Sphingosine 1-phoshate regulates intestinal B cell trafficking for the subsequent intestinal secretory IgA production

腸管分泌型IgAの産生におけるスフィンゴシン1リン酸介在型腸管B細胞の遊走制御メカニズムの解明

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Contents

Non standard abbreviation used

AFC, antibody-forming cell

CMIS, common mucosal immune system

DCs, dendric cells

GC, germinal centre

iLP, intestinal lamina propria

NIK, NFκB inducing kinase

OVA, ovalbumin

PCs, plasma cells

PC, phosphorylcholine

PerC, peritoneal cavity

PNA, peanut agglutinin

PPs, Peyer's patches

$S1P_1$, type 1 S1P receptor

TD, T cell-dependent

TI, T cell-independent

Introduction

Secretory IgA (S-IgA) in the intestinal lumen acts as the gateway controller against pathogenic and commensal microorganisms, and their production is achieved by two distinct subsets of B cells, termed B1 and B2 B cells (Fig. 1) (1-3). B1 B cells can be distinguished from B2 B cells by origin, growth properties and cell surface markers (e.g., B220, IgM, IgD, CD5, and Mac-1) (4). B1 B cells exist mainly in the peritoneal cavity (PerC) (3, 5, 6), and have been considered to play an important role in the protective peritoneal immunity by producing antibody in response to T cell-independent (TI) antigen, such as phosphorylcholine (PC), a hapten-like antigen associated with the many pathogenic bacteria (7, 8). In addition to their role in the peritoneal immunity, B1 B cells migrate to the intestinal lamina propria (iLP), where they further differentiate to IgA-secreting plasma cells (PCs) to produce S-IgA against TI antigens in the intestine (9-11). In contrast, mucosal B2 B cells are derived mainly from organized inductive tissues, such as Peyer's patches (PPs), where B2 B cells are primed and switched from the μ to α chain by immunological interactions with dendritic cells and T cells. These interactions results in differentiation of B2 B cells 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) (1, 2, 12-14). Expression of gut-tropic molecules allow the precursors of IgA^+ PCs to traffic specifically distant effector sites, iLP, where they finally differentiate into PCs to produce S-IgA against T cell-dependent (TD) antigen, such as viral protein and bacterial toxins in the intestine (15, 16). In spite of the importance of both B cell subsets in the intestinal IgA production, there is little

information about migration pathways of B1 and B2 B cells to iLP.

Accumulating evidence has revealed that sphingosine 1-phosphate (S1P) regulates lymphocyte trafficking, especially emigration from the organized lymphoid tissues, such as the thymus and secondary lymphoid organs (Fig. 2B) (16, 17). Five types of S1P receptors have been identified. The type 1 S1P receptor ($S1P_1$) primarily expressed on lymphocytes (16, 17). Several key findings about the involvement of S1P in the regulation of lymphocyte trafficking were obtained in experiments using FTY720. FTY720 acts as an agonist for S1P receptors, except type 2 S1P receptor ($S1P_2$) (18-20) and blocks S1P-mediated signaling by inducing internalization of receptors (Fig. 2C) (21, 22). Thus, treating mouse with FTY720, decrease number of circulating lymphocytes in both blood and lymph by inhibiting their emigration from the secondary lymphoid organs and thymus (18). These experiments on S1P-mediated trafficking were performed mainly using T cells (23-25). It was shown that $S1P_1$ expression on T cells was cyclically modulated during their cellular trafficking with sensitivity to the S1P gradient which allows lymphocyte retention into lymph nodes or exit into blood and lymph (23-25). In addition, several lines of evidence have identified that S1P is also involved in the regulation of B cell trafficking. For instance, FTY720 impairs plasma Ab production, especially against T-dependent Ag due to the abolishment of germinal center (GC) formation (26-28). S1P is also important in the determination of PC tropism into bone marrow (29). These data collectively suggest that S1P plays important roles of T and B cell trafficking in the systemic immune compartments. Furthermore, our group

has recently demonstrated that S1P also contributes to the regulation of mucosal cell trafficking, such as naive $\alpha\beta TCR^+$ intraepithelial T lymphocytes in naïve mice (30) and pathogenic T and mast cell trafficking into the intestine under intestinal allergic conditions (31). These findings provide strong evidence that S1P plays an essential role in the regulation of lymphocyte trafficking in both systemic and mucosal immunity. However, only minimal evidence was observed in S1P dependence in mucosal B cell trafficking at intestine. In this study, I focus on the involvement of S1P in the intestinal B cell trafficking from PerC and PPs and subsequent intestinal IgA production.

Material and Methods

Mice and experimental treatment

Female Balb/c, C57BL/6, *aly*, ICR and SCID mice (7–9 weeks) were purchased from Japan Clea (Tokyo, Japan) and were provided with sterile food and water ad libitum. For treatment with FTY720 (Novartis Pharma, Basel, Switzerland), mice were injected intraperitoneally with FTY720 (1 mg/kg/time) (31-33). 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.

Immunization

For immunization with heat-killed pepsin-treated *S. pneumoniae* strain R36A (gift

of Dr. John Kearney, University of Alabama, Birmingham) (8, 34), mice were immunized intraperitoneally with 10^7 R36A alone or orally with 2 x 10^8 R36A together with 10 μg of mucosal adjuvant, cholera toxin (List Biological Laboratories, Campbell, CA) (35). For oral immunization, mice were deprived of food for 15 h and then given a solution of sodium bicarbonate to neutralize stomach acid 30 min before oral immunization (36). The peritoneal immunization was conducted once and the oral immunization was conducted 3 times on days 0, 7, and 14. In the FTY720-treated group, mice were injected intraperitoneally with FTY720 six hours before the immunization and then again once per day during the experiment. Five days after the final immunization, serum and fecal extracts were prepared for the analysis of PC-specific IgM and IgA production by ELISA, as described below (43, 44).

For oral immunization with ovalbumin (OVA), the oral immunization procedure followed by the case of immunization with R36A. After neutralize stomach acid, mice were orally immunized with 1 mg of OVA (Sigma-Aldrich, St. Louis, MO) with 10 μg of cholera toxin. This oral immunization procedure was conducted on days 0, 7, and 14. Seven days after final immunization, serum and fecal extracts were prepared for the analysis of OVA-specific IgG and IgA production by ELISA, as described below (43, 44).

Lymphocyte isolation

Lymphocytes were isolated from the PerC, spleen, PPs, and iLP as previously

described (5, 31, 33, 36-38). Briefly, PerC cells were obtained by flushing the peritoneum with 8 ml of ice-cold PBS (5). 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, Osaka, Japan) was employed. To isolate lymphocytes from the iLP, after removing PPs and isolated lymphoid follicles, small intestines were cut into 2-cm pieces and stirred in RPMI1640 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. Lymphocytes were collected form the omentum, parathymic lymph nodes, and blood in accordance with previously established protocol (34, 39).

Flow cytometry and cell sorting

Flow cytometry and cell sorting were performed as previously described (31-33). Cells were preincubated with 10 μg/ml of anti-CD16/32 Ab (BD Biosciences, San Jose, CA) and then stained with fluorescent Abs specific for B220, CD5, CD11b, CD138, $\alpha_4\beta_7$ integrin, IgA, IgM (BD Biosciences), PNA (Vector Laboratory, Burlingame, CA), and CCR9 (R&D Systems, Minneapolis, MN). A Viaprobe (BD Biosciences) was used to discriminate between dead and living cells. Cytofix and Cytoperm kit (BD Biosciences) and ethidium monoazide bromide (Invitrogen) 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 FACS Calibur (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, Sunnyvale, CA). These T cell–depleted PP cells were subjected to cell sorting using FACSAria (BD Biosciences).

Adoptive cell transfer

For tracing cells *in vivo*, peritoneal cells (1 x 10^7 cells) were incubated with 0.25 μM 5-(and-6)-carboxyfluorescein diacetate, succinimidyl ester (CFSE) (Molecular Probes, Eugene, OR) in the dark for 10 min at 37°C, washed with PBS twice according the previously described method (40). The labeled cells were transferred into SCID or *aly* mice intraperitoneally (4 x 10^6 cells) or intravenously (1 x 10^7 cells), and FTY720 was simultaneously administered intraperitoneally. In the case of intravenously transfer of the labeled cells into *aly* mice, before transfer, *aly* mice were intraperitoneally injection with 2 ml PBS, and then PBS containing abdominal fluid was depleted by aspiration using 18 G needle. After 8 h from depletion of peritoneal cells, CFSE-labeled WT Peritoneal B cells were transferred intravenously and simultaneously were treated with FTY720. After 12 hrs, peritoneal cells were collected for FACS analysis.

For experiments involving stromal cell transfer, stromal cells were isolated from the small intestines of wild-type mice as previously described (41). Briefly, LP cells were isolated and cultured on 10 cm culture plates in complete RPMI1640 medium. After 1h,

nonadherent cells were removed by washing with PBS, and remaining adherent cells were cultured overnight in complete RPMI1640 medium. After overnight culture, the plates were washed with PBS, and remaining adherent cells were cultured in complete RPMI1640 medium. After two times subculture, confluent cells were used as stromal cells. The stromal cells were transferred (1×10^7 cells) into the peritoneal cavities of *aly* mice. Two weeks after stromal cells transfer, mice were intraperitoneally treated with FTY720.

For the analysis of antibody production from peritoneal B cells, SCID mice were intraperitoneally reconstituted with normal peritoneal B cells (5×10^6 cells) and treated with FTY720 every 2 days. Two weeks after the adoptive transfer, serum and fecal extracts were collected for the measurement of total Ig levels by ELISA as described below. Simultaneously, mononuclear cells were isolated from the intestinal lamina propria for enumeration of AFCs by ELISPOT as described below.

ELISA

Total and antigen-specific Ig level in serum and fecal extract was determined by ELISA as previously described (6) (34, 36-38). To measure total antibody concentration, purified murine isotype-specific antibodies (BD Biosciences) were used as standards for the quantification. For the detection of PC- and OVA-specific antibody, microtiter plates were coated with 5 μg/ml of PC-BSA (Biosearch Technologies Inc., Novato, CA) in bicarbonate buffer (pH 9.6) and 1 mg/ml of OVA (Sigma) in PBS, respectively.

Following blocking with 5 (for PC) or 1% (for OVA) BSA in PBS, serum and fecal dilutions were incubated in the coated wells for 2 hrs at room temperature. And bound antibodies were determined by HRP-conjugated anti-mouse IgA and IgG (Southern Biotechnology Associates, Birmingham, AL) and 3,3',5,5'-tetramethylbenzidine (Moss, Pasadena, CA) as previously described (34, 37).

Enumeration of antibody forming cells (AFCs) by ELISPOT

To measure AFCs producing IgM and IgA, ELISPOT assay was employed as previously described (6). Briefly, various concentrated mononuclear cells were cultured in 96-well nitrocellulose membrane plates (Millititer HA; Millipore, Bedford, MA) coated with 5 μg/ml affinity-purified goat anti-Ig (Southern Biotechnology Associates) at 37°C for 4 h (for the enumeration of IgA- or IgM-AFCs) or 1 mg/ml OVA-PBS at 4°C for 15 h (for the detection of OVA-specific IgA-AFCs). After vigorous washing with PBS and PBS containing 0.05% Tween 20, HRP-conjugated Abs specific for mouse IgM or IgA (Southern Biotechnology Associates) were added and incubated overnight at 4°C. The spots of AFCs were developed by 2-amino-9-ethylcarbazole (Polysciences, Warrington, PA) containing hydrogen peroxide.

