. s.c. priming could expand Ag-specific $CD4^+$ T cell responses by subsequent IR immunization. *a*, s.c. primed or naive mice were adoptively transferred with CFSE-labeled OVA-specific TCR transgenic $CD4^+$ T cells of DO11.10 mice and then immunized with OVA plus CT by the IR route. *b*, Mice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

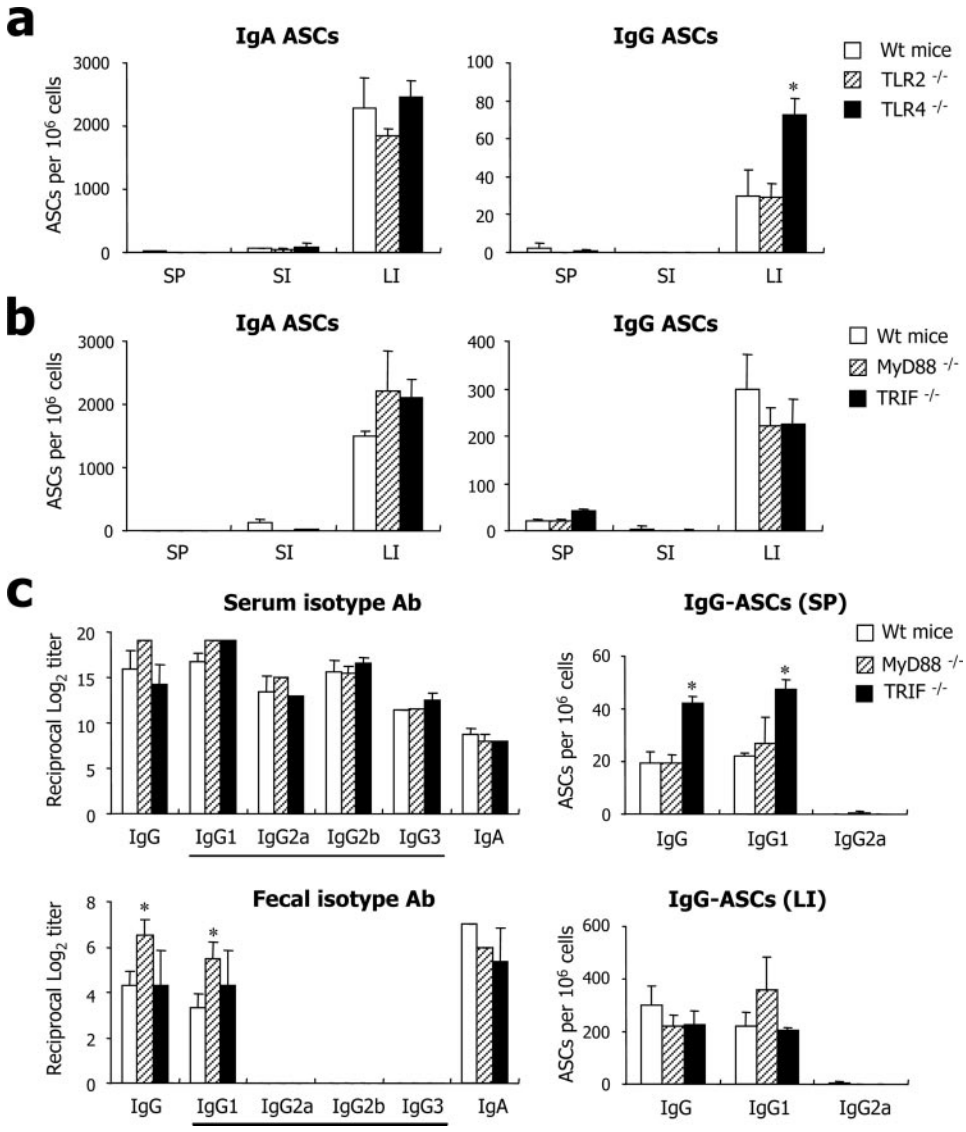

FIGURE 6. Innate immunity may not direct the cross-talk between the large intestine and systemic compartments following s.c.-IR immunization. To examine the involvement of TLR signaling for the induction of Ag-specific ASCs in the large intestine following s.c.-IR immunization, TT-specific IgA and IgG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