Sphingosine 1-Phosphate Regulates the Egress of IgA Plasmablasts from Peyer's Patches for Intestinal IgA Responses[1]

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It is well established that Peyer's patches (PPs) are sites for the differentiation of IgA plasma cell precursors, but molecular and cellular mechanisms in their trafficking remain to be elucidated. In this study, we show that alterations in type 1 sphingosine 1-phosphate (S1P) receptor expression during B cell differentiation in the PPs control the emigration of IgA plasma cell precursors. Type 1 S1P receptor expression decreased during the differentiation of IgM^+B220^+ B cells to IgA^+B220^+ B cells, but recovered on IgA^+B220^- plasmablasts for their emigration from the PPs. Thus, IgA^+B220^- plasmablasts migrated in response to S1P in vitro. Additionally, IgA^+ plasmablasts selectively accumulated in lymphatic regions of PPs when S1P-mediated signaling was disrupted by FTY720 treatment. This accumulation of IgA^+ plasmablasts in the PPs led to their reduction in the intestinal lamina propria and simultaneous impairment of Ag-specific intestinal IgA production against orally administered Ag. These findings suggest that S1P regulates the retention and emigration of PP B cells and plays key roles in the induction of intestinal IgA production. ***The Journal of Immunology*****, 2008, 180: 5335–5343.**

Secretory IgA (S-IgA)[4] in the intestinal lumen acts as the gateway controller against pathogenic and commensal microorganisms (1–3). S-IgA production is achieved by two distinct subsets of B cells, termed B1-B and B2-B cells (3). B1-B cells can be distinguished from B2-B cells by cell surface molecules (e.g., B220, IgM, IgD, CD5, and Mac-1), origin, and growth properties (4). B1-B cells migrate from nonorganized tissues such as the peritoneal cavity to the intestinal lamina propria (iLP), where they further differentiate to IgA-secreting plasma cells (PCs) (3). In addition to the peritoneal B cells, the common mucosal immune system (CMIS) acts as an alternative pathway for S-IgA production (1, 2). In the CMIS-dependent pathway, Peyer's patches (PPs) act as inductive tissues, where B cells are primed and switched from the μ- to α-chain by immunological interactions with dendritic cells (DCs) and T cells, and differentiate to the precursors of IgA^+ PCs with the expression of gut-tropic chemokine receptors (e.g., CCR9) and adhesion molecules (e.g., $\alpha_4\beta_7$ integrin) (5–7). These changes allow the precursors of IgA^+ PCs to traffic specifically to the iLP, where they further differentiate to IgA-secreting PCs.

The tissue-specific homing of Ag-primed lymphocytes is tightly regulated by a combination of adhesion molecules and chemokines (8, 9). In addition to these molecules, sphingosine 1-phosphate (S1P) also regulates lymphocyte trafficking, especially emigration from the organized lymphoid tissues, such as the thymus and secondary lymphoid organs (9, 10). Five types of S1P receptors have been identified, with the type 1 S1P receptor ($S1P_1$) primarily expressed on lymphocytes (10, 11). $S1P_1$ expression on T cells is cyclically modulated during their circulation, which determines their retention in lymph nodes or exit into the blood and lymph in response to the S1P gradient (12–14). T cells also control $S1P_1$ expression during their development and activation (15–17). Thus, treatment with FTY720, a $S1P_1$ modulator, decreases the number of circulating T cells in both blood and lymph by inhibiting their emigration from the secondary lymphoid organs and thymus (18).

In addition to T cells, S1P is also involved in the regulation of B cell trafficking. FTY720 impairs plasma Ab production, especially against T-dependent Ag due to the abolishment of germinal center (GC) formation (19–21). S1P is also important in the determination of PC tropism into bone marrow (22). Furthermore, we have recently demonstrated that S1P contributes to the regulation of peritoneal B cell trafficking into the intestine and subsequent intestinal S-IgA production (23), as well as naive $\alpha\beta TCR^+$ intraepithelial T lymphocytes in naive mice (24) and pathogenic T and mast cell trafficking into the intestine under intestinal allergic conditions (25). These findings provide strong evidence that S1P plays an essential role in the regulation of lymphocyte trafficking in both systemic and mucosal humoral immunity. However, it is still unclear whether S1P is involved in the regulation of intestinal S-IgA

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[4] Abbreviations used in this paper: S-IgA, secretory IgA; AFC, Ab-forming cell; CMIS, common mucosal immune system; CSR, class switch recombination; DC, dendritic cell; GC, germinal center; iLP, intestinal lamina propria; int, intermediate; KLF2, Kruppel-like factor 2; PC, plasma cell; PNA, peanut agglutinin; PP, Peyer's patch; S1P, sphingosine 1-phosphate; $S1P_1$, type 1 S1P receptor; FAE, follicle-associated epithelium.

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production via the CMIS, a core pathway for the development of Ag-specific IgA B cells and subsequent Ag-specific mucosal Ab production. Additionally, although the details of $S1P_1$ expression during T cell differentiation and activation are well studied (9–11, 15–17), the contribution of $S1P_1$ expression during B cell differentiation still remains to be elucidated.

PPs could potentially be used to investigate these two unresolved, but immunologically important questions simultaneously because PP is the main inductive tissue for CMIS-mediated intestinal humoral immunity and shows spontaneous class switching recombination (CSR) from μ- to α-chain to supply IgA-committed B cells to distant effector sites such as the iLP (26). This feature of PPs allowed us to investigate the involvement of S1P in intestinal IgA production via the CMIS, as well as to elucidate $S1P_1$ expression during B cell differentiation from IgM^+ to IgA^+ B cells. Thus, we first investigated $S1P_1$ expression during B cell differentiation in the PPs, and then assessed the involvement of S1P in Ag-specific intestinal S-IgA production. These findings provide new evidence that the level of $S1P_1$ expression correlates with distinct stages of IgA-committed B cell development in the PPs, and hence, S1P regulates the retention and emigration of IgA-committed B cells for the subsequent S-IgA production via the CMIS-mediated pathway.

Materials and Methods

Mice and experimental treatment

Female BALB/c mice (7–9 wk) were purchased from Japan CLEA or Japan SLC and provided with sterile food and water ad libitum. For treatment with FTY720 (Novartis Pharmaceuticals), mice were injected i.p. with FTY720 (1 mg/kg/time) (23–25). For oral immunization, mice were deprived of food for 15 h and then given a solution of sodium bicarbonate to neutralize stomach acid before oral immunization (27). Thirty minutes later, mice were orally immunized with 1 mg of OVA (Sigma-Aldrich) with 10 μg of cholera toxin (List Biological Laboratories). This oral immunization procedure was conducted on days 0, 7, and 14. All animals were maintained in the experimental animal facility at the University of Tokyo, and experiments were conducted in accordance with the guidelines provided by the Animal Care and Use Committee of the University of Tokyo.

Lymphocyte isolation

Lymphocytes were isolated from the spleen, PPs, and iLP, as previously described (23–25). Briefly, single-cell suspensions were prepared from the spleen by passing them through a 70-μm mesh filter. To isolate lymphocytes from the PPs, an enzymatic dissociation protocol with collagenase (Nitta gelatin) was used. To isolate lymphocytes from the iLP, after removing PPs and isolated lymphoid follicles, small intestines were cut into 2-cm pieces and stirred in RPMI 1640 containing 1 mM EDTA and 2% FCS. The tissues were then stirred in 0.5 mg/ml collagenase before undergoing a discontinuous Percoll gradient centrifugation. Lymphocytes were isolated at the interface between the 40 and 75% layers.

Flow cytometry and cell sorting

Flow cytometry and cell sorting were performed, as previously described (23–25). Cells were preincubated with 10 μg/ml anti-CD16/32 Ab (BD Biosciences) and then stained with fluorescent Abs specific for B220, CD138, $\alpha_4\beta_7$ integrin, IgA, IgM (BD Biosciences), peanut agglutinin (PNA; Vector Laboratories), and CCR9 (R&D Systems). A Viaprobe (BD Biosciences) was used to discriminate between dead and living cells. Cytofix and Cytoperm kit (BD Biosciences) and ethidium monoazide bromide (Invitrogen Life Technologies) were used for intracellular IgA staining and for discriminating between dead and live cells, respectively. Stained cells were then subjected to flow cytometric analysis using FACSCalibur (BD Biosciences). For B cell purification, T cells were depleted using biotin-conjugated Abs specific for CD4 and CD8α together with streptavidin-conjugated magnetic-activated cell sorter beads (Miltenyi Biotec). These T cell-depleted PP cells were subjected to cell sorting using FACSAria (BD Biosciences).

In vitro migration assay

In vitro migration assays using PP B cells were performed according to a previously established method (16, 22). Briefly, PP B cells were isolated from mock- or FTY720-treated mice. These cells were stained with appropriate fluorescence-conjugated anti-IgA, IgM, and B220 Abs; washed three times with RPMI 1640 medium containing 0.5% fatty acid-free BSA (Calbiochem); and applied to the upper chambers of Transwell plates (pore diameter, 5 μm; Corning-Costar). Various concentrations of S1P (0, 1, 10, or 100 nM; Sigma-Aldrich) were added to the lower wells. After a 3-h incubation, the B cells that had migrated into the lower wells were enumerated by flow cytometry.

Analysis of OVA-specific Ab responses and total Ab levels by ELISA and ELISPOT

One week after the last immunization, fecal samples were collected and lymphocytes were isolated for the enumeration of OVA-specific Ab responses by ELISA and ELISPOT, respectively. Standard OVA-specific ELISA and ELISPOT were performed, as previously described (28, 29). In separate experiments, the total numbers of blood-circulating IgA-forming cells and the amounts of serum IgA in nonimmunized mice were determined by ELISPOT and ELISA, as described previously (23).

Immunohistochemical analysis

Immunohistochemical analysis was performed, as previously described (24). Briefly, PPs were fixed in 4% paraformaldehyde for 15 h at 4°C, washed, and treated in 20% sucrose for 12 h at 4°C. The tissues were embedded in OCT compound (Sakura Finetechnical). Cryostat sections (7 μm) were preblocked with an anti-CD16/CD32 Ab for 15 min at room temperature and stained with fluorescent-conjugated PNA lectin or Abs specific for IgA and B220 for 15 h at 4°C. Counterstaining was performed using 4′,6′-diamidino-2-phenylindole (Sigma-Aldrich). Podoplanin, CD4, and CD11c were stained using the TSA-Direct kit (PerkinElmer), according to the manufacturer's instructions (30). Briefly, cryostat sections (7 μm) were treated with 3% H_2O_2 for 10 min to quench endogenous peroxidase activity. Sections were blocked with anti-CD16/CD32 Ab in TNT buffer (0.1 M Tris-HCl (pH 7.5), 0.15 M NaCl, 0.05% Tween 20) for 15 min at room temperature. Next, sections were stained with purified anti-podoplanin Ab (Acris Antibodies) plus biotin-conjugated anti-hamster IgG mixture (BD Biosciences), biotin-conjugated anti-CD4 Ab (BD Biosciences), or biotin-conjugated anti-CD11c Ab (BD Biosciences) overnight, followed by incubation with HRP-conjugated streptavidin (Pierce) for 30 min at 4°C and amplification of the fluorescent signal with Cy5-tyramide. The specimens were analyzed using a confocal laser-scanning microscope (TCS SP2; Leica Microsystems).