Immunohistochemical analysis

Immunohistochemical analysis was performed as previously described (5, 32).

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 Co., Tokyo, Japan). 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. Counter-staining was performed using 4',6'-diamidino -2-phenylindole (Sigma-Aldrich). Podoplanin and CD11c were stained using the TSA-Direct kit (PerkinElmer, Waltham, MA) according to the manufacturer's instructions (42). 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. Sections were stained with purified anti-podoplanin Ab (Acris Antibodies GmbH, Hiddenhansen, Germany) plus biotin-conjugated anti-hamster IgG cocktail (Becton Dickinson) or biotin-conjugated anti-CD11c Ab (BD Biosciences) overnight, followed by incubation with HRP-conjugated streptavidin (Pierce, Rockford, IL) 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, Wetzlar, Germany).

In vitro migration assay

In vitro migration assays using purified B1 and B2 cells were performed according to a previously established method (21). Briefly, peritoneal B cells were applied to the

upper chambers (pore diameter, 5 μm; Invitrogen, Carlsbad, CA) and 0, 20, 200, or 2000 nM S1P was added in the lower wells. After 6-hour incubation, the B cells that had migrated into the lower wells were counted with the aid of trypan blue staining.

***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 of murine IL-6 (R&D Systems) for 72 h (5). The amount of IgA in the culture supernatant was determined by ELISA, as described above (5, 33).

Quantitative and conventional RT-PCR

To measure mRNA expression for S1P receptors, quantitative RT-PCR using LightCycler (Roche Diagnostics, Mannheim, Germany) was performed (6). Briefly, total RNA was collected by TRIzol reagent (Invitrogen) and cDNA was synthesized using Powerscript Reverse transcriptase (BD Biosciences). The oligonucleotide primers and probes specific for $S1P_1$ (forward primer, TACACTCTGACCAACAAGGA; reverse primer, ATAATGGTCTCTGGGTTGTC; FITC-probe, TGCTGGCAATTCAAGAGGCCCATCATC; LCRed 640-probe, CAGGCATGGAATTTAGCCGCAGCAAATC), $S1P_2$ (forward primer, CATCGTACTGGGTGTTTTC; reverse primer, CCACGTATAGATGACAGGA;

FITC-probe, AATAGTGGGCTTTGTAGAGGACAGGGCAGG; LCRed 640-Probe, CCGAACGGGACAGGTGGAGTCTAAGAGAAG), $S1P_3$ (forward primer, TCCTCTTCCTCATCGACGTG; reverse primer, CCTTGCCCTTGACTAGACAG; FITC-probe, TTCATCATGCTGGCTGTCCTCAACTCGG; LCRed 640-Probe, CATGAACCCTGTCATCTACACGCTGGCC), $S1P_4$ (forward primer, CATCTTTAGAGTGGTCCGAG; reverse primer, GCCCAGACATTAGAACCAA; FITC-probe, CCGCAGGCTACTCAACACCGTGCTGAT; LCRed 640-Probe, ATCTTGGTGGCCTTTGTGGTGTGCTGG), and GAPDH (forward primer, TGAACGGGAAGCTCACTGG; reverse primer, TCCACCACCCTGTTGCTGTA; FITC-probe, CTGAGGACCAGGTTGTCTCCTGCGA; LCRed 640-probe, TTCA ACAGCAACTCCCACTCTTCCACC) were designed and synthesized by Nihon Gene Research Laboratory (Sendai, Japan). Conventional RT-PCR was performed to measure pIgR J-chain and AID, using specific primers (pIgR forward, AGTATTCAG GCAGAGCCAAC; pIgR reverse, ATTCATCCGGCACAGATATT; J-chain forward, ATGAAGACCCACCTGCTTCTCTGG; J-chain reverse, AGGGTAGCAAGAAT CGGGGGTCAA; AID forward, GGCTGAGGTTAGGGTTCCATCTCAG; AID reverse, GAGGGAGTCAAGAAAGTCACGCTGGA) (43).

Statistical analyses

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

Results

1. The S1P and NFκB inducing kinase (NIK) signaling collaborate to regulate peritoneal B cell trafficking for subsequent intestinal IgA production.

1.1 Rapid and reversible disappearance of peritoneal B cell by FTY720 treatment

The initial aim of this study is to examine an involvement of S1P in the regulation of peritoneal B cell trafficking (Fig. 3). To accomplish this, mice were administered intraperitoneally with FTY720, a modulator for S1P receptors, and cellular population in PerC was examined. Flow cytometric analyses revealed that both $B220^{+}CD11b^{+}$ B1 and $B220^{+}CD11b^{-}$ B2 B cells were dramatically reduced in the frequency in FTY720-treated mice (Fig. 4A). Because total peritoneal cell number remained unchanged even after the FTY720 treatment (data not shown), the changes in the cellular percentage directly reflected absolute cell number of each population (Fig. 4B). Thus, I found significant decreases in B1 and B2 B cell numbers after the FTY720 treatment (Fig. 4B).

I next sought to analyze the kinetics of the change and recovery of the peritoneal B cells after the treatment with FTY720. Marked reduction of both B1 and B2 B cells was found only 3 hrs after FTY720 treatment (Fig. 4C). And a partial recovery was detected 24 hrs after the injection, with full recovery observed 7 days after the administration (Fig. 4D). These data suggest that the effect of FTY720 on peritoneal B cells was rapid and reversible.

1.2 Specific and equal expression of type 1 S1P receptor ($S1P_1$)-encoding mRNA by peritoneal B cells

As might be expected by the comparable effects of FTY720 on the removal of peritoneal B cells, both peritoneal B1 and B2 B cells were revealed by quantitative RT-PCR to express similar levels of mRNA encoding type 1 S1P receptor ($S1P_1$) with no or only a dim expression of the other subtype of S1P receptors ($S1P_2$-$S1P_4$) (Fig. 5A). Since B1 B cells are further divided into B1a and B1b B cells based on CD5 expression (4, 7, 44), I then compared the $S1P_1$ expression of B1a and B1b B cells, finding that the level of $S1P_1$-specific mRNA expression was similar both cell groups (Fig. 5B).

1.3 FTY720 inhibits B cell migration into and enhances B cell emigration out of the PerC

To determine whether FTY720 reduced peritoneal B cells by promoting their emigration from the PerC, peritoneal B cells were isolated from normal mice, labeled them with CFSE adoptively transferred them into the PerC of SCID mice, and compared their emigration in mice receiving FTY720 treatment significantly decreased the numbers of peritoneal $CFSE^+$ cells in the PerC and blood of treated mice (Fig. 6A). Since it has been considered that lymphocytes pass through the omentum and parathymic lymph nodes on their way to the blood from the PerC (45, 46), I next

examined the cell population in these tissues. Flow cytometric analysis revealed that the number of $CFSE^+$ $B220^+$ cells was reduced in the omentum but increased in the parathymic lymph nodes of FTY720-treated mice (Fig. 6A). These data indicate that FTY720 treatment induces the accumulation of peritoneal B cells in the parathymic lymph nodes, leading to a reduction of these cells in the PerC and the blood.

Under similar experiments, I also examined the effect of FTY720 on their migration from the blood into the PerC because it was previously reported that mature B cells could home to the PerC from the blood (34) and thus it was possible that FTY720 also inhibit peritoneal B cell migration into the peritoneal cavity from the blood. I transferred CFSE-labeled peritoneal cells to SCID mice via the intravenous route and compared their migration into PerC of mice receiving FTY720 and in those that did not. The number of $CFSE^+$ cells was significantly lower in the PerC when mice were treated with FTY720, indicating that FTY720 inhibited the migration of peritoneal B cells from the blood circulation into the PerC (Fig. 6B). In these mice, $CFSE^+$ cell numbers were reduced in the blood, omentum, and parathymic lymph nodes but increased in the bone marrow (Fig. 6B and data not shown). These data suggest that FTY720 directs the circulating B cells to migrate to the bone marrow rather than to the PerC. Collectively, these findings indicate that FTY720 removes the peritoneal B cells both by enhancing their emigration from the PerC and by inhibiting their immigration from the blood into the PerC.

1.4 Decreased sensitivity to FTY720 in *aly* mice in spite of normal $S1P_1$ expression and reactivity to S1P

Previous study suggested that *aly* mice, NFκB inducing kinase (NIK) mutant mice, showed the accumulation of peritoneal B1 and B2 B cells in PerC due to impaired the migration of peritoneal B cell (58). Because a previous report showed that NIK-mediated signaling was linked to G-coupled protein that S1P receptors use (58), I speculated that NIK mutation abolished $S1P_1$-mediated signaling. To test this hypothesis, I administered intraperitoneally FTY720 in PerC of *aly* mice.

Consistent with the data shown above (Figs. 4A, B), a single injection of FTY720 induced rapid reductions in the percentages and absolute cell numbers of peritoneal B1 and B2 cells in WT mice (Fig. 7A). In contrast, *aly* mice showed scant reduction in peritoneal B cells after a single injection of FTY720 (Fig. 7B). To elucidate whether the FTY720 reactivity of *aly* mice was complete or partial, mice were injected repeatedly with FTY720 and their peritoneal B cells were examined. Although treating WT mice with multiple injections of FTY720 did not increase its effect on peritoneal B cells (Fig. 7A), repeated FTY720 treatment significantly ($p<0.05$) reduced the peritoneal B1 and B2 B cell populations of *aly* mice (Fig. 7B). These findings suggest that the peritoneal B cells of *aly* mice showed reduced sensitivity to FTY720 but still reactive to FTY720.

To examine the possibility that the decreased reactivity of *aly* peritoneal B cells to

FTY720 was due to their minimal expression of S1P receptors, especially $S1P_1$. To test this possibility, I performed quantitative RT-PCR analysis and found that the levels of $S1P_1$ in peritoneal B1 and B2 B cells and splenic B cells were comparable between *aly* and WT mice (Fig. 8A). I next investigated the *in vitro* migration of peritoneal B cells toward S1P to test whether $S1P_1$-mediated signaling in *aly* peritoneal B cells was functional. I found that, like the peritoneal B cells isolated from WT mice, both peritoneal B1 and B2 cells from *aly* mice migrated to the gradient of S1P (Fig. 8B).

To further confirm that *aly* peritoneal B cells show normal reactivity to S1P *in vivo*, we isolated *aly* peritoneal B cells, labeled them with CFSE, and adoptively transferred them into SCID mice. Because FTY720 inhibited B cell immigration into the peritoneal cavity and enhanced their emigration from it (Fig. 6A, B), I transferred the labeled B cells through two different routes, intraperitoneal and intravenous injection. When *aly* peritoneal B cells were adoptively transferred into the peritoneal cavities of SCID mice, FTY720 treatment resulted in a marked reduction in B cell numbers (Fig. 8C). Further, the immigration of *aly* B cells from the blood into the PerC was also impaired when SCID mice were treated with FTY720 after the intravenous transfer of *aly* peritoneal B cells (Fig. 8C). Taken together with the *in vitro* and *in vivo* data, our findings convincingly show that *aly* peritoneal B cells can react to S1P and FTY720. However, the sensitivity to FTY720 is lower in *aly* mice than in WT mice.

1.5 Lack of secondary lymphoid organs did not cause the unresponsiveness to

FTY720

I next addressed a question why *aly* mice showed the decreased reactivity to FTY720 in spite of the normal reactivity of *aly* B cells to FTY720. A previous study has demonstrated that S1P lyase, an enzyme degrading S1P into phosphoethanolamine, is abundantly present in secondary lymphoid organs, which establishes the S1P gradient of lower S1P concentration in the secondary lymphoid organs than in the blood and lymph. These findings led to a hypothesis that a lack of secondary lymphoid organs in aly mice might lead to their decreased sensitivity to FTY720 due to the impaired S1P gradient. To test this hypothesis, I employed another secondary lymphoid organ-deficient mouse, which lacked Id2, negative regulator of basic helix-loop-helix transcription factors. Although secondary lymphoid organs were deficient in Id2 KO mice, peritoneal B cells in Id2 KO mice showed normal reactivity to FTY720 (Fig. 7A, B). Therefore, the reduction of both peritoneal B1 and B2 B cells was noted in Id2 KO mice after FTY720 treatment. These data suggest that the deficient secondary lymphoid organ structure is not attributable to the impaired reactivity of aly mice to FTY720.

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

In light of our current findings that *aly* peritoneal B cells react to FTY720 and S1P, we hypothesized that their decreased sensitivity to FTY720 was due to the NIK

mutation in the non-B cells. We therefore examined the FTY720 reactivity of WT peritoneal B cells adoptively transferred into SCID and *aly* mice. Regardless of the injection route, WT peritoneal B cells showed normal reactivity to FTY720 and thus were decreased after treatment with FTY720 when they were adoptively transferred into SCID mice (Fig. 10A and B). In contrast, WT peritoneal B cells transferred intraperitoneally into *aly* mice lacked reactivity to FTY720. Indeed, the numbers of peritoneal B cells were similar with or without FTY720 treatment (Fig. 10C).

We next addressed whether NIK mutation affected B cell immigration from the blood into the peritoneal cavity. In this experiment, we removed the peritoneal cells from recipient *aly* mice 8 hours before adoptive transfer, because the *aly* peritoneal B cells were too numerous to allow detection of intravenously transferred B cells. Twenty-four hours after depletion of the peritoneal B cells, the peritoneal cavities of *aly* mice contained more B2 cells than untreated *aly* peritoneal B cells (data not shown), demonstrating that peritoneal B cells were removed and that many cells were derived from the blood. Removing peritoneal cells from recipient mice before adoptive transfer enabled us to detect intravenously injected WT peritoneal B cells in *aly* mice (Fig. 10D). Unlike the case with intraperitoneally transferred WT B cells (Fig. 10C), FTY720 prevented the immigration of intravenously transferred WT peritoneal B cells from the blood into the peritoneal cavity (Fig. 10D). The number of adoptively transferred B cells was decreased consistently and significantly ($p<0.05$) in the peritoneal cavities of FTY720-treated *aly* mice (Fig. 10D). These findings suggest that NIK-mediated

signaling in non-B cells participates in the regulation of S1P-mediated emigration of B cells from the peritoneal cavity but not in their immigration from the blood.

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

Because both *aly* and WT B cells showed normal emigration from the peritoneal cavities of SCID mice (Figs. 8C and 10A, respectively), T cells likely do not play a role in this pathway. We therefore speculated that NIK-mediated signaling in stromal cells was involved in the emigration of peritoneal B cells. To test this hypothesis, we transferred WT stromal cells into the peritoneal cavities of *aly* mice treated with FTY720 and noted a subsequent reduction in the number of peritoneal B cells (Fig. 11A, right panels). In contrast, FTY720 had no discernible effect when *aly* stromal cells were transferred into *aly* mice (Fig. 11A, left panels). These data suggest that NIK-mediated signaling in stromal cells participates in the regulation of S1P-mediated peritoneal B cell emigration.

To investigate the mechanisms of peritoneal B cell trafficking mediated by NIK in stromal cells, we compared the expression of VCAM-1 and ICAM-1, adhesion molecules regulating peritoneal B cell trafficking, between WT and *aly* stromal cells. The expression of these adhesion molecules was lower on *aly* stromal cells than on WT stromal cells (Fig. 11B). A previous report that S1P regulated the expression of

VCAM-1 on endothelial cells led us to hypothesize that S1P affects the expression of VCAM-1 and ICAM-1 on stromal cells. However, unlike endothelial cells, WT and *aly* stromal cells showed negligible expression of all types of S1P receptors (data not shown). Therefore, treatment of stromal cells with S1P or FTY720 influenced neither VCAM-1 nor ICAM-1 expression, although IFNγ increased the expression of both of these molecules in both WT and *aly* stromal cells (Fig. 11B). These findings collectively indicate that, in S1P-mediated peritoneal B cell trafficking, S1P directly affects peritoneal B cells, not stromal cells, but stromal cells are involved in the S1P-mediated pathway through the expression of adhesion molecules. Decreased expression of VCAM-1 and ICAM-1 on stromal cells in *aly* mice may explain the impaired S1P-mediated trafficking of peritoneal B cells in *aly* mice.

Taken together, FTY720 promotes the egress of peritoneal B cells from PerC via adhesion molecules expressed on stromal cells in NIK-mediated signaling, but inhibits the emigration from parathymic lymph nodes into blood circulation. FTY720 also promotes the trafficking of peritoneal B cell form blood circulation into bone marrow, and subsequent immigration of peritoneal B cells into PerC in NIK independent manner (Fig. 13).

1.8 Comparable serum antibody production under natural conditions and after immunization with killed bacteria

In the next experiment, I set out to determine whether the FTY720-induced disappearance of peritoneal B cells exerted any influence on systemic and mucosal antibody production (Fig. 3). Since peritoneal B cells are well characterized as a source of natural antibody production (4, 7, 44), I examined the total serum antibody production in SCID mice following adoptive transfer of normal peritoneal cells and continuous treatment with FTY720. Comparable productions of serum IgG and IgM were detected in mock- and FTY720-treated mice, while serum IgA production decreased partly in mice treated with FTY720 (Fig. 14A). FTY720 did not affect IgG subclasses, and so TI-antigen associated subclasses of IgG2b and IgG3 were prevalent in both mock- and FTY720-treated mice (Fig. 14A). These data indicate that FTY720 induces B1 B cell alteration in the PerC but does not affect the generation of natural serum antibody production.

To investigate the effects of FTY720 on the induction of bacterial antigen-specific antibody production, I employed PC, a main TI antigen on the bacterial wall, as a model antigen, since B1 B cells have been shown to be a major source of PC-specific antibodies (31, 35). Accordingly, I intraperitoneally immunized mock- or FTY720-pretreated mice with R36A, a heat-killed, pepsin-treated *S. pneumoniae* strain (31, 35) and continued to treat everyday for 5 days with mock or FTY720, respectively. Although repeated treatment with FTY720 were meant to maintain the low number of peritoneal B1 B cells during the experiment, in the end similar levels of PC-specific IgM production were detected in both mock- and FTY720-treated mice (Fig. 14B).

These findings suggest that the alteration of B cell trafficking induced by FTY720 in the PerC did not affect either natural or bacterial antigen-specific serum antibody production.

1.9 Reduction of intestinal IgA production by the treatment with FTY720

I investigated whether the alteration of peritoneal B cell trafficking affected intestinal antibody production since peritoneal B cells are thought to migrate into iLP and contribute to subsequent S-IgA production (10, 11, 48). When peritoneal B cells were adoptively transferred into SCID mice, considerable IgA and IgM production was noted in the feces (Fig. 15A) and consistent with such antibody production, IgA- or IgM-AFCs were detected in the iLP (Fig. 15B). In contrast, when SCID mice were continuously treated with FTY720 after the adoptive transfer of peritoneal B cells, fecal IgA production was significantly impaired in FTY720-treated mice, while fecal IgM production was comparable to that seen in the mock-treated mice (Fig. 15A). ELISPOT analysis confirmed this finding, demonstrating that IgA AFCs levels were significantly reduced in the iLP of mice treated with FTY720, while IgM AFCs levels were to mock-treated group (Fig. 15B). Additionally, RT-PCR analyses revealed that mock- and FTY720-treated mice expressed identical poly Ig receptors (pIgR) in intestinal epithelial cells and J-chain in intestinal B cells, both are key molecules for the transport of IgA into the intestinal lumen (49, 50). Thus, the reduction of fecal IgA could not be due to a defect in the formation of polymeric IgA or its subsequent transport via epithelial cells

into the lumen (Figs. 15C and D).

I next examined the contribution, if any, made to S1P-dependent intestinal IgA production by B1 and B2 B cells. To address this issue, I adoptively transferred purified peritoneal B1 or B2 B cells into SCID mice, treated with or without FTY720, and then examined intestinal B cells. Flow cytometric analysis revealed that both B1 and B2 B cells equally developed IgA^+ and IgM^+ cells in the intestine (Fig. 15E). As might be expected from the observed selective reduction in intestinal IgA production after FTY720 treatment (Figs. 15A and B), FTY720 was found to reduce IgA^+ cells regardless of the subset of B cells from which they originated, suggesting that FTY720 equally affects B1 and B2 B cells. Thus, both B1 and B2 B cell-derived IgA^+ cells were equally decreased in the intestine of FTY720-treated mice (Fig. 15E). These findings were further confirmed by showing that FTY720 treatment reduced both B1- and B2-derived fecal IgA production (data not shown).

We then examined whether S1P regulated B1 B cell-originated intestinal IgA responses against microbial infection. To address this issue, we enumerated PC-specific intestinal IgA production against orally administered R36A and found that PC-specific fecal IgA production was impaired when mice received FTY720 after oral immunization with R36A (Fig. 15F). These results suggest that the cell trafficking of intestinal IgA-committed B1 and B2 B cells from the PerC is under the regulation of S1P.

2. S1P signaling regulates egression of IgA^+ plasmablasts from PPs for the B2 B cell-mediated intestinal IgA production

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

To examine the role of S1P in PP dependent IgA induction pathways, I investigated $S1P_1$ expression at different B cell developmental stages based on the expression pattern of Ig heavy chain in the PPs. High level of class switch recombination to IgA occurred due to the direct exposure of intestinal antigen via subepithelial dome region and this class switch recombination is mediated partly by IL-4 and TGF-β (Fig. 16). Therefore, B cells isolated from the PPs of naïve mice consisted of three distinct Ig expression patterns: $IgM^+ IgA^-$ (70%), $IgM^+ IgA^+$ (1%), and $IgM^- IgA^+$ (4%; Fig. 17A, left top 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. 17A, bottom left 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. 17A, right panels). 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. 17A, bottom right panel and right panels). These findings suggest that naïve $IgM^+ B220^+$ B cells switch to $IgM^- IgA^+ B220^+$ cells through $IgM^+ IgA^+ B220^+$ B cells under the control of class switch recombination (CSR) and then $IgM^- IgA^+ B220^+$ cells further differentiated to $IgM^- IgA^+ B220^-$ PC precursors in the PPs (12).

To determine the $S1P_1$ expression at different stages of B cell differentiation in the PPs, I performed $S1P_1$-specific quantitative RT-PCR using purified PP B cells. Naïve $IgM^+ IgA^- B220^+$ B cells expressed high levels of $S1P_1$, and the $S1P_1$ expression was markedly decreased (about 40-fold) in PP B cells that underwent IgA commitment, such as $IgM^+ IgA^+ B220^+$ B cells (Fig. 17B). 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. 17B). These results demonstrate that $S1P_1$ expression was altered during B cell differentiation to IgA^+ cells in the PPs.