In vitro culture of PP B cells

To measure IgA production by IgA^+B220^- B cells, purified PP IgA^+B220^- B cells (10^4 cells/well) were cultured in U-bottom, 96-well microtiter plates with 500 pg/ml murine IL-6 (R&D Systems) for 72 h (31). The amount of IgA in the culture supernatant was determined by ELISA, as described previously (23, 31). To induce differentiation of PP B cells, stromal cells were isolated from iLP and cocultured (4000 cells/well) with purified IgM^-B220^+ PP B cells (10^5 cells/well) in the presence of 1 μg/ml anti-CD40 Ab (BD Biosciences), 100 ng/ml murine IL-5 (R&D Systems), and 1 ng/ml human TGF-β (PeproTech) for 6 days with a protocol previously described (32). These differentiated B cells were purified by FACSAria for subsequent RT-PCR analysis.

Quantitative RT-PCR

To measure mRNA expression for $S1P_1$, quantitative RT-PCR using LightCycler (Roche Diagnostic Systems) was performed, as previously described (23–25). Total RNA was prepared using TRIzol reagent (Invitrogen Life Technologies), and cDNA was synthesized using Powerscript reverse transcriptase (BD Biosciences). The oligonucleotide primers and probes specific for $S1P_1$ (forward primer, 5′-TACACTCTGACCAACAAGGA-3′; reverse primer, 5′-ATAATGGTCTCTGGGTTGTC-3′; FITC probe, 5′-TGCTGGCAATTCAAGAGGCCCATCATC-3′; LCRed 640 probe, 5′-CAGGCATGGAATTTAGCCGCAGCAAATC-3′) and GAPDH (forward primer, 5′-TGAACGGGAAGCTCACTGG-3′; reverse primer, 5′-TCCACCACCCTGTTGCTGTA-3′; FITC probe, 5′-CTGAGGACCAGGTTGTCTCCTGCGA-3′; LCRed 640 probe, 5′-TTCAACAGCAACTCCCACTCTTCCACC-3′) were designed and produced by Nihon Gene Research Laboratory.

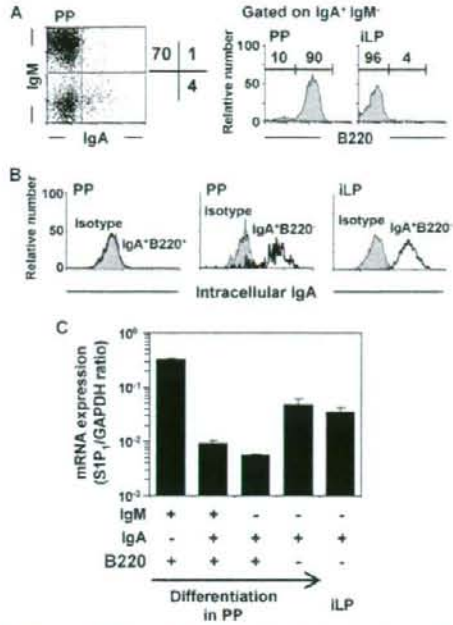

FIGURE 1. Alteration of $S1P_1$ expression during B cell differentiation in PPs. *A* and *B*, Flow cytometry was performed to characterize the cell populations in the PPs and iLP using Abs specific for IgM, IgA, B220 (*A*), and intracellular IgA (*B*). Data are representative of four independent experiments. *C*, Quantitative RT-PCR analysis for $S1P_1$ was performed using RNA isolated from IgM^+B220^+, $IgA^+IgM^+B220^+$, IgA^+B220^+, and IgA^+B220^- cells sorted from PPs and iLP IgA^+B220^- cells. The relative amount of specific mRNA is expressed as the ratio to GAPDH. Data are expressed as mean ± SD of three independent experiments.

Statistical analyses

The results for treatment vs control groups were compared using Student's *t* test or Welch's *t* test. Statistical significance was established at $p < 0.05$.

Results

Alteration of $S1P_1$ expression during B cell differentiation in the PPs

We first investigated $S1P_1$ expression at different B cell developmental stages based on the expression pattern of Ig H chain in the PPs. B cells isolated from the PPs of naive mice consisted of three distinct Ig expression patterns, as follows: IgM^+IgA^- (70%), IgM^+IgA^+ (1%), and IgM^-IgA^+ (4%; Fig. 1*A*, *left panels*). The first two populations exclusively expressed B220, whereas IgM^-IgA^+ B cells were composed of IgA^+B220^+ B cells and IgA^+B220^- B cells (Fig. 1*A*, *right panel*). Staining of intracellular IgA showed that no expression of intracellular IgA was noted in IgA^+B220^+ cells, whereas high levels of intracellular IgA were expressed in IgA^+B220^- cells (Fig. 1*B*). These intracellular IgA expressions in PP IgA^+B220^- cells were similar to those in IgA^+B220^- B cells in the iLP, which are predominant population of iLP B cells (Fig. 1*A*, *right panel*, and Fig. 1*B*). These findings suggest that naive IgM^+B220^+ B cells switch to $IgM^-IgA^+B220^+$ cells through $IgM^+IgA^+B220^+$ B cells under the control of CSR and then $IgM^-IgA^+B220^+$ cells further differentiated to $IgM^-IgA^+B220^-$ PC precursors in the PPs (6).

To determine the $S1P_1$ expression at different stages of B cell differentiation in the PPs, we performed $S1P_1$-specific quantitative RT-PCR using purified PP B cells. Naive $IgM^+IgA^-B220^+$ B cells expressed high levels of $S1P_1$, and the $S1P_1$ expression was markedly decreased (~40-fold) in PP B cells that underwent IgA commitment, such as $IgM^+IgA^+B220^+$ B cells (Fig. 1*C*). Whereas the $IgM^-IgA^+B220^+$ B cells retained a low level of $S1P_1$ expression, the expression on $IgM^-IgA^+B220^-$ B cells increased to a level similar to that of $IgM^-IgA^+B220^-$ B cells located in the iLP (Fig. 1*C*). These results demonstrate that $S1P_1$ expression was altered during B cell differentiation to IgA^+ cells in the PPs.

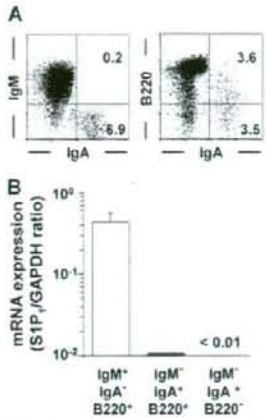

FIGURE 2. In vitro differentiated IgA^+ B cells reduced, but did not recover the expression of $S1P_1$. *A*, Purified PP IgM^+B220^+ cells were cultured for 6 days with stromal cells plus IL-5, TGF-β, and anti-CD40 Ab, and their differentiation was examined by flow cytometry. *B*, $S1P_1$ expression on each in vitro differentiated B cells was measured by quantitative RT-PCR. Data are expressed as means ± SD of three independent experiments.

Regulation of $S1P_1$ expression in in vitro differentiated PP B cells

We next investigated whether S1P expression is similarly regulated in in vitro differentiated PP B cells. To address this issue, we used in vitro B cell differentiation model using purified PP $IgM^+IgA^-B220^+$ B cells (32). Six days coculture of $IgM^+IgA^-B220^+$ B cells with iLP stromal cells plus IL-5, TGF-β, and anti-CD40 Ab induced $IgM^-IgA^+B220^+$ B cells (3.6%) and further differentiated $IgM^-IgA^+B220^-$ plasmablasts (3.5%) (Fig. 2*A*). Like in vivo differentiated B cells (Fig. 1), in vitro differentiated $IgM^-IgA^+B220^+$ B cells showed decreased levels of $S1P_1$ (Fig. 2*B*). However, unlike in vivo differentiated $IgM^-IgA^+B220^-$ B cells (Fig. 1), the $S1P_1$ expression was not recovered on in vitro differentiated $IgM^-IgA^+B220^-$ cells (Fig. 2*B*). These data suggested that the recovery of $S1P_1$ expression was not simply coincided with the differentiation to $IgM^-IgA^+B220^-$ cells and required additional unknown factor(s).

FTY720 treatment causes accumulation of IgA^+B220^- plasmablasts in PPs

We next investigated whether the alteration of $S1P_1$ expression during B cell differentiation in the PPs was truly associated with B cell commitment to CMIS-mediated cell trafficking, especially emigration from the PPs. To address this issue, we used FTY720, an immunomodulator of S1P receptors (18). When we analyzed IgM^+ and/or IgA^+ B cells in the PPs of mice treated i.p. with FTY720 for 5 days, no significant difference was noted among IgM^+IgA^-, IgM^+IgA^+, and IgM^-IgA^{low} B cells, whereas the relative abundance and total number of IgM^-IgA^{high} B cells were

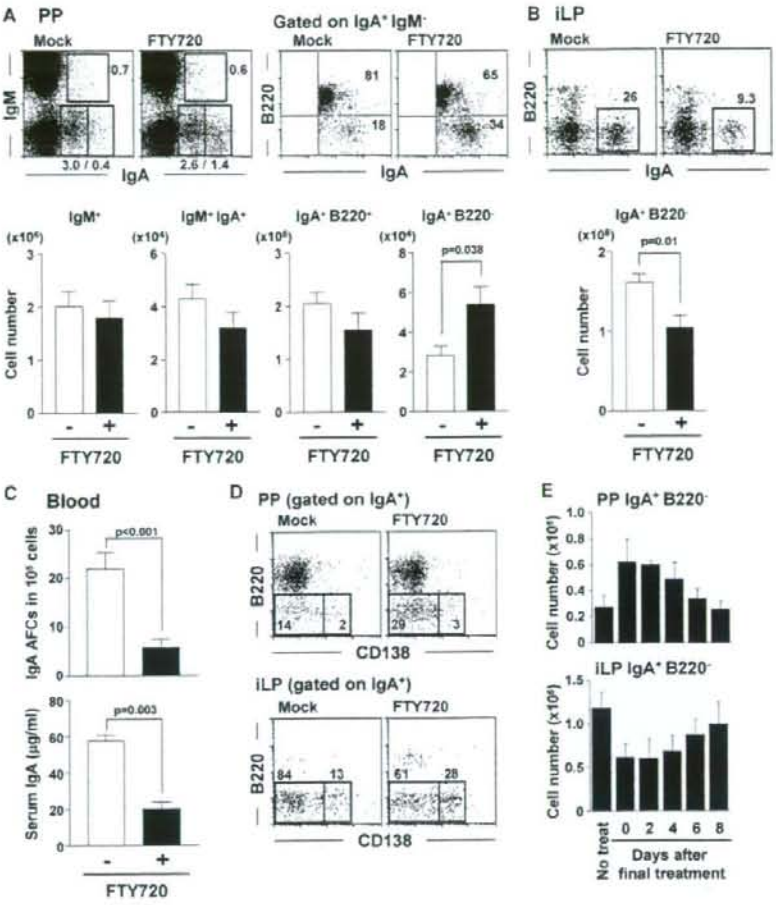

FIGURE 3. FTY720 induces accumulation of IgA^+B220^- plasmablasts in PPs and their reduction in iLP. *A* and *B*, Lymphocytes were isolated from the PPs (*A*) and iLP (*B*) of mice treated with mock (*left*) or FTY720 (*right*) for 5 days, and their expression of IgA, IgM, and B220 was analyzed by flow cytometry. Cell numbers for each population were calculated using the total cell number and flow cytometric data. Error bars are SEM ($n = 5$). *C*, IgA AFCs in blood (*upper*) and the amount of serum IgA (*bottom*) were determined by ELISPOT and ELISA, respectively. Error bars are SEM ($n = 5$). *D*, Cell population was examined by flow cytometry for analysis of plasmablasts ($IgA^+B220^-CD138^{-/low}$) and PCs ($IgA^+B220^-CD138^{high}$). Data are representative of five independent experiments. *E*, At each time point after the fifth FTY720 injection, cell numbers of IgA^+B220^- B cells in the PPs and iLP were measured. Data represent mean ± SEM ($n = 8$).