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

To investigate whether the alteration of $S1P_1$ expression during B cell differentiation in the PPs was truly associated with B cell commitment to PP-mediated cell trafficking, especially emigration from the PPs, I analyzed IgM^+ and/or IgA^+ B cells in the PPs of mice treated 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 $IgM^- IgA^{hi}$ B cells were increased (Fig. 18A). These increased $IgM^- IgA^{hi}$ B cells were $B220^-$ (Fig. 18A), which was consistent with the high expression of $S1P_1$ on $IgA^+ B220^-$ B cells (Fig. 17B). The accumulation of $IgA^{hi} B220^-$ B cells in the PPs was coincident with the reduction of the same population in the iLP (Fig. 18B).

I then 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. 18C). Partial recuperation occurred by day 4, with full recovery observed 8 days after the termination of FTY720 treatment (Fig. 18C). These data suggested that like S1P-mediated peritoneal B cell trafficking (Figs. 6, 15B), the inhibition of S1P-mediated signaling by FTY720 reversibly hampered the migration of B cells from the PPs into the iLP.

Because $IgA^+ B220^-$ cells include both plasmablasts and PCs, I next examined which of these cell types FTY720 targeted. FTY720 did not change the frequency of IgA^+ PCs ($CD138^{hi} B220^-$) in the PPs, but did cause accumulation of IgA^+ plasmablasts ($CD138^{int/-} B220^-$) in the PPs (Fig. 18D). 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. 18D). These data demonstrated that S1P plays a key role in regulating the emigration of $S1P_1{}^+ IgA^+$ plasmablasts from the PPs.

2.3 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 expression of gut-homing-associated adhesion molecules and chemokine receptor. Therefore, I examined the expression of

the adhesion molecule, $\alpha_4\beta_7$ integrin, and the chemokine receptor, CCR9, which determine gut tropism (51, 52). Flow cytometric analysis demonstrated that accumulated IgA^+ $B220^-$ plasmablasts expressed high levels of $\alpha_4\beta_7$ integrins and CCR9 (Fig. 19A), indicating that accumulation of IgA^+ $B220^-$ plasmablasts in the PPs and their simultaneous decrease in the iLP was not due to a lack of migration ability through these gut-homing molecules. In addition, these accumulated IgA^+ $B220^-$ plasmablasts were capable of differentiating further into IgA-producing cells. 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. 19B). Consistent with these results, I also found that FTY720 did not affect the distribution of $B220^+$ B cells, $CD4^+$ T cells, or $CD11c^+$ DCs in the PPs (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, 13, 14).

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

I next performed confocal microscopic analysis to determine the sites where 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. 20A). In FTY720-treated mice, however, IgA^+ B cells accumulated only on the basal side of PPs (Fig. 20B, arrows). Some IgA^+ B cells bound to

lymph-expressing podoplanin (Fig. 20B 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.

2.5 FTY720-mediated inhibition of IgA^+ plasmablast emigration from the PPs abolishes the subsequent induction of Ag-specific intestinal IgA production without affecting GC formation

I 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 ovalbumin (OVA) plus cholera toxin, a mucosal adjuvant. An ELISPOT assay revealed that OVA-specific IgA AFCs were induced in the iLP after oral immunization in control mice (Fig. 21A). 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. 21A). Consistent with the results obtained from the analysis of non-immunized mice treated with FTY720 (Fig. 18D), 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. 21B). I 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. I found that OVA-specific fecal IgA was markedly decreased in the FTY720-treated mice (Fig. 21C). 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 (26-28). Thus, I next examined GC formation in the PPs and spleen of immunized mice. Consistent with the previous findings (26-28), reduced numbers of peanut agglutinin $(PNA)^{hi}$ $B220^+$ GC B cells were seen in the spleen of FTY720-treated mice (Fig. 21D). In contrast, comparable numbers of PNA^{hi} $B220^+$ GC B cells were detected in the PPs of mice receiving FTY720 during the oral immunization period (Fig. 21D). Histological analysis further 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. 21E). These results indicate that, unlike systemic immune compartments (Fig. 21D) (26-28), 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.

Discussion

The current study provides direct evidence that S1P plays important roles in the intestinal S-IgA production through the regulation of peritoneal and PP B cell trafficking (Fig. 22). Adoptive transfer experiments using peritoneal B cells revealed that FTY720-mediated disappearance of peritoneal B cells via at least two distinct pathways (Fig. 6): 1) their migration into PerC from the blood circulation and 2) inhibition of their egress from the parathymic lymph nodes on their way to blood circulation from the PerC. The first observation on B cell immigration from the blood into PerC support current idea that there are the hierarchy between S1P and chemokines. The impairment of S1P signaling or chemokine signaling induce redistribution of B cells (26). My results revealed that the defect of S1P signaling induce the accumulation of peritoneal B cell in bone marrow from blood circulation. In contrast, the other group suggested that FTY720 did not affect the homing of PCs from blood circulation into bone marrow in intravenously transfer experiments (29). The difference of FTY720 sensitivity may cause the expression levels of CXCR4 between peritoneal B cells and PCs. PCs expressed comparable high levels of CXCR4 to peritoneal B cells, so the high expression of CXCR4 enable PCs to migrate into bone marrow regardless of the intensity of S1P signaling. The second observation on peritoneal B cell emigration accords well with the current conclusion that lymphocytes can emigrate from the peritoneal cavity to the blood through omentum and parathymic lymph nodes (45, 46) and FTY720 treatment results in the accumulation of cells in the lymph nodes (53-55). Additionally, using NIK-mutated *aly* mice, my current findings indicate that the

NIK-mediated pathway of stromal cells is an additional regulatory mechanism in the control of S1P-mediated peritoneal B cell emigration, but not immigration (Figs. 10, 11). My current data showed that S1P receptors were not expressed on stromal cells (data not shown), suggesting that S1P did not directly regulate stromal cell function (e.g. adhesion molecules expression) in the S1P-mediated peritoneal B cell emigration. Related with these issues, recently it was reported that ICAM-1 and VCAM-1 were important molecules for peritoneal B cells to egress from PerC (56), therefore, ICAM-1 and VCAM-1-deficent mice showed the accumulation of peritoneal B cells in PerC. Based on my data, one of the reason for the impairment of S1P-mediated migration in *aly* mice might be due to the reduction of ICAM-1 and VCAM-1 expression on *aly* stromal cells. It was also reported that TLR-mediated stimulation induced down-regulation of the S1P receptor (26, 57), and that CXCL13 played the major role in the TLR signaling-induced B cell emigration from the PerC (57). In addition, a previous study indicated that *aly* mice showed the impaired CXCL13 production (58), and another study showed that S1P signaling overcame the recruiting activity of CXCL13 in the regulation of marginal zone B cell localization (26). Taken all findings together, I hypothesis that S1P signaling is dominant in the retention of peritoneal B cells in PerC than CXCL13 (Fig. 12). Therefore, CXCL13 does not work in the peritoneal B cell emigration in the presence of functional S1P-mediated signaling. In contrast, in the absence of S1P-mediated retention pathway by inducing down regulation of S1P receptors (*e.g.*, TLR-mediated signaling and FTY720 treatment), CXCL13 produced by stromal cells through NIK-mediated pathway facilitate the emigration of B cells out of

PerC (Fig. 12). The detailed analysis is a subject of future experiments.

Another important question is whether FTY720 acts as an agonist or functional antagonist in my experiments. Previous study indicated that FTY720 acted as an agonist in vivo, and eventually prevented the migration of naïve T cells into afferent lymphatic vessels from peripheral tissues due to the persistent interaction between naïve T cells and adhesion molecules in peripheral organs (59). However, from my data, it was considered that FTY720 might act as functional antagonist with inhibiting of the S1P-mediated retention signal (Fig. 12). My data indicated the FTY720 induced peritoneal B cells migration into regional lymph nodes (Fig. 6A), and these effects might implicate that FTY720 acted as not agonists but functional antagonists to S1P receptors. To clarify the detail mechanisms in this point, further experiments using S1P1-deficient mice are required.

This study showed that FTY720 treatment of SCID mice adoptively transferred with normal PerC cells did not influence the natural antibody production in serum. Since SCID mice lack functional T cells, the preferential production of IgG2b and IgG3, well known subclasses dominantly reactive to TI antigen, was detected in mock- and FTY720-treated mice (Fig. 14A). This observation was consistent with previous reports demonstrating that FTY720 did not influence antibody production against soluble TI antigen (e.g., TNP-Ficoll and NP-Ficoll) (27, 28). Additionally, because FTY720 did not affect the serum PC-specific serum IgM production in intraperitoneally immunized with killed bacterial antigen, R36A (Fig. 14B), These data further suggested that FTY720-mediated alteration of peritoneal B cell distribution did not affect antibody

production against TI antigen regardless of antigen form (e.g., soluble or particulate).

In contrast to the inhibitory effects of FTY720 on GC formation in the spleen after systemic immunization with TD antigens (26-28), I showed that FTY720 did not affect GC formation in the PPs (Figs. 21D, E). A major difference between the spleen and PPs is that spleen is located in germ-free condition while PPs are exposed to continuous stimulation by environmental Ags (e.g., microbial and food Ags), which may account for the 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. 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.

Although FTY720 did not inhibit GC formation of PPs in oral immunization, the amounts of OVA-specific fecal IgA were decreased by inhibiting the emigration of IgA^+ plasmablasts from the PPs. This study extend the idea that lymphocytes biologically regulate S1P receptor expression (12, 60) by showing that alternation of $S1P_1$ during PP B cell differentiation regulates their retention in and emigration from the PPs (Figs. 17B, 18). It seems that reduction of $S1P_1$ expression during CSR enables IgA^+ B cells to stay in the PPs for efficient differentiation to the IgA^+ plasmablasts. The IgA^+ plasmablasts then recover $S1P_1$ expression together with the expression of gut-homing molecules (e.g., $\alpha4\beta7$ integrin and CCR9), allowing them to exit from the PPs and migrate into the

iLP for the final differentiation to IgA-producing PCs. This study further provides 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 in S1P dependent manner to the iLP for the final differentiation to IgA synthesis.

Unresolved questions in both B cell trafficking pathways is why FTY720 selectively affects IgA^+ B cells, but not IgM^+ B cells although both cells express high levels of $S1P_1$ (Figs. 17, 18). This discrepancy may by explained by the mutual interaction between S1P and chemokines as mentioned above (26, 29, 60-62). Indeed, Kunkel et al. 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 (63). Therefore, the expression of an unidentified chemokine receptor on IgM^+ or IgA^+ B cells may determine their dependency (or lack thereof) on S1P for emigration from the PPs. These points need to further study about the involvement of gut-associated cytokines and chemokines in the regulation of S1P-mediated intestinal B cell trafficking.

Additional novel finding is the presence of S1P-independet IgA^+ B cells in the PPs. In contrast to the S1P-dependent IgA^+ plasmablasts, the few cells showing IgA^+ PC phenotypes (e.g., $CD138^{hi}$) in the PPs were not affected by FTY720 treatment (Fig. 18D), suggesting the presence of S1P-independent differentiation, an alternative pathway to IgA^+ PCs in the PPs. In this context, previous studies 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 (13, 14). IgA^{hi} cells in the follicle-associated epithelium were barely affected by FTY720 treatment (Fig. 20), and DCs are abundant in the follicle-associated epithelium (42). Thus, the DCs may induce IgA^{+} PCs in the follicle-associated epithelium in an 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 (64). Hence, S1P-independent IgA^{+} cells in the follicle-associated epithelium might provide a rapid response against newly-arriving Ags for creating local immunity in the PPs.

Taken together, this study indicated that S1P is a key molecule in determining the PP and PerC B cell trafficking whether they stay or emigrate. This pathway is essential for the efficient S-IgA production in the versatile intestinal immune system.

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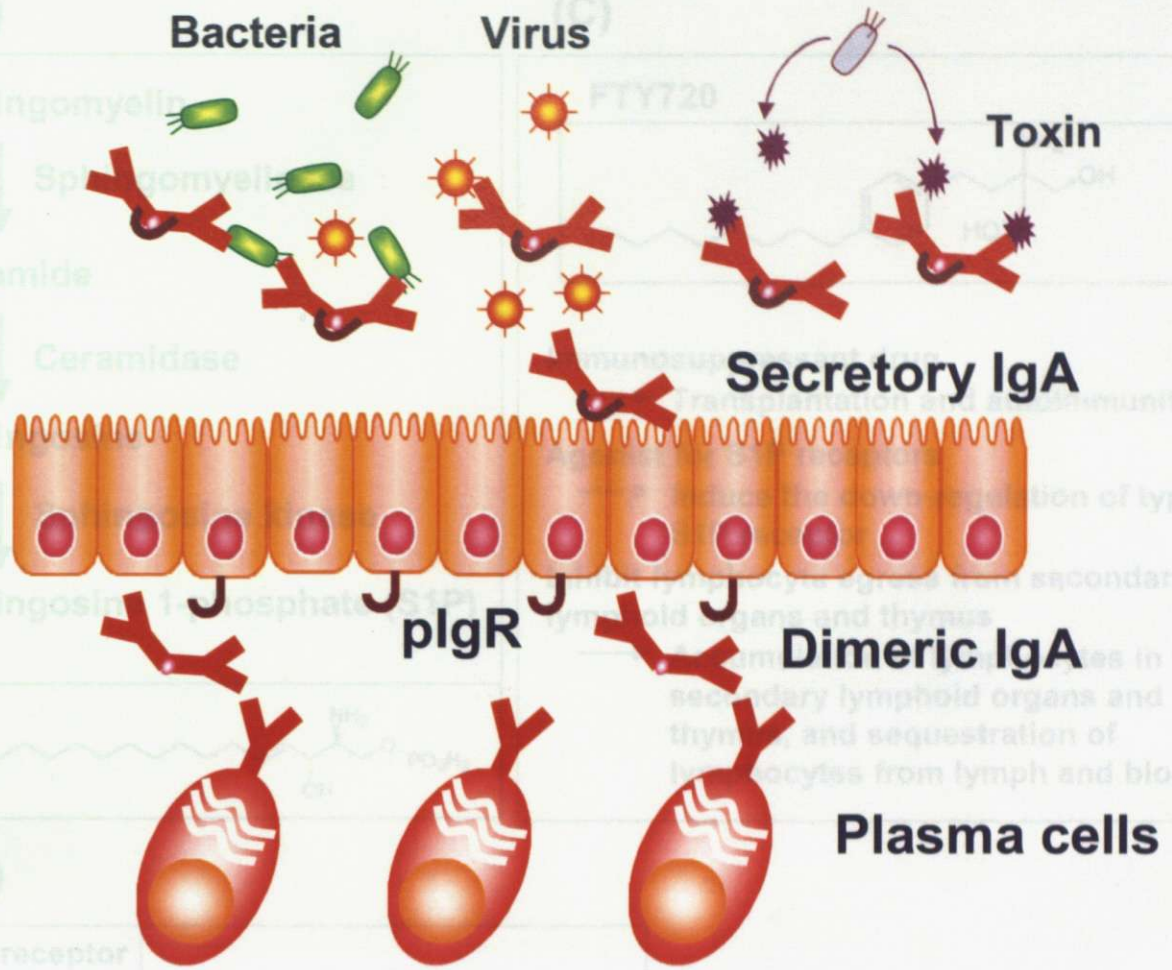

	B1 cells	B2 cells
Distribution	Peritoneal cavity	Peyer's patch
T cell dependency	T cell-independent	Mainly T cell-dependent
Antigen	Lipid, polysaccharide	Protein
Marker	$B220^{+}CD11b^{+}$	$B220^{+}CD11b^{-}$

Figure 1 Pivotal roles of secretory IgA (S-IgA) in the maintenance of immunological homeostasis in the gut.

Mucosal immunity is the first line of defense against pathogens by equiping various kinds of immunocompetent cells. Among them, S-IgA plays an important role in preventing the attachment of pathogenic and commensal microbiota, and neutralization of bacterial toxins. Dimeric IgA is produced by plasma cells (PCs) in iLP. These PCs are derived from distinct B cell subsets, B1 and B2 B cells, which can be discriminated by tissue distribution, antigen specificity and cell surface markers.

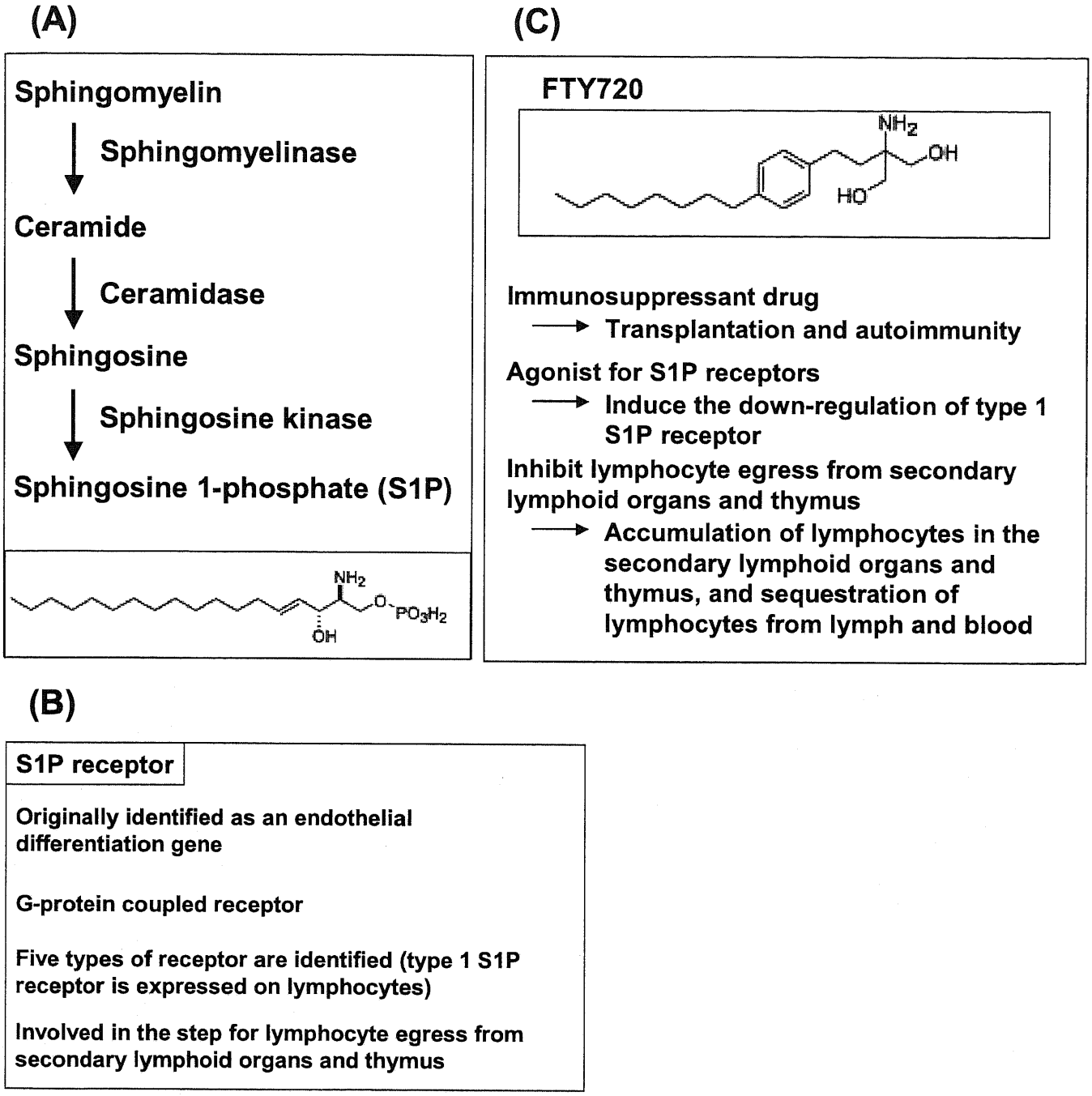

Figure 2 Sphingosine 1-phosphate (S1P) is a novel key molecule for the regulation of lymphocytes trafficking and immunomodulator, FTY720, prevents S1P-mediated signaling by inducing down-regulation of S1P receptors.

(A) S1P is a lipid mediator, generated from sphingomyelin which is a one of the major components of cellular membrane. Sphingomyelinase and ceramidase are involved in the generation of sphingosine, and sphingosine is phosphorylated by sphingosine kinase to generate S1P. (B) Five types of S1P receptors have been identified and type 1 S1P receptor ($S1P_1$) is expressed on lymphocytes. (C) Phosphorylated FTY720 binds to S1P receptors and induce down-regulation of S1P receptor expression, eventually leading to the blockade of S1P-mediated signaling.