increased (Fig. 3*A*). These increased IgM^-IgA^{high} B cells were $B220^-$ (Fig. 3*A*), which was consistent with the high expression of $S1P_1$ on IgA^+B220^- B cells (Fig. 1*C*). The accumulation of IgA^{high} $B220^-$ B cells in the PPs was coincident with the reduction of the same population in the iLP and IgA Ab-forming cells (AFCs) in the blood (Fig. 3, *B* and *C*). The 5-day treatment with FTY720 did not affect the amount of serum IgA, probably due to the $t_{1/2}$ of IgA and IgA-forming cell (our unpublished data), whereas the 4-wk treatment with FTY720 resulted in decreased amounts of serum IgA (Fig. 3*C*). Because IgA^+B220^- cells include both plasmablasts and PCs, we next examined which of these cell types FTY720 targeted. FTY720 did not change the frequency of IgA^+ PCs ($CD138^{high}B220^-$) in the PPs, but did cause accumulation of IgA^+ plasmablasts ($CD138^{int/-}B220^-$) in the PPs (Fig. 3*D*). In agreement with this observation, the number of $CD138^{int/-}IgA^+$ plasmablasts was reduced in the iLP of FTY720-treated mice without affecting the number of IgA^+ PCs (Fig. 3*D*).

We next analyzed the kinetics of cell recovery after FTY720 treatment. Five treatments with FTY720 resulted in the accumulation of IgA^+B220^- B cells in the PPs and simultaneous reduction in the iLP (Fig. 3*E*). Partial recuperation occurred by day 4, with full recovery observed 8 days after the termination of FTY720 treatment (Fig. 3*E*). These data suggest that the inhibition of S1P-mediated signaling by FTY720 reversibly hampered the migration of B cells from the PPs into the iLP.

To directly investigate whether IgA^+ plasmablasts migrated in response to S1P and whether this response was inhibited by FTY720 treatment, we performed an in vitro Transwell migration assay. Consistent with $S1P_1$ expression (Fig. 1*C*), $S1P_1^+$ IgM^+B220^+ B cells and $S1P_1^+IgA^+B220^-$ plasmablasts, but not $S1P_1^-IgA^+B220^+$ B cells isolated from mock-treated mice migrated toward S1P (Fig. 4). In addition, both $S1P_1^+IgM^+B220^+$ B cells and $S1P_1^+IgA^+B220^-$ plasmablasts from FTY720-treated mice failed to migrate in response to S1P (Fig. 4), which is in accord with previous data that FTY720 treatment abolished the reactivity to S1P in lymphocytes (16, 22). These data suggested that the altered $S1P_1$ expression on B cells in the PPs could be a key biological determinant of whether PP B cells stay or emigrate from the PPs, and that S1P plays a key role in regulating the emigration of $S1P_1^+IgA^+$ plasmablasts from the PPs.

FTY720 does not influence the expression of gut-homing molecules on and IgA production by IgA^+ plasmablasts

An obvious explanation for the effect of FTY720 on the emigration of IgA^+ plasmablasts would be the influence of S1P on the

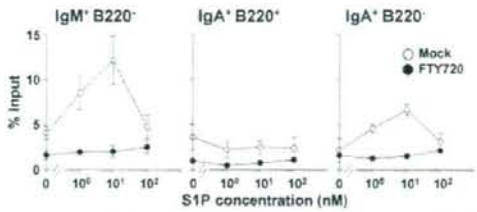

FIGURE 4. FTY720-sensitive chemotactic responses of IgA^+B220^- plasmablasts and IgM^-B220^+ B cells to S1P. Lymphocytes were isolated from the PPs of mice treated with mock (○) or FTY720 (●) for 5 days, and applied to in vitro Transwell migration assay. The percentage of the input cell population that migrated into the lower wells in response to various concentrations of S1P (0, 1, 10, or 100 nM) is shown. Error bars are SEM ($n = 3$), and the data are representative of two separate experiments.

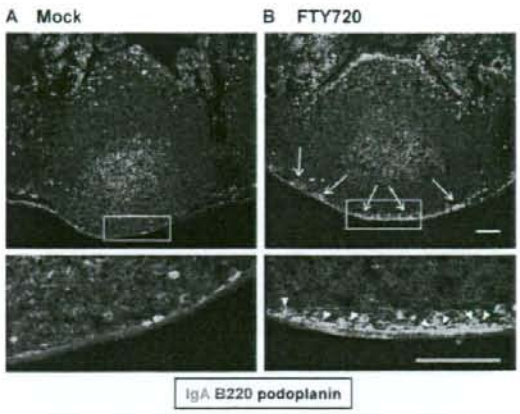

FIGURE 6. FTY720 causes accumulation of IgA^+B220^- plasmablasts on basal side of PPs. Confocal microscopic analysis was performed to examine the distribution of cells expressing IgA (green), B220 (red), and podoplanin (blue) in the PPs of mock-treated (*A*) or FTY720-treated (*B*) mice. Data are representative of five independent experiments. Bars indicate 100 μm.

expression of gut-homing-associated adhesion molecules and chemokine receptor. Therefore, we examined the expression of the adhesion molecule, $\alpha_4\beta_7$ integrin, and the chemokine receptor, CCR9, which determine gut tropism (33, 34). Flow cytometric analysis demonstrated that accumulated IgA^+B220^- plasmablasts expressed high levels of $\alpha_4\beta_7$ integrins and CCR9 (Fig. 5*A*), indicating that accumulation of IgA^+B220^- plasmablasts in the PPs and their simultaneous decrease in the iLP were not due to a lack

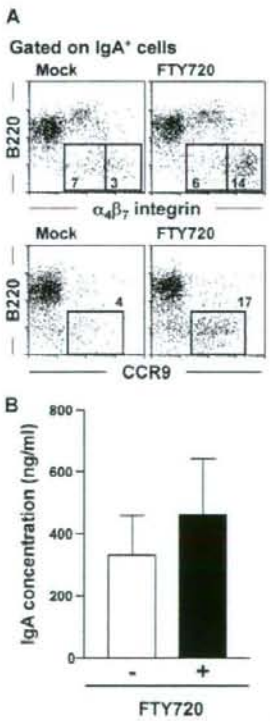

FIGURE 5. FTY720 did not affect expression of gut-homing molecules and IgA-producing ability of PP B cells. *A*, The expression of $\alpha_4\beta_7$ integrin (*upper panels*) and CCR9 (*lower panels*) on IgA^-B220^- cells was examined by flow cytometry. Data are representative of five independent experiments. *B*, IgA^+B220^- B cells were purified from PPs of mock-treated (□) or FTY720-treated (■) mice, and their IgA production in the culture supernatant was examined after 72-h culture with 500 pg/ml IL-6. Error bars are SEM ($n = 3$).

of migration ability through these gut-homing molecules. In addition, these accumulated IgA^+B220^- plasmablasts were capable of producing substantial amounts of IgA. Indeed, IgA^+B220^- plasmablasts isolated from the PPs of mock- or FTY720-treated mice showed comparable levels of IgA production induced by IL-6 treatment (Fig. 5*B*). Consistent with these results, we also found that FTY720 did not affect the distribution of $B220^+$ B cells, $CD4^+$ T cells, or $CD11c^+$ DCs in the PPs (our unpublished data), which are all involved in the appropriate induction of IgA-committed B cells, including CSR and expression of gut-homing molecules (1, 2, 7). These findings suggested that FTY720 did not influence the immunological nature of IgA-committed B cells, including their gut-homing molecules, and differentiation to Ab production ability; FTY720 solely affected their egress from the PPs.

FTY720 inhibits emigration of IgA^+ plasmablasts from the lymphatic area of the basal side of PPs

We next performed confocal microscopic analysis to determine the sites in which IgA^+ plasmablasts accumulated after FTY720 treatment. In mock-treated mice, IgA^+ B cells were found in the GCs, follicle-associated epithelium, and lymphatic area of the basal side of PPs (Fig. 6*A*). In FTY720-treated mice, however, IgA^+ B cells accumulated only on the basal side of PPs (Fig. 6*B*, arrows). Some IgA^+ B cells bound to $podoplanin^+$ lymph (Fig. 6*B*, *bottom*, arrowheads). These data clearly indicate that S1P regulates the emigration of IgA^+ plasmablasts from the lymph around the basal side of PPs without affecting other immunological functions, including expression of gut-homing molecules, class switching to IgA^+ B cells, and the ability to differentiate to IgA-producing cells.

FTY720-mediated inhibition of IgA^+ plasmablast emigration from the PPs abolishes the subsequent induction of Ag-specific intestinal IgA production

We next examined whether the S1P-mediated regulation of IgA^+ plasmablast emigration from the PPs is crucial for the induction of efficient Ag-specific Ab responses against orally administered Ag. To address this issue, mice were orally immunized with OVA plus cholera toxin, a mucosal adjuvant. An ELISPOT assay revealed that OVA-specific IgA AFCs were

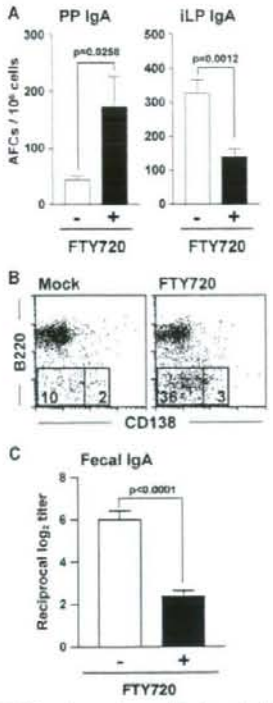

FIGURE 7. FTY720 reduces Ag-specific intestinal S-IgA production against orally administered OVA. *A*, One week after the final oral immunization, an ELISPOT assay was performed to enumerate OVA-specific AFCs in the PPs and iLP of mock-treated (□) and FTY720-treated (■) mice. Error bars are SEM ($n = 5$). *B*, Cell population was examined by flow cytometry for analysis of plasmablasts ($IgA^+B220^-CD138^-$) and PCs ($IgA^+B220^-CD138^+$) in the PPs of mice orally immunized with OVA plus cholera toxin, with (*right*) or without (*left*) FTY720 treatment. Data are representative of five independent experiments. *C*, OVA-specific fecal IgA production in mock-treated (□) and FTY720-treated (■) mice was determined by ELISA. Error bars are SEM ($n = 5$).

induced in the iLP after oral immunization in control mice (Fig. 7*A*). In contrast, daily treatment with FTY720 during immunization resulted in a decreased number of OVA-specific IgA AFCs in the iLP, which was associated with the accumulation of OVA-specific IgA AFCs in the PPs (Fig. 7*A*). In nonimmunized control mice, negligible levels of OVA-specific IgA AFCs were detected (mock PP, 0.5 ± 0.14; FTY720 PP, 1.4 ± 0.22; mock LP, 3.5 ± 0.29; FTY720 LP, 3.5 ± 0.28 cells/10^6 cells). Consistent with the results obtained from the analysis of nonimmunized mice treated with FTY720 (Fig. 3*D*), the increase of OVA-specific IgA AFCs in the PPs was coincident with the accumulation of $IgA^+B220^-CD138^{-/low}$ plasmablasts in the PPs (Fig. 7*B*). We also measured the levels of OVA-specific IgA in feces to examine whether the altered trafficking of IgA^+ plasmablasts affected actual Ab production in the intestinal lumen. We found that OVA-specific fecal IgA was markedly decreased in the FTY720-treated mice (Fig. 7*C*). These data clearly indicate that the migration of Ag-specific IgA^+ plasmablasts from the PPs into the iLP is a prerequisite for the efficient production of Ag-specific S-IgA Abs in the intestinal lumen.