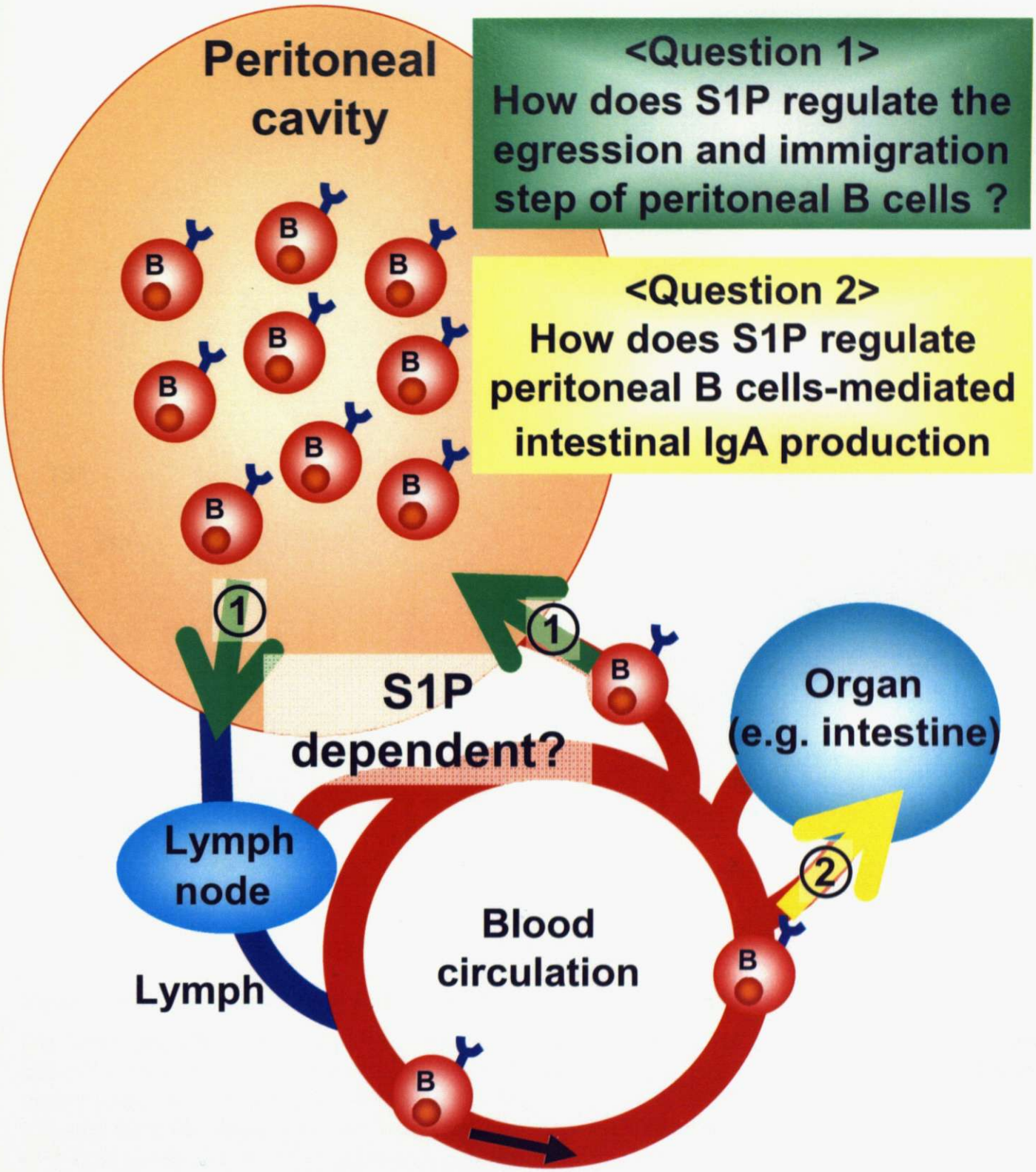

Figure 3 The questions related to S1P-mediated peritoneal B cell migration from PerC into the intestine.

The purpose of this study is to clarify the mechanism of S1P-mediated peritoneal B cell migration. The first issue is whether S1P regulates the egression and immigration steps of peritoneal B cells. The second one is to understand whether S1P signaling regulates the migration of peritoneal B cells into the intestine and subsequent secretory IgA production.

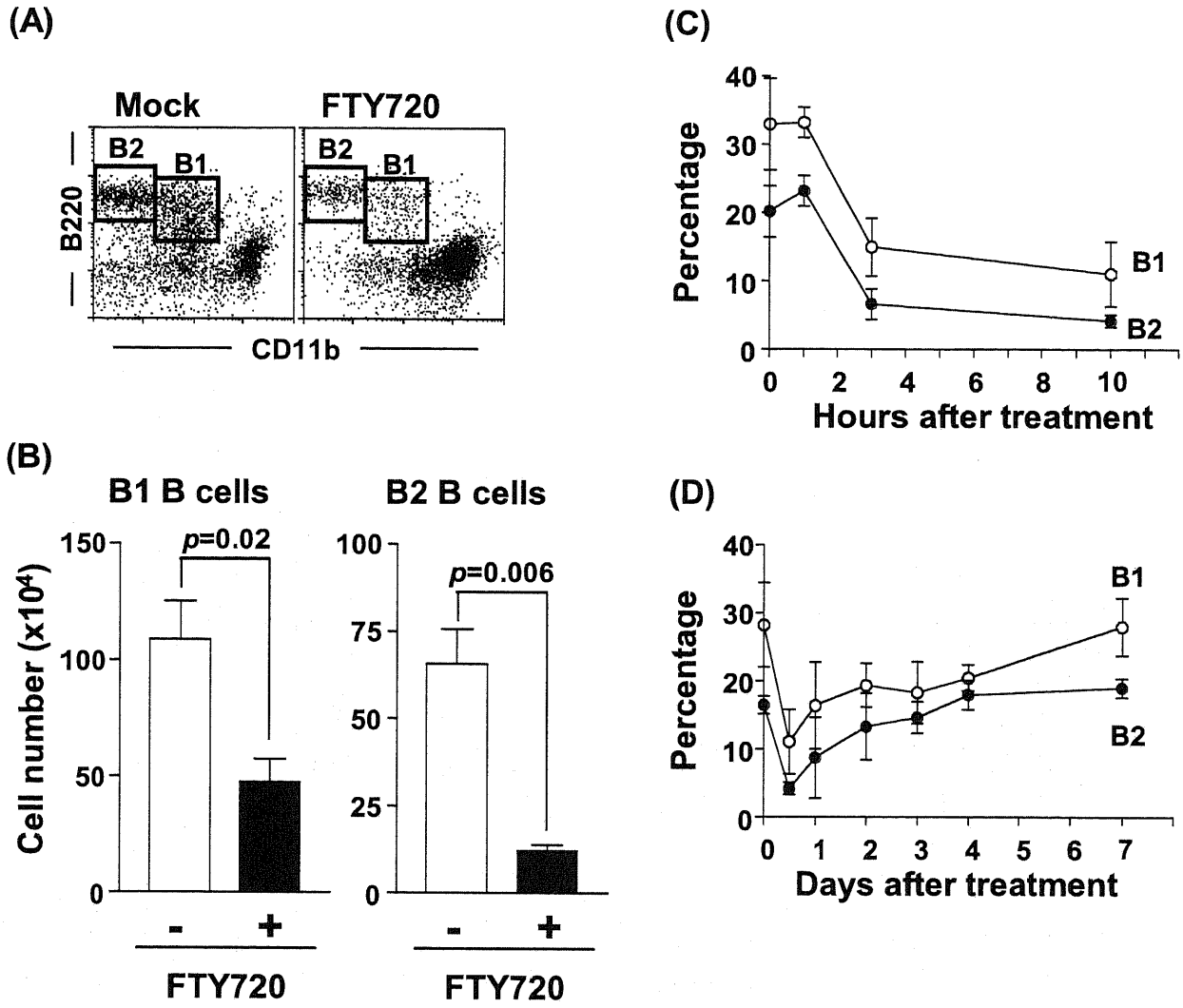

Figure 4 FTY720 induces rapid and reversible disappearance of peritoneal B cells.

(A) Peritoneal cells were isolated 10 hr after intraperitoneal injection of FTY720 (right) or mock (left), and cell populations were analyzed using flow cytometry. The data are representative of five independent experiments. (B) Cell numbers of $B220^{+}CD11b^{+}$ B1 B cells and $B220^{+}CD11b^{-}$ B2 B cells were calculated by using the total cell number and flow cytometric data. The error bars are ± SEM (n=5). (C and D) At each time points after FTY720 injection, peritoneal cells were analyzed by flow cytometry (open circle, $B220^{+}CD11b^{+}$ B1 B cells; closed circle, $B220^{+}CD11b^{-}$ B2 B cells). The data represent the mean ± SD (n=4).

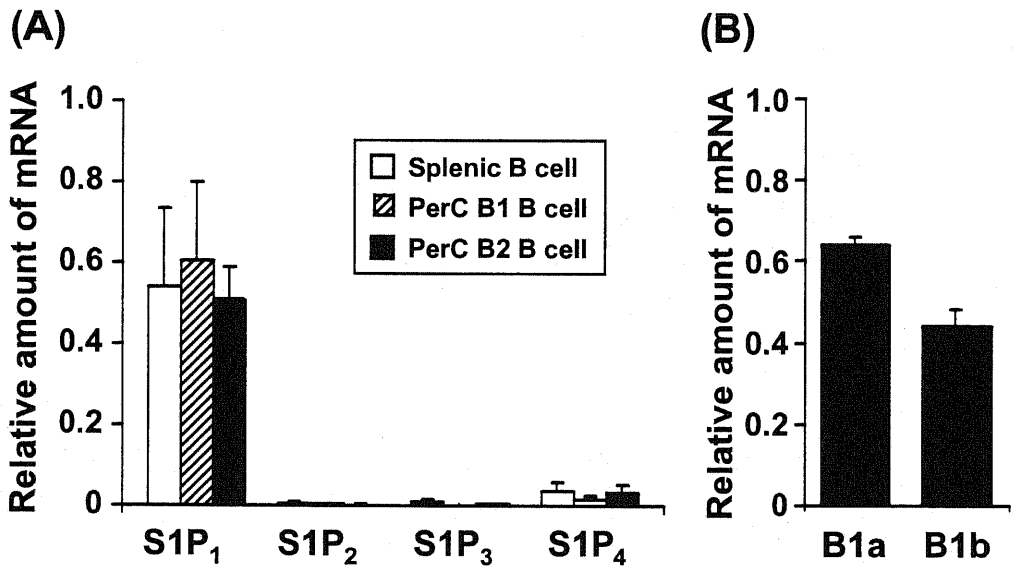

Figure 5 Equal expression of $S1P_1$ by peritoneal B1 and B2 B cells.
(A) Quantitative RT-PCR analysis for S1P receptors was performed using RNA isolated from sorted splenic B (open), peritoneal B1 (slashed) and B2 B (closed) cells. The relative quantity of specific mRNA was expressed as a ratio to GAPDH. The data are expressed as mean ± SD from four mice. (B) $S1P_1$ expression in B1a and B1b cells was determined by quantitative RT-PCR analysis.

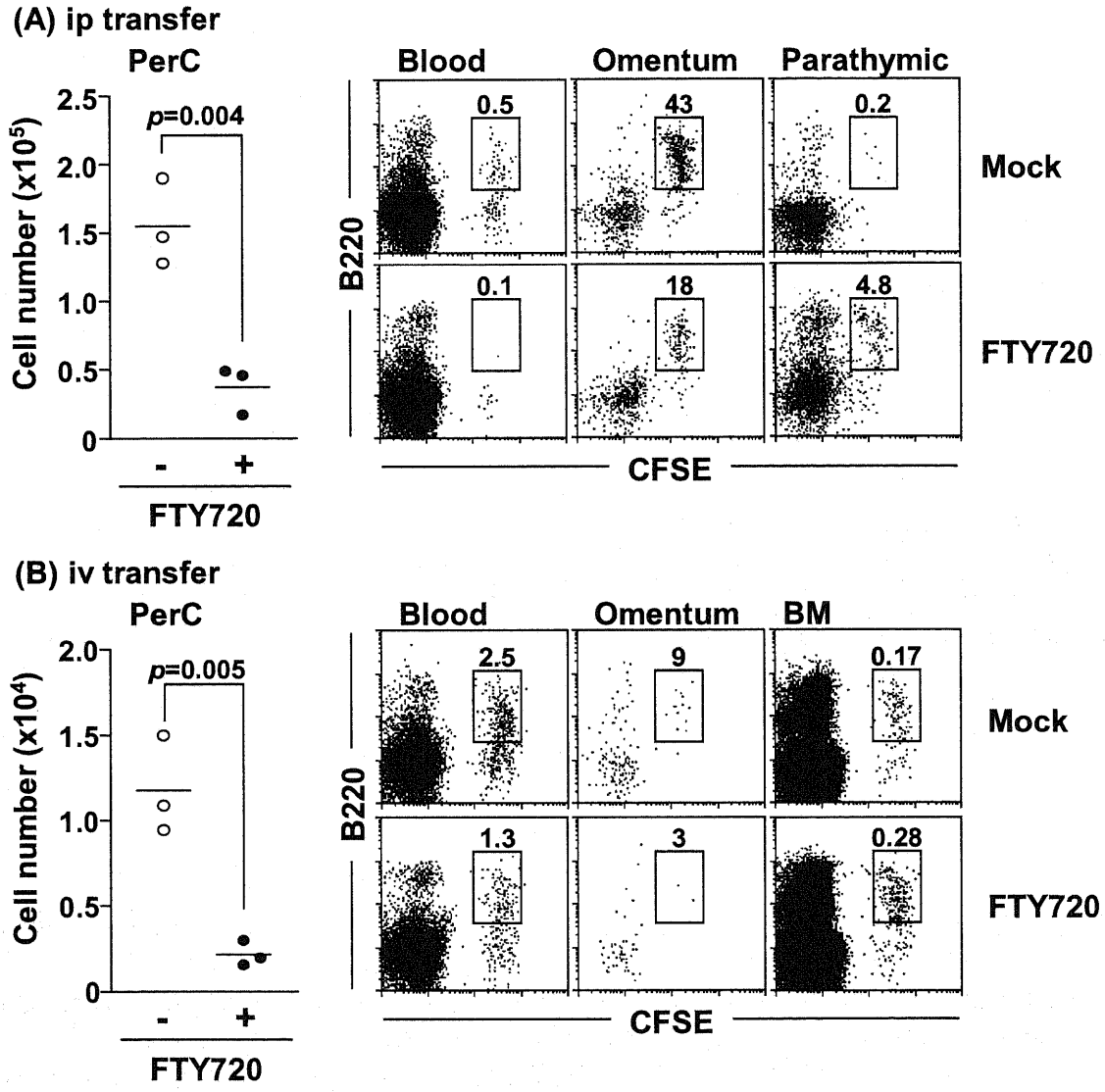

Figure 6 FTY720 simultaneously inhibits peritoneal B cells emigration from the parathymic lymph nodes and entrance from the blood into the PerC.

SCID mice were adoptively transferred with CFSE-labeled normal peritoneal B cells via the intraperitoneal (A) or the intravenous (B) routes, and simultaneously treated with (closed circle and bottom panels) or without (open circles and top panels) FTY720. After 12 hrs, cells were isolated from the PerC, blood, omentum, parathymic lymph nodes, and bone marrow (BM) for the analysis of $CFSE^+$ cells.

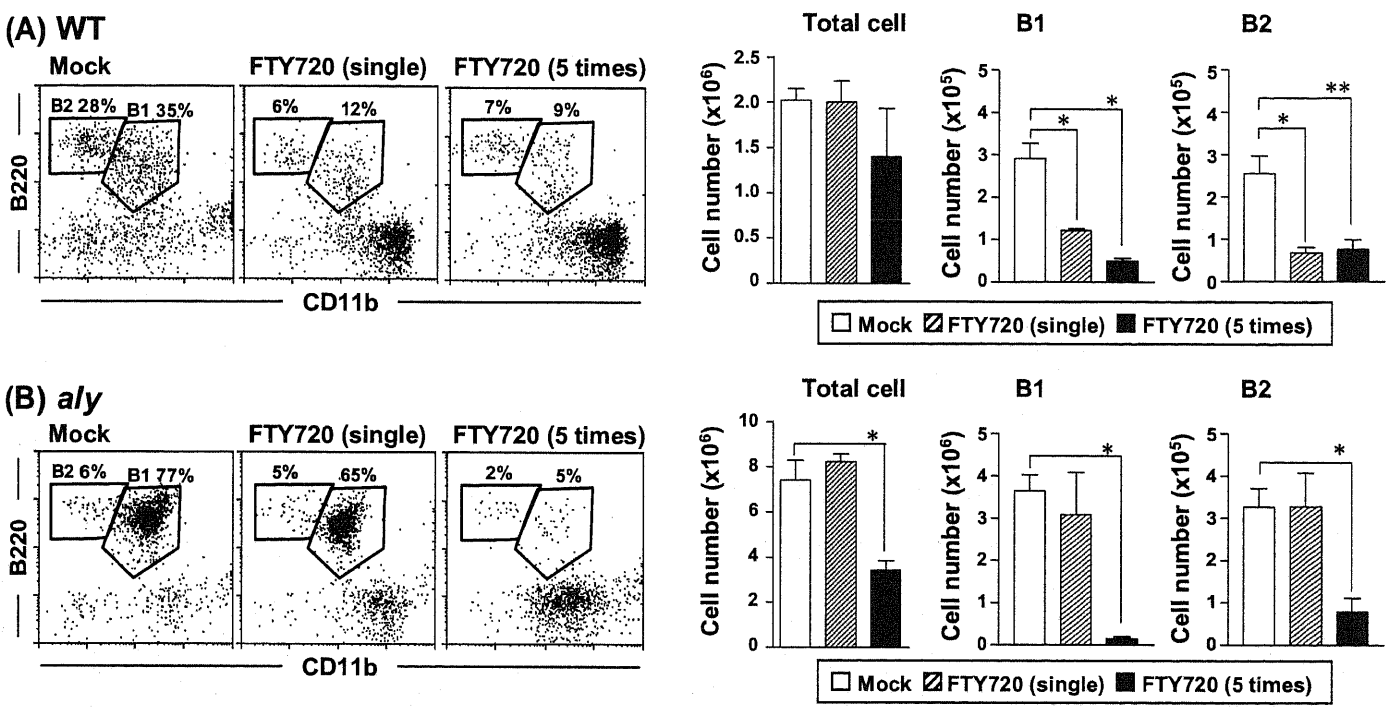

Figure 7 Decreased reactivity of peritoneal B cells to FTY720 in *aly* mice.

(A, B) Cells were isolated from the PerC of WT (A) or *aly* (B) mice 12 hours after single or multiple (that is 5) injections of FTY720 (right) or mock (left), and cell populations were analyzed by flow cytometry. The data are representative of at least four independent experiments. The numbers of total peritoneal cells, and $B220^{+}CD11b^{+}$ B1 cells and $B220^{+}CD11b^{-}$ B2 cells were calculated by using the total cell number and flow cytometric data. Data are presented as mean ± SEM (n=4). * $p< 0.01$, ** $p< 0.05$

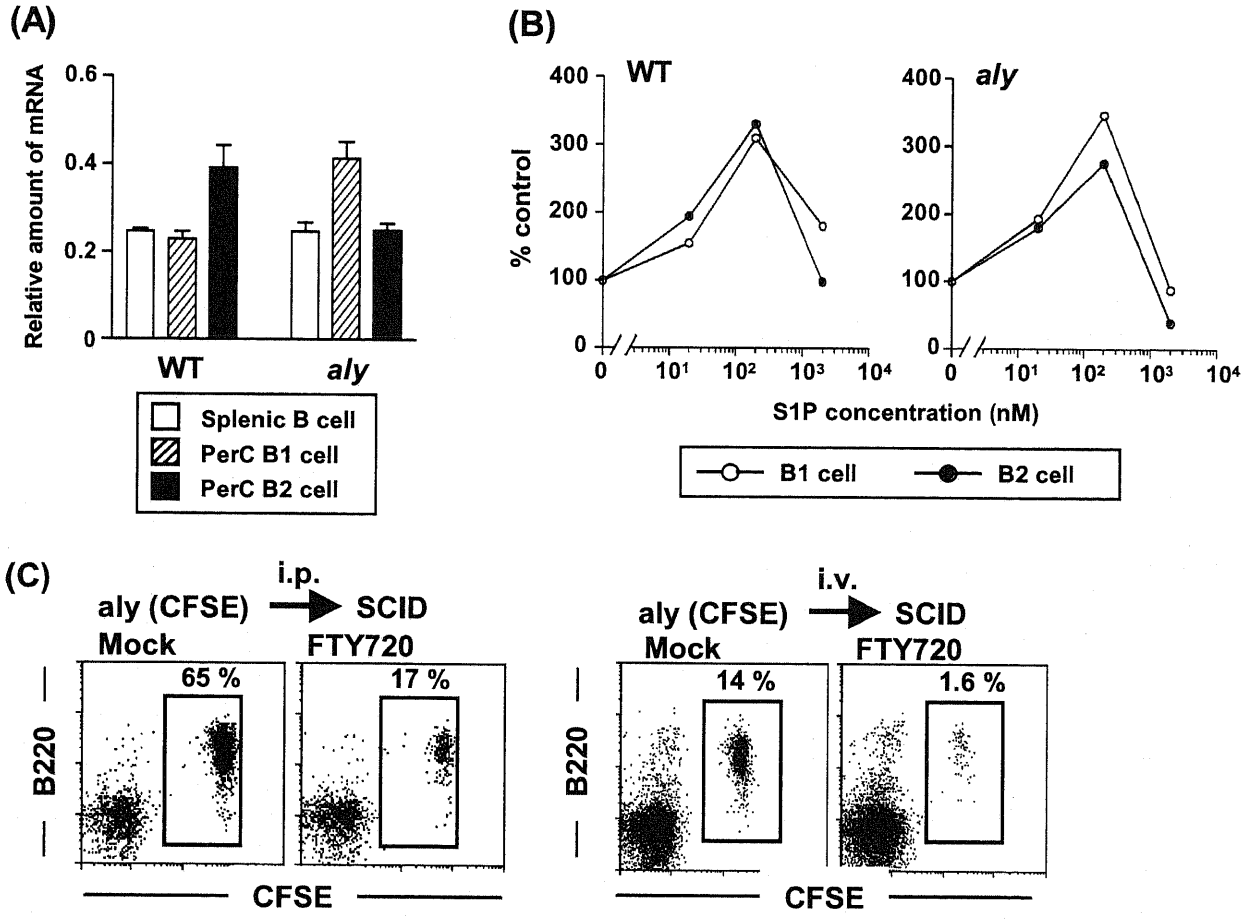

Figure 8 Similar expression of $S1P_1$ and biological reactivity to S1P among various populations of *aly* peritoneal B cells *in vitro* and *in vivo*.

(A) Quantitative RT-PCR analysis of $S1P_1$ expression was performed by using RNA isolated from sorted splenic B (open), peritoneal B1 (hatched) and B2 (solid) cells. The relative quantity of specific mRNA encoding $S1P_1$ was shown as a ratio to GAPDH. The data are indicated as the mean ± SD (n=4 per group). (B) *In vitro* migration assay was performed with peritoneal B1 (open circles) and B2 (solid circles) B cells purified from WT (left) and *aly* (right) mice. Peritoneal B cells were added to the upper chamber of a transwell plate in the presence of 0, 20, 200, or 2000 nM S1P in the lower chamber. Six hours later, the number of cells that had migrated into the lower chamber were counted by flow cytometry. The data are representative of three independent experiments. (C) Peritoneal B cells were isolated from *aly* mice, labeled with CFSE, and adoptively transferred via the intraperitoneal (i.p.) (left) or intravenous (i.v.) (right) routes into SCID mice. The reconstituted mice were treated simultaneously with or without FTY720. After 12 hours, cells were isolated from the PerC for the analysis of $CFSE^+$ $B220^+$ cells.