Previous studies reported that FTY720 treatment inhibited Ab responses against systemically immunized T-dependent Ag by abolishing GC formation in the systemic lymph nodes (19–21). Thus, we next examined GC formation in the PPs and spleen. In naive mice, there were few PNA^{high} $B220^+$ GC B cells in the spleen, and 4% of lymphocytes were PNA^{high} $B220^+$ GC B cells in the PPs, and FTY720 barely affected these populations in naive condition (our unpublished data). When we examined mice orally immunized with OVA plus cholera toxin, the numbers of PNA^{high} $B220^+$ GC B cells were increased in the spleen, but not significantly changed in the PPs compared with naive mice (Fig. 8*A*). Consistent with the previous findings (19–21), reduced numbers of PNA^{high} $B220^+$ GC B cells were seen in the spleen of mice receiving FTY720 during the oral immunization period (Fig. 8*A*). In contrast, comparable numbers of PNA^{high} $B220^+$ GC B cells were detected in the PPs of FTY720-treated mice (Fig. 8*A*). Histological analysis confirmed that GC formation in the PPs was not impaired by FTY720 treatment. Thus, comparable GC formation containing $B220^+$ PNA^+ cells was noted in the FTY720-treated mice (Fig. 8*B*). These results indicate that, unlike systemic immune compartments (19–21), FTY720 reduced intestinal S-IgA production against orally inoculated Ag by inhibiting IgA^+ plasmablast emigration from the PPs without affecting GC formation in the PPs.

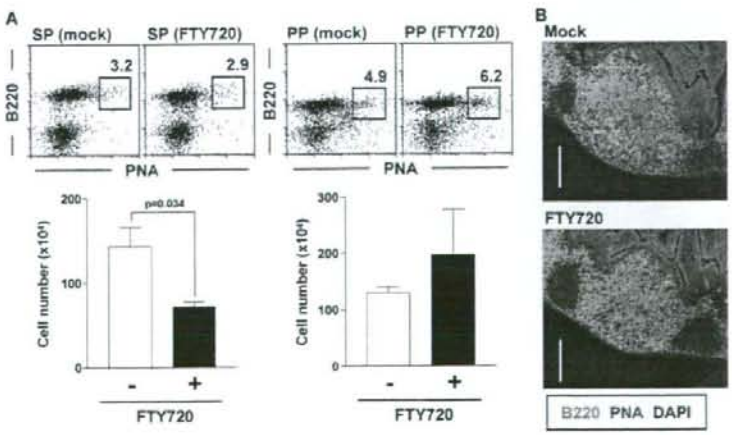

FIGURE 8. FTY720 barely affected GC formation in the PPs. *A*, GC B cells in the PPs were determined by flow cytometry using anti-B220 Ab and PNA lectin. Data are representative of four independent experiments. Cell numbers of GC B cells were calculated using the total cell number and flow cytometric data. The error bars represent ± SEM ($n = 4$). *B*, Confocal microscopy analysis was performed to detect the GC formation in the PP of OVA-immunized mice treated with mock (*left*) or FTY720 (*right*). Data are representative of three independent experiments. Bar indicates 200 μm.

Discussion

More than 30 years ago, PPs were discovered to be the induction sites of precursor of IgA-producing PCs (5). The current study indicates that PPs are the main sites for the induction of IgA^+ plasmablasts, which selectively use S1P for emigration from the PPs by changing their $S1P_1$ expression during differentiation to IgA^+ B cells in the PPs. Because a previous study showed that activation of B cells by LPS or BCR stimulation induced the reduction of $S1P_1$ expression (19–21), it is likely that PP milieu containing abundant IL-4- or TGF-β-producing cells allows activated B cells to differentiate to IgA^+ B cells with simultaneous reduction of $S1P_1$. The reduction of $S1P_1$ expression enables IgA^+ B cells to stay at the PPs for efficient differentiation to IgA^+ plasmablasts. The IgA^+ plasmablasts then recover $S1P_1$ expression together with the expression of gut-homing molecules (e.g., $\alpha_4\beta_7$ integrin and CCR9), allowing them to exit from the PPs and migrate into the iLP for the final differentiation to IgA-producing PCs. This S1P-mediated regulation system observed in this study is in agreement with a previous data on the systemic immunization model (22). It was shown that differential expression of $S1P_1$ and Kruppel-like factor 2 (KLF2), a transcription factor that increases $S1P_1$ expression (35), in differentiating PCs is a key factor to determine their trafficking from spleen to the bone marrow. Indeed, high levels of $S1P_1$ and KLF2 were noted in $Blimp1^{int}$ $CXCR4^{int}$ IgG AFCs in the spleen and blood (22).

In addition, our current results provide further evidence that the differentiation of IgM^+ B cells to IgA^+ plasmablasts in the PPs is not sufficient for effective intestinal IgA production against intestinal Ags, but that the cells require appropriate egress from PPs to the iLP for the final differentiation to IgA synthesis. This idea was previously proposed based on studies showing that intestinal S-IgA production was impaired in mice deficient in gut-homing molecules, such as $\alpha_4\beta_7$ integrin and CCR9 (34, 36). Our results convincingly demonstrate the role of lipid mediator for the egress of IgA-committed B cells from the inductive tissue by showing for the first time that intestinal S-IgA production against ingested Ag was impaired by inhibiting the S1P-mediated emigration of IgA^+ plasmablasts from the PPs, without affecting their expression of gut-homing molecules and their ability to produce IgA (Figs. 5 and 7). Our current findings do not exclude the possibility that FTY720 inhibits the emigration of IgA^+ plasmablasts via prevention of endothelial cell function, which was previously reported to express S1P receptors (37). However, our current in vitro data on $S1P_1$ expression on PP B cells (Fig. 1) and their migration in response to S1P (Fig. 4) and in vivo data on FTY720-treated mice (Fig. 3) collectively suggest that $S1P_1$ on PP B cells itself is responsible for the FTY720-sensitive emigration from the PPs.

Although the recovery of $S1P_1$ expression was noted in $IgM^-IgA^+B220^-$ plasmablasts in the PPs in vivo (Fig. 1), in vitro differentiated $IgM^-IgA^+B220^-$ cells did not show the $S1P_1$ recovery (Fig. 2*B*). It was possible that continuous stimulation of differentiated $IgM^-IgA^+B220^-$ cells with the cytokines and anti-CD40 Ab might inhibit the recovery of $S1P_1$ expression because previous studies indicated that several hours were required for the full recovery of $S1P_1$ (38, 39). To test this possibility, we removed the CSR-related molecules after their differentiation to $IgM^-IgA^+B220^-$ cells, but we found that $S1P_1$ expression was still low after removing the CSR-related molecules (our unpublished data). Additionally, coculture with whole PP cells in the presence of the CSR-related molecules resulted in the induction of the $IgM^-IgA^+B220^-$ cells, but they still did not recover the $S1P_1$ expression (our unpublished data). Thus, it seems that well-organized structure of PP and/or some unknown factors are required for the $S1P_1$ recovery during differentiation to $IgM^-IgA^+B220^-$ plasmablasts in the PPs. Related to the imprinting system in the gut-associated lymphocyte trafficking, accumulating evidence has shown that the expression of gut-homing molecules (e.g., $\alpha_4\beta_7$ integrin and CCR9) is induced by the interaction with gut-associated DCs producing retinoic acid (7). In contrast to the retinoic acid-mediated expression of $\alpha_4\beta_7$ integrin and CCR9, retinoic acid did not affect the $S1P_1$ expression of PP B cells in vitro (our unpublished data). Another possibility is that cytokines produced in the intestinal compartment may cooperatively regulate $S1P_1$ expression. In this context, the expression of KLF2, a transcription factor for the increase of $S1P_1$ expression (22), is regulated by several cytokines, including IL-7, which is abundantly produced by intestinal epithelial cells (40, 41). Thus, it is possible that gut-associated cytokines (e.g., IL-7) may enhance the re-expression of $S1P_1$ in PP IgA^+ B cells, but this circumstance is not established in vitro because freshly isolated epithelial cells cannot retain the ability in vitro due to their low viability. These points, including the effect of organized structure of PPs, represent challenges for future studies.

Our current findings suggest that S1P is not involved in cellular distribution in the PPs because FTY720 treatment did not affect the distribution of $CD4^+$ T cells, $B220^+$ B cells, and $CD11c^+$ DCs in the PPs, with the exception of the IgA^+ plasmablast emigration from the lymphatic area of the basal side of PPs (Fig. 6 and our unpublished data). In contrast, B cell distribution in the spleen was affected by FTY720 (12, 19). Moreover, unlike the inhibitory effects of FTY720 on GC formation in the spleen after systemic immunization (19–21), we show that FTY720 did not affect GC formation in the PPs (Fig. 8). A major difference between the spleen and PPs is that spleen is located in germfree condition, whereas PPs are exposed to continuous stimulation by environmental Ags (e.g., microbial and food Ags), which may account for these different effects of FTY720. B cells in the systemic immune compartments (e.g., spleen) are normally in a quiescent state, and thus, no GC formation is detected in intact mice. In contrast, PPs contain GCs in intact mice that are induced by stimulation from intestinal microbiota (26). Thus, FTY720 may inhibit the formation of new GCs, such as GC formation in the spleen induced by immunization, but does not interfere with established GCs in the PPs.

Another unresolved question is why IgA^+ plasmablasts selectively accumulated by FTY720 treatment, yet IgM^+ B cells, which express high levels of $S1P_1$ and show comparable reactivity to S1P in vitro, did not change (Figs. 1, 3, and 4). Similar selective effects of FTY720 on IgA^+ B cells were observed in our previous study on peritoneal B cell trafficking into the iLP (23). In that study, we found that IgA^+ B cells, but not IgM^+ B cells, were inhibited by FTY720 treatment from migrating from the peritoneal cavity into the iLP (23). There is a mutual interaction between S1P and chemokines in lymphocyte trafficking, with some levels of hierarchy (19, 22, 42–45), so a cooperative pathway mediated by both S1P and chemokines may determine the selective effects of FTY720 on IgA^+ B cells. Indeed, it was previously reported that CCR10 expression was prevalent on IgA^+ B cells with plasmablast and PC phenotypes in the blood and the intestine, but expression was negligible on IgA^- B cells (46). By contrast, IgM^+ B cells, but not IgA^+ B cells, predominantly expressed CCR7 (our unpublished data), which was reported to regulate S1P-mediated T cell trafficking together with additional Gαi-coupled receptors (45). Therefore,

the expression of identified and/or unidentified chemokine receptor(s) on IgM^+ or IgA^+ B cells may determine their dependency (or lack thereof) on S1P for emigration from the PPs. Our group is currently conducting research on the involvement of gut-associated cytokines and chemokines in the regulation of S1P-mediated intestinal B cell trafficking.

In addition to the IgA^+ plasmablasts, the few cells showing IgA^+ PC phenotypes (e.g., $CD138^{high}$) in the PPs were not affected by FTY720 treatment (Fig. 3*D*), suggesting the presence of an alternative, S1P-independent differentiation pathway to IgA^+ PCs in the PPs. In this context, a recent study demonstrated that PP-DCs produce retinoic acid, IL-5, and IL-6, which provide a milieu for class switching from μ- to α-chain, as well as IgA production (7). IgA^{high} cells in the follicle-associated epithelium (FAE) were barely affected by FTY720 treatment (Fig. 5), and DCs are abundant in the FAE (30). Thus, the DCs may induce IgA^+ PCs in the FAE in a S1P-independent manner. This idea is supported by a previous report that B cells near M cells generally situated in FAE region showed a memory cell phenotype (47). Hence, S1P-independent IgA^+ cells in the FAE might provide a rapid response against newly arriving Ags for creating local immunity in the PPs.

Our previous work indicated the pivotal role of S1P in the regulation of peritoneal B cell trafficking for intestinal S-IgA production (23), and our current findings show that B cells can alter $S1P_1$ expression to regulate their retention on and emigration from PPs. Together, these findings show that S1P is a key molecule in the versatile S-IgA production pathways in the intestinal immune system.

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Disclosures

The authors have no financial conflict of interest.

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Review

Cell PRESS

Immunological commonalities and distinctions between airway and digestive immunity

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Airway and digestive tissues are the frontlines of the body's defense, being continuously exposed to the outside environment and encountering large numbers of antigens and microorganisms. To achieve immunosurveillance and immunological homeostasis in the harsh environments of the mucosal surfaces, the mucosal immune system tightly regulates a state of opposing but harmonized immune activation and quiescence. Recently, accumulating evidence has revealed that although the respiratory and intestinal immune systems share common mucosa-associated immunological features that are different from those of the systemic immune system, they also show distinctive immunological phenotypes, functions, and developmental pathways. We describe here the common and distinct immunological features of respiratory and intestinal immune systems and its application to the development of mucosal vaccines.

Shared and divergent immunological features of mucosa-associated lymphoid tissues in respiratory and intestinal tracts

Mucosal immunological components can be divided into two main parts, organized mucosa-associated lymphoid tissues (MALTs), where antigen-specific immune responses are initiated, and diffuse lamina propria (LP) regions, which are effector sites for IgA production and T cell responses [1–3]. MALTs are situated along the surfaces of various kinds of mucosal tissues and include gut-associated lymphoid tissues (GALT) [e.g. Peyer's patches (PPs), isolated lymphoid follicles, and colonic patches], nasopharynx-associated lymphoid tissue (NALT), and bronchus-associated lymphoid tissue (BALT). The most extensively studied MALTs are PPs and NALT in the digestive and airway tissues, respectively [1–3]. PPs usually number 8 to 10 in the small intestine of mice and hundreds in humans. In rodents, NALT is found on both sides of the nasopharyngeal duct dorsal to the cartilaginous soft palate. Humans generally do not have NALT anatomically, except at an early age [4], but they possess oropharyngeal lymphoid tissues, including unpaired nasopharyngeal tonsils (adenoids) and bilateral tubular palatine, and lingual tonsils (Waldeyer's ring), which seem to correspond functionally to NALT [1–3].

Although the MALTs (e.g. PPs and NALT) share some common immunological features with systemic secondary lymphoid organs (e.g. the presence of follicular B cells and parafollicular T cells), they are also characterized by their unique mucosa-associated features that differ from those of systemic lymphoid organs (Table 1). For instance, both PPs and NALT contain efferent but not afferent lymphatics, therefore decreasing the opportunity for antigens to be transferred into these lymphoid organs. Instead, MALTs are uniquely covered with a specialized epithelial region, termed follicle-associated epithelium (FAE), containing specialized microfold or membranous cells (M cells) (Figure 1) [5]. Similar M cells are present in the crypt epithelium of human tonsils and adenoids [6]. The M cells are characterized by short microvilli, a thin mucus layer, small cytoplasmic vesicles, and efficient transcytosis activity, allowing the selective and efficient transfer of inhaled or ingested luminal antigens to antigen-presenting cells (APCs) in the MALTs [7]. As APCs, PPs and NALT contain dendritic cells (DCs), which are capable of processing and presenting antigens immediately for the initiation of antigen-specific immune responses (Figure 1). Several lines of evidence have revealed a unique distribution of DCs in the PPs (also see the article by Tsuji and colleagues in this issue). At least three distinct types of DCs are present. $CD11^+$ DCs are located in the subepithelial dome region (SED); $CD8\alpha^+$ DCs are present in the T cell-rich interfollicular regions (IFRs); and double-negative ($CD4^-CD8\alpha^-$) DCs exist in both the SED and IFRs [8,9]. These DCs are involved in the induction of immune responses against pathogenic [10,11] and commensal microbiota [12], as well in tolerance to food and self-antigens [13]. Unlike PP DCs, the phenotype of NALT DC has not been determined although reports have shown the presence and function of NALT DCs in the induction of antigen-specific immune responses [14,15]. In the human tonsil (equivalent to murine NALT), DCs consist of immature DCs in the lymphoepithelium and mature DCs in IFRs and they include at least five different subsets, showing different levels of HLA-DR, CD13, and CD123 [16–18], but the functional differences among them remain to be investigated.

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Table 1. Shared and unique immunological features of Peyer's patch and NALT

	Peyer's patch	NALT
Lymphatics	Efferent only (no afferent)	Efferent only (no afferent)
M cells	Present in FAE	Present in FAE
T cells	$CD4^+$ (15%)	$CD4^+$ (32%)
	$CD8^+$ (5%)	$CD8^+$ (8%)
B cells	IgM^+ $B220^+$ (70%)	IgM^+ $B220^+$ (50%)
	$IgM^+IgA^+B220^+$ (1%)	$IgM^+IgA^+B220^+$ (0.4%)
	IgA^+B220^+ (3%)	IgA^+B220^+ (< 0.1%)
	IgA^+B220^- (0.5%)	IgA^+B220^- (< 0.1%)
Germinal center	Present	Only following antigen priming
Tissue genesis	Time: prenatal	Time: postnatal
	Inducer cells: $CD3^-CD4^+$	Inducer cells: $CD3^-CD4^+$
	both Id2 and RORγt dependent	Dependent on Id2, but not RORγt
	LT dependent	LT independent
	IL-7Rα dependent	IL-7Rα independent

Among immunocompetent cells residing in the PPs, ~20% of PP cells consist of T cells (Table 1). Major T cell subsets are found in the IFRs of the PPs (Figure 1A). Within the PPs ~75% of the T cells are $CD4^+$ and the remaining 25% are $CD8^+$ (Table 1). Murine NALT and the human palatine tonsil contain more T cells than PPs. Indeed, ~40% of NALT cells are T cells, consisting of $CD4^+$ (~80%) and $CD8^+$ (~20%) cells (Table 1) [19]. Cytokine profile analyses have revealed that many of them are naïve T cells capable of becoming effector T cells (Th1, Th2, regulatory T [Treg], cytotoxic T lymphocytes [CTL], or Th17 cells) [19–21].

Formation of the cell cluster of DCs and T cells in the PPs leads to B cell stimulation through CD40, TGF-β, and

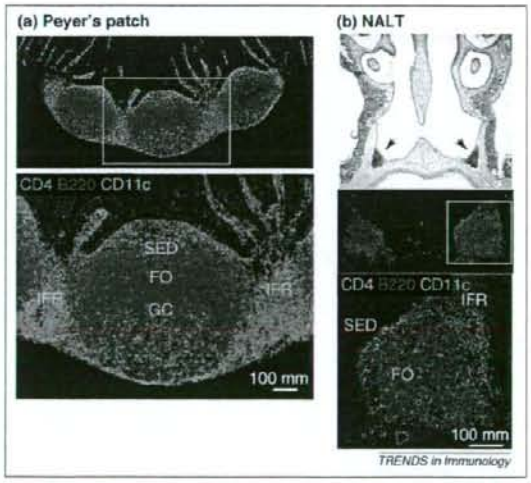

Figure 1. Organized microarchitecture of murine Peyer's patches and NALT. Immunohistochemical data on Peyer's patch **(a)** and NALT **(b)** is shown. Yellow boxes indicate the regions highlighted in the lower panels. $CD4^+$ T cells (green) are present mainly in the intrafollicular regions (IFRs), and B cells (red) are located in the subepithelial dome (SED), follicle (FO), and germinal center (GC). Dendritic cells (blue) are distributed in the SED, IFRs and GCs. T cells, B cells, and dendritic cells identified respectively with CD4, B220, and CD11c. (b) In the NALT, upper panel shows hematoxylin and eosin staining with the two regions of NALT indicated by arrows.

IL-4, creating an environment for promoting μ to α class switch recombination (CSR), i.e. B cells switch from IgM^+ to IgA^+[22]. A recent study has showed that PP DCs producing TNF-α and inducible nitric oxide synthase (iNOS), termed Tip-DCs, function to induce IgA-committed cells [23]. This nitric oxide increases TGF-β receptor expression on B cells, leading to the enhancement of CSR to IgA [23]. Therefore, PPs consist of B cells (~75%) at different maturation stages, including IgM^+B220^+ (~70%), $IgM^+IgA^+B220^+$ (~1%), IgA^+B220^+ (~3%), and IgA^+B220^- (~0.5%) B cells (Table 1) [24]. Under normal steady-state conditions, PPs contain several germinal centers (GCs) containing surface IgA-positive ($sIgA^+$) B cells (Figure 1A and Table 1), but, unlike in the spleen and peripheral secondary lymph nodes (LNs) in the systemic compartment, plasma cell (PC) development does not occur effectively. Alternatively, B cell development is generally terminated at the stage of IgA^+ plasmablasts (precursors of PCs in PPs) [24,25].

In contrast to PPs, the NALT of normal mice under steady-state conditions shows an absence of GC formation, however nasal immunization induces GC formation leading to the induction of high numbers of $sIgA^+$ B cells in NALT (Table 1). Another difference between PP B cells and NALT B cells is that B cell differentiation in NALT leads to the production of both IgA and IgG [26]. Similarly, human tonsils contain a high proportion of IgG B cells in addition to IgA [27]. The development of IgA-committed cells in the presence of TGF-β is characterized by a sequential class switch recombination from Cμ to Cα via Cγ [28]. This suggests that differences in the cytokine milieu of the intestinal and respiratory tracts result in their distinct patterns of B cell class switching. In addition to the GCs, the underlying dome regions of the PPs, NALT, and human palatine tonsil consist of sparse $sIgA^+$ or IgM^+ cells that might play a crucial role in immediate antibody production toward antigens taken up through M cells (Figure 1B) [29,30].

NALT and PP have distinct developmental programs

In spite of their anatomical and cell population similarities, PPs and NALT differ in their pattern of tissue genesis (Figure 2) [1–3]. A model of MALT tissue genesis has been proposed from evidence accumulated from well-studied murine PPs [31–33] (also see the article by Fagarasan and colleagues in this issue). In the PP developmental pathway, clustering of mesenchymal-lineage $VCAM\text{-}1^+ICAM\text{-}1^+$ cells occurs at the site of tissue anlagen in the upper small intestine beginning at embryonic day 15 to 16 [31–33]. These cells are termed PP organizer (PPo) cells and express lymphotoxin β receptor (LTβR) and artemin, a ligand for receptor tyrosine kinase (RET) (Figure 2) [34]. Subsequently, lymphoid-lineage $RET^+IL\text{-}7R^+CD3^-CD4^+CD45^+$ PP inducer (PPi) cells are recruited to the PP anlagen at embryonic day 17.5 [31–33]. Epithelial cells (ECs) secrete IL-7, which triggers PPi cells to express $LT\alpha_1\beta_2$; this then activates PPo cells through their LTβRs. PPo cells in turn produce chemokines such as CXCL13, CCL19, and CCL21 to further recruit PPi cells and lymphocytes expressing CXCR5 and CCR7, respectively (Figure 2). The reciprocal interaction between inducer and organizer cells is essential for the initiation of PP

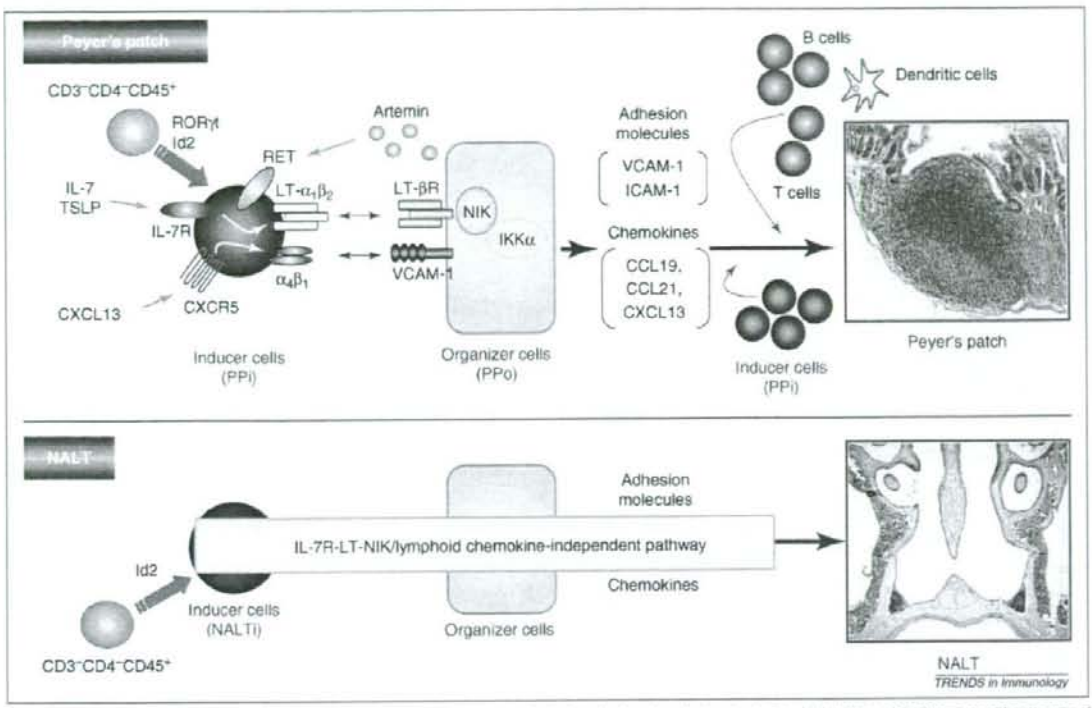

Figure 2. Distinct pathways for mucosa-associated lymphoid tissue development in the intestinal and respiratory tracts. The initial phase of the tissue genesis program of Peyer's patches (PP) operates during embryogenesis. RET⁺IL-7R⁺CD3⁻CD4⁺CD45⁺ PP-inducer cells (PPi) require Id2 and RORγt genes for their development and accumulation at the tissue anlagen for interaction with artemin⁺ VCAM-1⁺ PP organizer cells (PPo). This interaction induces subsequent activation of LTβR-associated molecules, such as NIK and IKKα, and induces the expression of adhesion molecules and chemokines by PP organizer cells; this leads to the further recruitment of T cells, B cells, and DCs. In comparison to the PP tissue genesis program, NALT organogenesis is initiated postnatally and is totally independent of the IL-7R-LT-LTβR-NIK-mediated pathway. The development of NALT-inducer cells (NALTi), like PPs, requires Id2 for the differentiation of CD3⁻CD4⁺CD45⁺ NALT-inducer cells but does not require RORγt.

formation, and thus the loss of any part of this signaling program results in the disruption or impairment of PP development (Table 1) [1–3]. Further evidence supports this model by showing that mice lacking $CD3^-CD4^+CD45^+IL\text{-}7R^+$ inducer cells due to genetic deletion of the transcription regulators Id2 or RORγt also completely lack PPs (Table 1) [35–37].

In contrast to the embryonically initiated program of PP development, the NALT formation program occurs postnatally (Figure 2 and Table 1) [38]. In addition to these chronological differences, the molecular requirements for PP and NALT tissue genesis are also different [1–3]. Indeed, normal NALT structure is observed in mice otherwise lacking PPs due to a deficiency in the LTβR-, IL-7Rα-, or chemokine (e.g. CCL13, CCL19, and CCL21)-mediated cascade (Table 1) [15,38,39]. Intriguingly, both PP and NALT structures are impaired in mice lacking Id2, whereas deletion of RORγt, results in the suppression of PP but not NALT development [38,40]. These findings suggest that although NALT and PP development depends on the phenotype of $CD3^-CD4^+CD45^+$ inducer cells, these inducer cells can be categorized into at least into two subsets, NALT inducer (NALTi) and PPi cells, on the basis of the transcriptional regulators, which are either dependent on Id2 alone or dependent on both Id2 and RORγt, respectively (Figure 2 and Table 1).

The mucosal decision for inflammatory versus quiescent immune responses

In the diffuse LP regions of the aero-digestive tract, a wide variety of T cell subsets in the effector tissues are prepared for induction of active or silent immune responses against continuously encountered inhaled or ingested antigens. Therefore, in addition to the active form of immune responses mediated by effector T cells, various regulatory T cell (Treg) subsets are present in the gut for the creation of an immunologically quiescent environment for food-derived antigens and commensal microbiota [1].

Recent studies have identified unique molecular mechanisms mediated by various cytokines in the determination of whether T cells differentiate into pathogenic Th17 or regulatory-type T cells (e.g. Tr1 and Treg) (Figure 3). For example, transforming growth factor-β (TGF-β), which is abundantly produced in intestinal tissues, is an essential molecule for induction of both $Foxp3^+$ Treg cells and Th17 cells; however, Th17 cells additionally require IL-6 for their development (Figure 3) [41,42]. A recent study showed that IL-23 plus IL-6 and TGF-β is an additional nonredundant factor in the full differentiation of Th17 cells (Figure 3) [43], whereas IL-27 plus IL-6 and TGF-β induced the differentiation of IL-10-producing T cells that resemble Tr1 cells (Figure 3) [44,45]. Several independent studies have identified retinoic acid (RA)

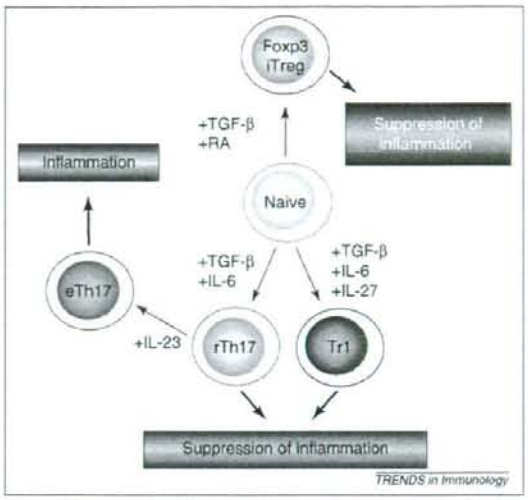

Figure 3. The generation of mucosal inflammatory and regulatory T cells is controlled by a complex network of signals. Naïve T cells are primed and stimulated via a molecular network with the recognition of peptide antigen presented by professional antigen-presenting cells (APC) and their associated cytokines and bioactive molecules. TGF-β and retinoic acid (RA) produced by dendritic cells lead to the default induction of Foxp3$^+$ regulatory T (Treg) cells. By contrast, IL-6 inhibits this pathway and alternatively enhances the induction of cells that differentiate into regulatory Th17 (rTh17) cells. IL-23 and IL-27 determine whether the rTh17 cells differentiate into effector Th17 (eTh17) or IL-10-producing type 1 regulatory (Tr1) cells, respectively.

produced by intestinal DCs as a key molecule to enhance the conversion of TGF-β-treated T cells to Treg cells and simultaneously suppresses their differentiation to Th17 cells (Figure 3) [46–49]. Because RA production is specifically observed in gut DCs, the respiratory tract likely employs other mechanisms for maintaining quiescence. In this regard, respiratory DCs alter their ability to induce inflammatory and/or noninflammatory responses [20,50–53] although the details of this process await elucidation. In addition, intestinal macrophages have been shown to behave as a member of mucosal regulatory cells by preferential production of IL-10 [54,55]. In a similar manner, the lung contains unique macrophages, known as alveolar macrophages, in the alveolar space. The alveolar macrophages by default inhibit immune responses through interaction with TGF-β activated by $\alpha_v\beta_6$ integrin$^+$ ECs [56], providing an additional pathway for inhibitory events in the lung. These findings collectively suggest the presence of a multivalent mucosa-associated regulatory system of unique mononuclear cells (e.g. DCs, macrophages, Th17, Tr1, and Treg cells), cytokines (e.g. TGF-β, IL-6, IL-10, IL-23, and IL-27), and other biological molecules (e.g. RA) that determine and control the qualitative and qualitative aspects of antigen-specific mucosal immune responses (Figure 3).

Regulation of secretory IgA (S-IgA) production in the aero-digestive tract

In addition to Th17, Tr1, and Treg cells, classical helper T cells producing IL-5, -6, and -10 allow IgA$^+$ plasmablasts to differentiate into PCs that produce polymeric forms of IgA (pIgA) joined by J-chains [57]. The polymeric IgA (pIgA) produced by PCs binds to polyimmunoglobulin receptors (pIgR) expressed on the mucosal ECs and are transported to the apical surface. Extracellular proteolytic fragments of the pIgR (secretory component, SC) and pIgA are secreted as secretory IgA (S-IgA) [58].

Mucosal IgA can be discriminated into two groups according to their affinities [1,59]. The high-affinity form of IgA plays an important role in neutralization of microbial proteins including toxins, and it is considered to originate from B2 (i.e. conventional) B cells, which are induced in MALTs [22]. The low-affinity type of IgA is thought to originate from T-independent B1 B cells in the peritoneal and pleural cavities, leading to the inhibition of adhesion of commensal microbiota [60,61]. Unlike the CD40-dependent differentiation of B2 B cells, several lines of evidence have revealed that B1 B cells use other molecular interactions for their development, such as B cell-activating factor of the TNF family (BAFF) and A-proliferation-inducing ligand (APRIL) [62]. Indeed, T-independent IgA responses are decreased in mice lacking the transmembrane activator and CAML interactor (TACI), a receptor for both BAFF and APRIL [63].

In addition to affinity, structural differences of human IgA separate it into two subclasses, known as IgA1 and IgA2 [64]. This difference is attributable to the deletion of 13 amino acids in the hinge region and is associated with the resistance of IgA2 to proteases. Distinct distributions of IgA1 or IgA2 are noted in different parts of the immunological tissues [64]. Systemic lymphoid tissues (e.g. spleen, lymph nodes, and bone marrow) are dominated by IgA1-producing cells. In most of the mucosal tissues (nasal, gastric, and small intestinal mucosa), IgA1 producers are still the major subclass, but these tissues have greater numbers of IgA2-producing cells than are found in the lymphoid tissues of the systemic compartments. Additionally, IgA2-producing cells are more frequent than IgA1 cells in the large intestine; this might account for the fact that naturally occurring S-IgA antibodies to bacterial endotoxin are associated with the IgA2 isotype [64]. To this end, a recent study revealed that intestinal ECs trigger sequential class switching from IgA1 to IgA2-expressing B cells in a T cell-independent but toll-like receptor (TLRs)- and APRIL-dependent manner [65]. Thus, it is likely that different circumstances (e.g. the nature of commensal microbiota) between respiratory and intestinal tracts might determine the different IgA1:IgA2 ratio.

Aero-digestive epithelium as a critical player in the mucosal immune system

In addition to their involvement in the transport of IgA, ECs are also involved in the regulation of mucosal immunity. Intraepithelial lymphocytes (IELs) are representative cells located among ECs, and consist mainly of T cells expressing either αβ TCRs or γδ TCRs, which allow the bridging innate and acquired immune responses at the surfaces of aero-digestive tracts through their interaction with classical and nonclassical MHC molecules expressed on ECs [66].

In addition to IELs, antigen-sampling M cells, termed villous M cells, are found in villous epithelium as an

alternative antigen-sampling pathway [67]. Additionally, DCs in the LP extend their dendrites into the lumen and sample antigen [68–70]. A recent study has suggested that these DCs are capable of initiating systemic IgG responses, whereas antigen transport by M cells into the PPs is required for the induction of intestinal IgA production [71], a finding that is consistent with another report that DCs in the PPs are responsible for intestinal IgA production [12]. Similar villous M cells and intraepithelial DCs have been reported in the respiratory tracts [72,73]. In addition, our recent studies have also suggested the presence of respiratory M cells in the nasal cavity (Kim *et al.*, submitted for publication). Taken together, these results suggest that the aero-digestive tissue is equipped with a diversified antigen-uptake and presenting system consisting of ECs, MALT M cells, villous M cells, and intraepithelial DCs.

Mucosal lymphocyte trafficking

Following T cell priming and CSR to IgA-committed B cells in the MALTs, lymphocytes move to distant mucosal effector compartments (especially the LP regions of the respiratory and gastrointestinal tracts). Accumulating evidence has revealed the existence of a highly sophisticated system regulating lymphocyte trafficking from MALTs into the LP regions of the aero-digestive tract [1] (also see the article by Agace in this issue).

A lipid mediator, sphingosine 1-phosphate (S1P) is now recognized as a general molecule in the regulation of lymphocyte trafficking [74]. We have recently shown that S1P contributes to the mucosal immune responses including intestinal S-IgA production from peritoneal and PP B cells as well as trafficking of IEL precursors from the thymus [24,75–77]. Other studies have demonstrated that S1P is also involved in the immune regulation of respiratory tissues [78]. Because the S1P-mediated system is also utilized in the trafficking of pathogenic cells, it is a potential target for the inhibition of inflammation in both respiratory and intestinal tracts [79–82].

In contrast to the shared function of S1P, aero-digestive tissue possesses a distinct pathway to achieve selective trafficking to its mucosa. Mucosal addressin cell adhesion molecule-1 (MAdCAM-1) expressed by high endothelial venules (HEVs) in the gastrointestinal tracts and $\alpha_4\beta_7$ integrin expressed by lymphocytes is the most important adhesion molecule in the cell trafficking to the intestine (Figure 4) [83]. Chemokines, especially CCR9, is selectively expressed on IgA-committed B cells and T cells activated in the PPs, and CCL25 (the ligand of CCR9) is produced dominantly by the intestinal epithelium, allowing PP-primed B and T cells to selectively migrate to the intestinal LP (Figure 4) [84]. Several lines of evidence suggest that PP DCs play a pivotal role in the induction of $\alpha_4\beta_7$ integrin and CCR9 on activated B and T cells through the production of RA from vitamin A [85,86]. Thus, significantly decreased numbers of intestinal T and B cells have been noted in the intestine of vitamin A-deficient mice and rats, which might in turn explain the increased level of child mortality arising from vitamin A deficiency induced diarrhea [85–87].

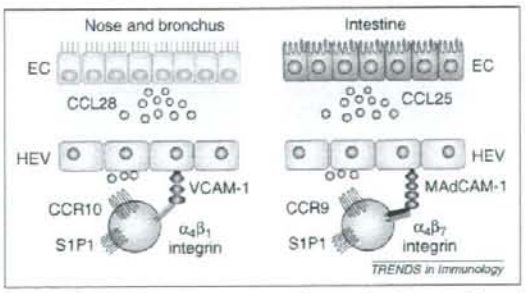

Figure 4. Mucosal T cell migration pathways for aero-digestive tracts. Mucosal lymphocyte migration is determined and controlled by the combination of adhesion molecules and chemokines. CCL28-CCR10 and VCAM-1-$\alpha_4\beta_1$ integrin interactions are involved in the trafficking of cells to the respiratory mucosa, whereas CCL25-CCR9 and MadCAM-1-$\alpha_4\beta_7$ integrin participate in the cell trafficking to intestinal tissues through high endothelial venules (HEV). S1P1 is a mucosal migration-associated molecule common to both respiratory and intestinal mucosa.

During lymphocyte trafficking to respiratory tissues, neither $\alpha_4\beta_7$ integrin nor MAdCAM-1 was expressed by human tonsil mononuclear cells or the tonsils and adenoids of rodent NALT HEVs, respectively. However, nasal immunization induces upregulation of $\alpha_4\beta_1$ integrin and CCR10, allowing selective trafficking of lymphocytes to LP regions of the nose, trachea and bronchus, where their ligands, VCAM-1 and CCL28, are strongly expressed (Figure 4) [88]. Several lines of evidence show that lymphocyte trafficking into the lung is regulated by adhesion molecules (VLA-1 and LFA1) and chemokines (e.g. CCL5) [89,90].

Leading bench mucosal immunology toward a new generation of self-administered vaccines

One of the major missions of basic research is of course the translation of bench discoveries to the bedside and public health. Understanding the molecular and cellular functions of the mucosal immune system associated with the aero-digestive tract has allowed us to create a general consensus that nasal or oral administration is an effective regimen for the induction of localized antigen-specific mucosal immune responses mediated by both S-IgA and CTL as well as systemic immune responses (e.g. IgG) [91] (also see the article by Belyakov and colleagues in this issue). Because mucosal immunization can effectively induce both layers of antigen-specific immunity, it has been considered that mucosal vaccination is an ideal strategy for the global control of mucosal infectious diseases [91]. In general, thanks to the above-described elegance of the imprinting system for tissue tropism of mucosally activated lymphocytes, the antigen-specific humoral and cellular immune responses induced by mucosal immunization most effectively occur at the site of antigen-deposited mucosal tissues with some mobility to the other mucosa-associated tissues. Therefore, a general view is that antigen administration via oral and nasal routes will be a suitable method for vaccination against gastrointestinal and respiratory infections, respectively [92].

Currently several oral vaccines, such as polio (OPV) and rotavirus vaccine (Rotarix® and RotaTeq®), are approved for human use (Table 2) [93,94]. These vaccines are forms of live-attenuated viruses that stimulate the gastrointes-

Table 2. Currently approved oral/nasal vaccine against mucosal infectious diseases

Pathogen	Administration route	
	Oral	Nasal
Polio virus	Live attenuated polio virus (strain: Sabin)	–
Vibrio cholera	Heat or formalin inactivated *V. cholerae* O1 (strain: Inaba and Ogawa) and cholera toxin B subunit (CT-B) (Dukoral®)	–
Rotavirus	Live attenuated rotavirus Strain: human rotavirus 89-12 (RotaRix®) Bovine rotavirus WC3 (RotaTeq®)	–
Influenza virus	–	Live attenuated influenza virus (strain: Fashion species) (FluMist®)

tinal immune system upon oral immunization, resulting in the effective induction of high levels of virus-specific protective immunity. In addition to oral vaccines, a nasal spray vaccine against influenza (FluMist®), composed of live-attenuated, cold-adapted influenza virus, is now approved and used in the United States (Table 2) [95]. Nasally administered FluMist® effectively induces influenza virus-specific protective immune responses via the airway mucosal immune system. In particular, the hemagglutinin-specific S-IgA secreted into the lumen of the respiratory tract plays a pivotal role in the inhibition of the virus entry through the epithelial surface of the respiratory tract. Despite their effectiveness as mucosal vaccines, it should be noted that these live-attenuated mucosal vaccines occasionally cause side effects because of their live nature. For instance, attenuated polio virus can, albeit very rarely, acquire neurovirulence after oral immunization and cause poliomyelitis [93]. In addition, nasal vaccination with the virus attenuated FluMist® is more likely to cause wheezing than the nasally administered inactivated influenza virus, especially in young children with a history of asthma [96]. For this reason, FluMist® is not yet approved for the immunologically infirm populations of infants and the elderly [97]. Therefore improvements to the safety of live-attenuated mucosal vaccines are an important prerequisite for their practical use.

By contrast, mucosal vaccines composed of inactivated viruses or bacteria killed by heat or formalin treatment have been energetically developed because they rarely cause side effects. An oral cholera vaccine (Dukoral®) composed of the O1 and B subunits of cholera toxin (CT-B) from inactivated *Vibrio cholerae* induces protective immunity, especially in the gastrointestinal tract (Table 2) [98]. By contrast, an injectable type of inactivated cholera vaccine is available, but it fails to fully protect against *V. cholerae* infection because it induces only systemic, and not mucosal immune responses [98]. Therefore, the development of an oral rather than injectable cholera vaccine is still a prudent approach and currently the subject of a worldwide effort. In addition to oral vaccines, the development of inactivated nasal vaccines against respiratory infectious diseases has been extensively investigated. It was recently reported that nasal vaccination with formalin-inactivated influenza virus (strain H5N1) induces protective immunity in both the systemic and mucosal compartments of nonhuman primates [99]. In addition, co-administration of the TLR3 agonist polyI:polyC$_{12}$U (Ampligen®) in combination with the H5N1 vaccine enhanced virus-specific S-IgA responses in the respiratory tract [100]. H5N1 is the highly pathogenic avian influenza virus, and these findings should facilitate the development of a nasal influenza vaccine for the global control of potentially catastrophic zoonoses caused by this strain of influenza virus.

Injectable vaccines are currently and commonly used in both developing and industrialized countries, despite our scientific knowledge of the advantages of mucosal immunization. One of the major practical obstacles to vaccination in the field, especially in developing countries, is storage of the vaccine under refrigeration (known as the cold-chain). In addition, injection of a vaccine with a needle and syringe requires skilled medical professionals at the time of inoculation and disposal of the used needles and syringes from mass vaccinations, which is now a major concern because medical waste can contaminate the environment. To overcome these practical concerns, a new generation of 'self-administrable cold-chain and needle and syringe-free vaccines' will need to be developed. To accomplish this goal, a plant-based vaccine is considered to be one attractive strategy, because plants can be used as natural bioreactors and transporters of vaccine antigens. Progress in plant genetic technology has enabled the development of plant-based oral subunit vaccines [91]. Among the several plant-based vaccines developed so far, a rice-based oral vaccine (or MucoRice^{TM}) has recently attracted interest as a vaccine production and delivery system because of its practical advantages [101]. For instance, a unique protein-storage organelle in the rice seed, named the protein body, provides a suitable vehicle for expression of vaccine antigens that are not only stable at room temperature for several years without loss of immunogenicity, but are also protected from digestive enzymes in the gastrointestinal tract [101]. In this context, we have recently reported that oral immunization with MucoRice^{TM}-expressed CT-B subunit in mice effectively induced antigen specific intestinal and systemic immune responses with a protective function against an oral challenge with cholera toxin [101]. By contrast, it should be noted that MucoRice^{TM}-expressed CT-B did not induce any detectable level of immune response against rice storage proteins. This discrepant but advantageous result might be due to the high antigenicity of MucoRice^{TM}-expressed CT-B compared with that of the rice storage protein. The MucoRice^{TM} system therefore opens up novel avenues for developing both human and environmentally friendly vaccines as cold-chain-, needle- and syringe-free self-administered vaccines that will benefit both developing and developed countries.

The other recent progress in the development of new-generation mucosal vaccines is the creation of a delivery system that targets M cells. As discussed above, M cells in

FAE regions of PPs and NALT enable the selective transport of luminal antigens from the gastrointestinal or respiratory lumen to their respective MALTs for the initiation of antigen-specific immune responses [7]. However, because the number of M cells in mucosal epithelium is limited, the development of M cell-targeting systems for antigen delivery might facilitate the efficacy of mucosal vaccines for the induction of antigen-specific systemic and mucosal immune responses. Several approaches incorporating an M cell-specific lectin or peptide [e.g. *Ulex europaeus* agglutinin-1 (UEA-1) [102,103] and YQCSYTMPHPPV [104]] or microbial invasion molecules known to target M cells (e.g. reovirus σ1 protein and *Yersinia*-derived invasin) into the vaccine [91,105] have been examined as the cell-targeted delivery vehicles for nasal or oral vaccines, but their binding to other neighboring cells (e.g. UEA-1 binding to goblet cells [106]) has hampered their effective delivery of antigens to M cells. To overcome such obstacles, we have generated a monoclonal antibody (NKM 16-2-4), which specifically binds M cells but not other cells of the intestinal and respiratory tract [107]. NKM 16-2-4 also reacts with villous M cells, which are known to be alternative antigen-sampling cells located in the intestinal villous epithelium [67,107]. Thus, the use of NKM 16-2-4 as a delivery molecule for mucosal vaccines makes it possible to effectively target a vaccine antigen to both PP-associated and villous M cells, and the subsequent induction of antigen-specific immune responses in both mucosal and systemic compartments is more efficient than that of other M cell-targeted vaccines (e.g. UEA-1-conjugated vaccine). Indeed, we have shown that potent levels of antigen-specific protective immunity were induced when mice were orally immunized with small amounts of tetanus-toxoid- or botulinum-toxoid-conjugated NKM 16-2-4 [107]. Thus far we have not tested the application of NKM 16-2-4 to a nasal vaccine and several other issues, e.g. the requirement of a mucosal adjuvant and reactivity to human M cells, await future study. Development of an M cell-targeted mucosal vaccine using an M cell-specific antibody is a hopeful strategy for the development of a safe and effective mucosal vaccine.

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Chapter 31

The Mucosal Immune System

Hiroshi Kiyono, Jun Kunisawa, Jerry R. McGhee, and Jiri Mestecky

Add H1 "Introduction"?

The most important source of stimulation of the entire immune system is the external environment comprising the indigenous mucosal microbiota, potential pathogenic microorganisms, abundant food antigens (Ags), and allergens, all of which are encountered mainly at the vast surface areas of mucosal membranes. This enormous and highly variable antigenic load has resulted in a strategic distribution of cells involved in the uptake, processing and presentation of Ags, production of antibodies (Abs), secretion of cytokines, and cell-mediated immune (CMI) defenses at the front line of defense—mucosal tissues and associated secretory glands. Quantitative data concerning the distribution of phagocytic cells, T and B lymphocytes, and Ab-producing cells illustrate the point: mucosal tissues, particularly those of the intestinal tracts, contain more macrophages (MΦ), plasma cells (PCs), and T cells than any other lymphoid tissue in the entire immune system.

Notwithstanding the global importance of systemically acquired infections such as malaria and neonatal tetanus, the majority of infectious diseases worldwide either directly afflicts or is acquired through mucosal surfaces of the gastrointestinal (GI), respiratory, and genital tracts. Consequently, innate and adoptive immune mechanisms operational at mucosal surfaces are of great importance to the protection and survival in a hostile environment. The induction of preventive and protective immune responses to mucosal infectious agents, and to ingested food Ags and environmental allergens that would limit their absorption, is usually the most emphasized functional aspect of the mucosal immune system. Yet recently revived interest in the induction of systemic unresponsiveness to Ags applied first by the mucosal route, so called *oral* or *nasal* (mucosal) *tolerance,* has directed the attention of immunologists working in the field of autoimmunity, transplantation, and hypersensitivity to the exploitation of this fundamental

principle. Although there are limited numbers of clinical successes, the phenomenon of mucosal tolerance is an essential feature and critical functional component that efficiently prevents and suppresses otherwise unavoidable overstimulation of the entire immune system by the most common environmental Ags primarily of food and indigenous bacterial origins. The enhancement of protective mucosal immune responses to infectious agents sought by vaccinologists, and the desired suppression of systemic immune responses to autoAgs and transplantation Ags, may seem paradoxical. Yet such outcomes are not mutually exclusive due to the hierarchy in the quality of immune responses induced by mucosal Ag delivery: Mucosal immunity manifested by the appearance of secretory Abs and systemic tolerance evaluated by diminished CMI-responses may be concomitantly induced. Thus, the fundamental objectives of the mucosal immune system—containment of the vast onslaught of environmental Ags without compromised integrity of mucosal barriers and prevention of overstimulation of the systemic compartment—are achieved by concerted interactions of lymphoid and nonlymphoid cells, epithelial cells (ECs) in particular, and their respective products as a mucosal internet of communication. Thus, an orchestrated mucosal immune system consisting of innate immunity as well as acquired immunity including secretory IgA (S-IgA) Abs and mucosal cytotoxic T lymphocytes (CTLs), adds additional layers of host defense.

INNATE MUCOSAL IMMUNE SYSTEM

Epithelial Cells

Physical Barrier Function of ECs

The epithelium of the mucosa-associated lymphoid tissues (MALTs) of the lung, gut and genitourinary tracts, and, likely, others have been clearly shown to play an active role in both innate and adaptive types of mucosal immunity. Given the physical proximity of the ECs to the external milieu and, therefore, the primary site of initial Ag exposure, ECs may be a central cell type in both defining the Ags with which the mucosal immune system is confronted and regulating the ultimate responses to these antigenic exposures. Initially, prevention of luminal Ag transport is through a thick layer of mucus. Mucin 2 (MUC2) is a dominant intestinal mucus-formation molecule that is abundantly expressed by goblet cells located at the intestinal villous epithelium (1). Mucus not only provides a physical and biological protective barrier, but also ensures maintenance of an appropriate concentration of Abs at the mucosal surface by preventing Ag-specific S-IgA Abs from being physically carried away. Additionally, paracellular transport of luminal Ag is prevented by the juncture between adjacent ECs that is mediated by physical structures associated with the epithelium including the tight junctions (TJs) and the subjacent desmosomes and adherence junctions (2). The TJs are composed of a number of interacting cellular proteins, which include claudin, occuludin, ZO-1, ZO-2, and cingulin, among others. Under normal circumstances, the TJs exclude Ags greater than 6–12 Å (> 500–900 Daltons) in molecular diameter.

In addition to these physical barrier functions of ECs, the epithelium of the MALTs of the lung, gut and genitourinary tracts, and, likely, others have been clearly shown to play an active role in both innate and adaptive types of mucosal immunity by collaboration with adjunct neighboring ECs as well as subjacent parenchymal cells (fibroblasts and mesenchymal cells and their connective tissue substances) and hematopoietic cells (MΦ, dendritic cells (DCs), polymorphonuclear (PMN) lymphocytes and lymphocytes) and likely microbial components in the lumen (3).

Antimicrobial Peptides

The epithelium also secretes a variety of antimicrobial peptides (defensins, cathelicidins, crytdin-related sequence [CRS] peptides) and bacteriolysis enzymes (lysozyme, secretory phospholipase-A2 [PLA2], peroxidase, and lactoferrin), and others (Figure 31.1). In the intestinal epithe- **Fig. 31.1**
lium, ECs, Paneth cells, and PMNs mainly produce these molecules (4,5). Paneth cells reside at the base of the crypt regions of the small intestine, but not the stomach or colon. They produce α-defensins constitutively. In contrast, β-defensins are produced by ECs of the whole intestine, which requires microbial stimulation (4,5). Both defensins are cationic small peptides with a characteristic β-sheet–rich fold and a framework of six disulphide-linked cysteines and exhibit antimicrobial activity by damaging and permeabilizing the bacterial cell membrane by pore formation. Defensins also inhibit viral infection (e.g., human immunodeficiency virus [HIV], herpes simplex virus [HSV], vesicular stomatitis virus, and influenza virus) by interupting their invasion at an early step, such as receptor binding (4,5). In addition to the antimicrobial properties, defensins have chemotactic activities for monocytes, T cells, and B cells, implying that defensins may bridge between mucosal innate and acquired immunity via the augmentation of T and B cell interactions (6). The cathelicidin is also a cationic small peptide containing a cathelin-like domain produced by ECs, PMNs, and keratinocytes (4,5). The expression of cathelicidin by ECs is regulated by butyrate and other short-chain fatty acids produced by fermenting bacteria. The CRS peptide is produced by Paneth cells and shows antimicrobial activity through its cationic feature (4,5).

Antimicrobial enzymes are other molecules showing antimicrobial activities (Figure 31.1). PLA2 is a small enzyme produced by Paneth cells and PMNs, which