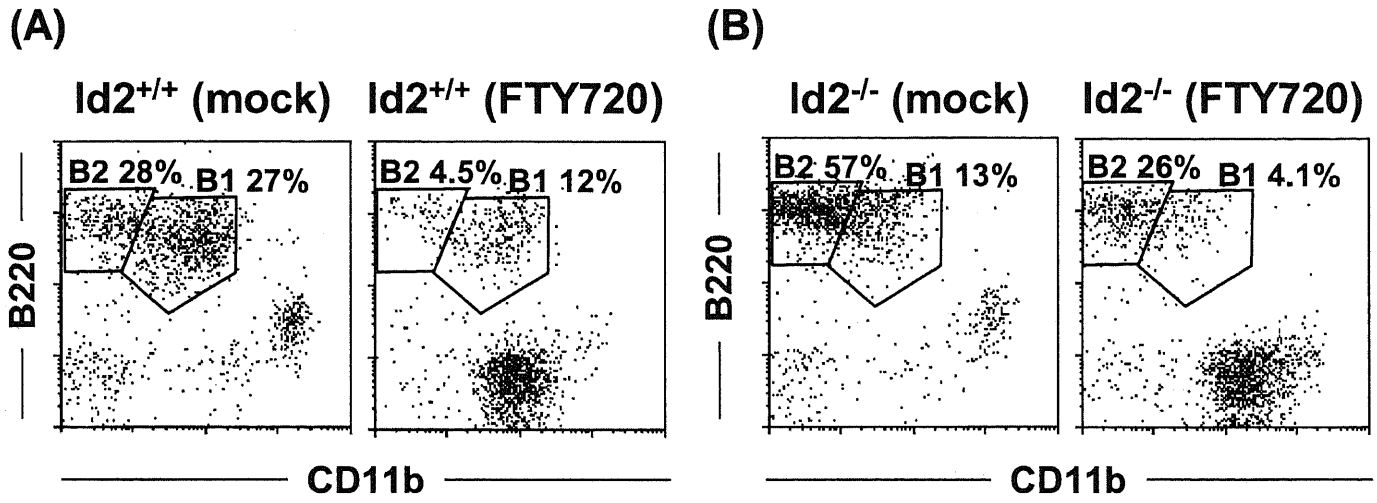

Figure 9 Defective lymphoid organ structure is not responsible for the unresponsiveness of *aly* peritoneal B cells to S1P.

Peritoneal cavity cells were isolated from the WT (A) or Id2-deficient (B) mice 12 hr after single injection of mock (right) or FTY720 (right), and cell populations were analyzed using flow cytometry. The data are representative of three independent experiments.

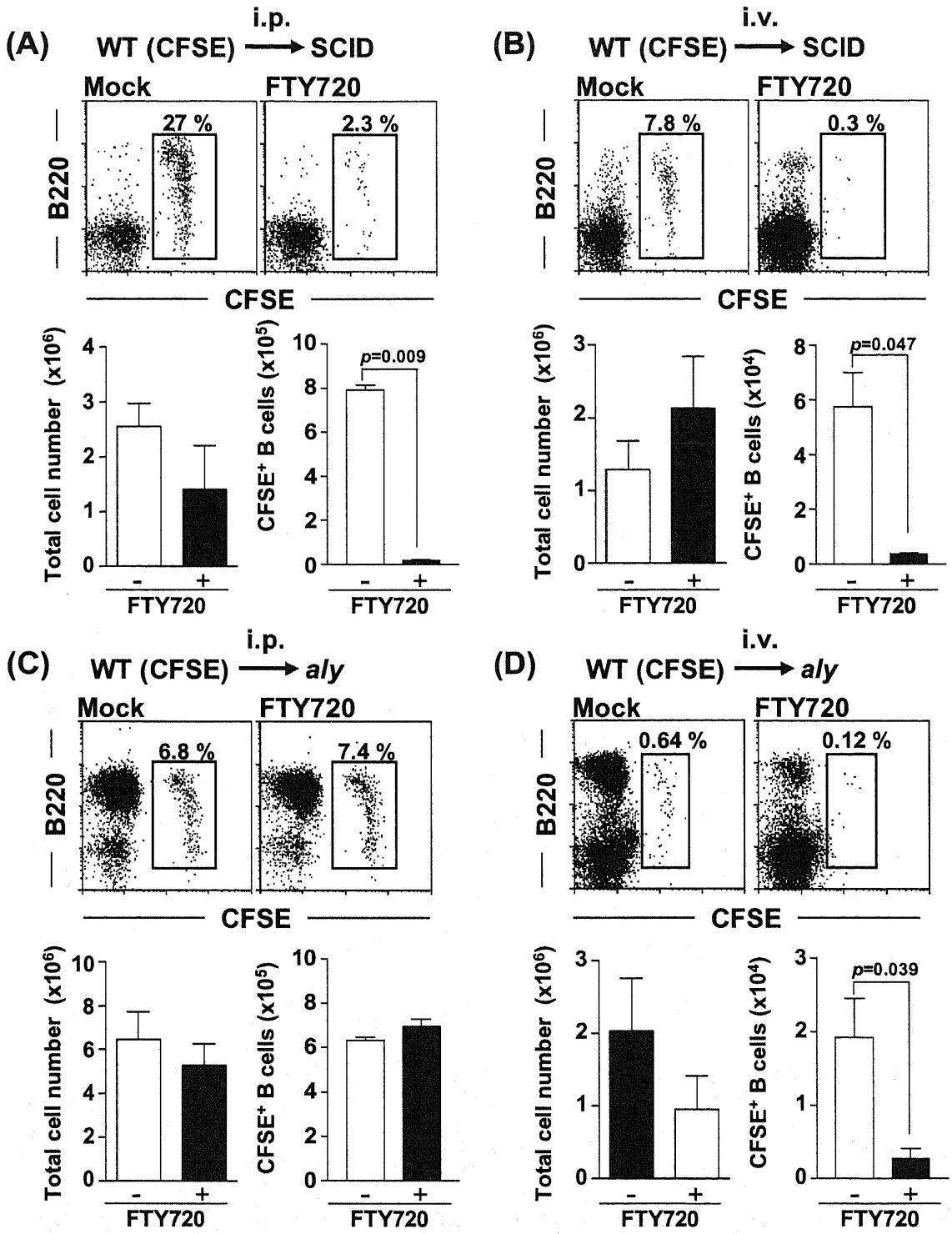

Figure 10 NIK-mediated signaling in non-B cells controls S1P-mediated peritoneal B cell emigration from PerC , but not immigration.

(A and B) Peritoneal B cells were isolated from WT mice, labeled with CFSE, and adoptively transferred via the intraperitoneal (i.p.) (A) or intravenous (i.v.) (B) routes into SCID mice. (C and D) Similarly, CFSE-labeled peritoneal WT B cells were transferred in to *aly* mice from which peritoneal cells were removed 8 hours before transfer. The reconstituted mice were treated simultaneously with (right panels) or without (left panels) FTY720. After 12 hours, cells were isolated from the PerC for the analysis of total cell number (left graph) and $CFSE^+$ $B220^+$ cells (right graph). Flow cytometric data are representative of three independent experiments and are presented as mean ± SEM (n=3).

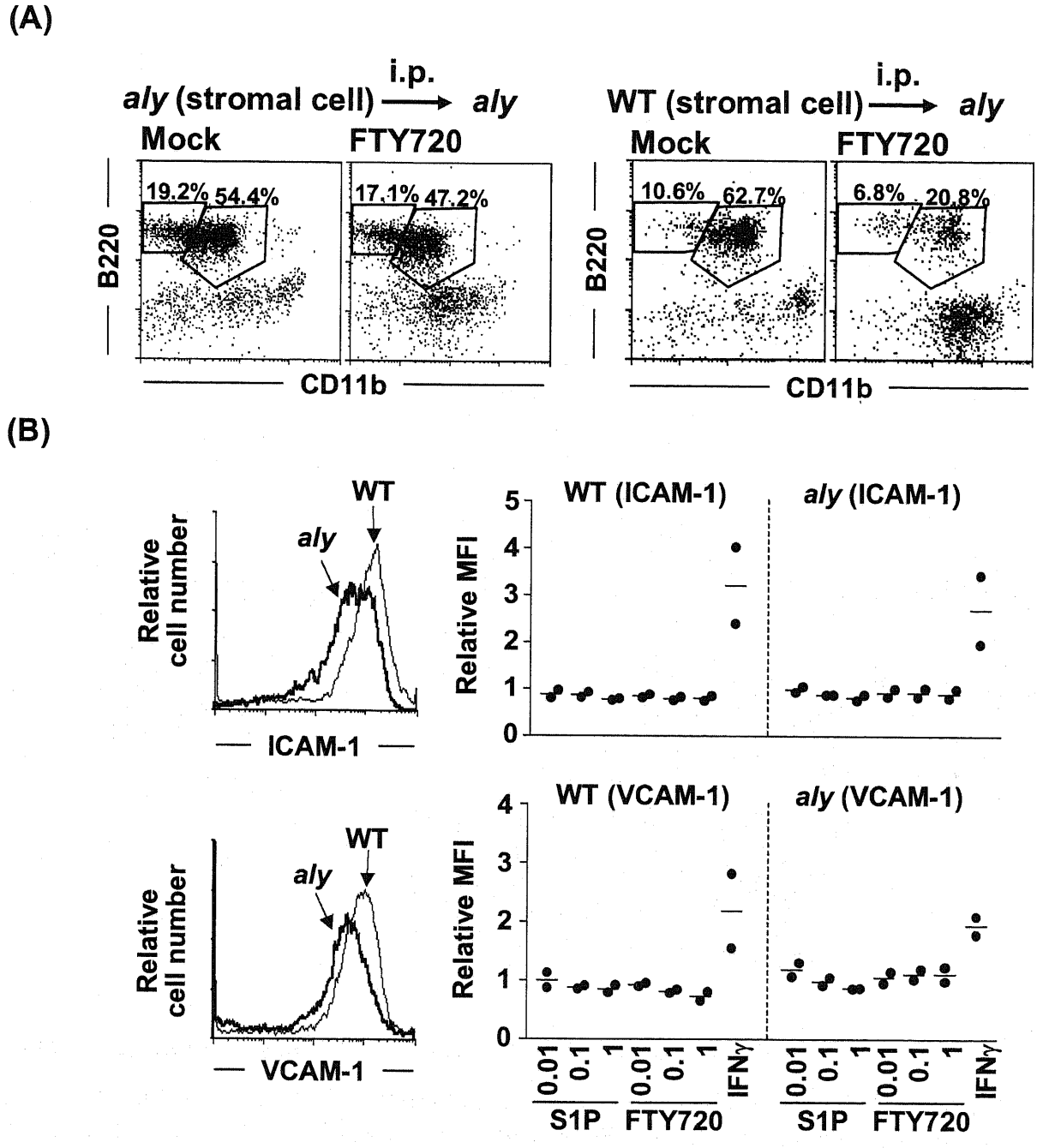

Figure 11 Requirement of NIK-mediated signaling in stromal cells for the emigration of peritoneal B cells.

(A) *aly* mice were intraperitonealylly (i.p.) transferred with WT (right panels) stromal cells or *aly* (left panels) stromal cells. Two weeks after cell transfer, mice were treated with FTY720 for the analysis of peritoneal B cell populations. The flow cytometric data are representative of three independent experiments and are presented. (B) Expression of ICAM-1 (upper panels) and VCAM-1 (lower panels) on WT (thin lines) and *aly* (thick lines) stromal cells was determined by flow cytometry (left). Twenty-four hours after treatment of stromal cells with various concentrated S1P, FTY720 or IFNγ (50 units/ml), expression of ICAM-1 and VCAM-1 was determined by flow cytometry. Relative mean fluorescence intensity (MFI) was expressed as a ratio to MFI of untreated cells. Date are representative of two independent experiments, and bars indicate mean values.

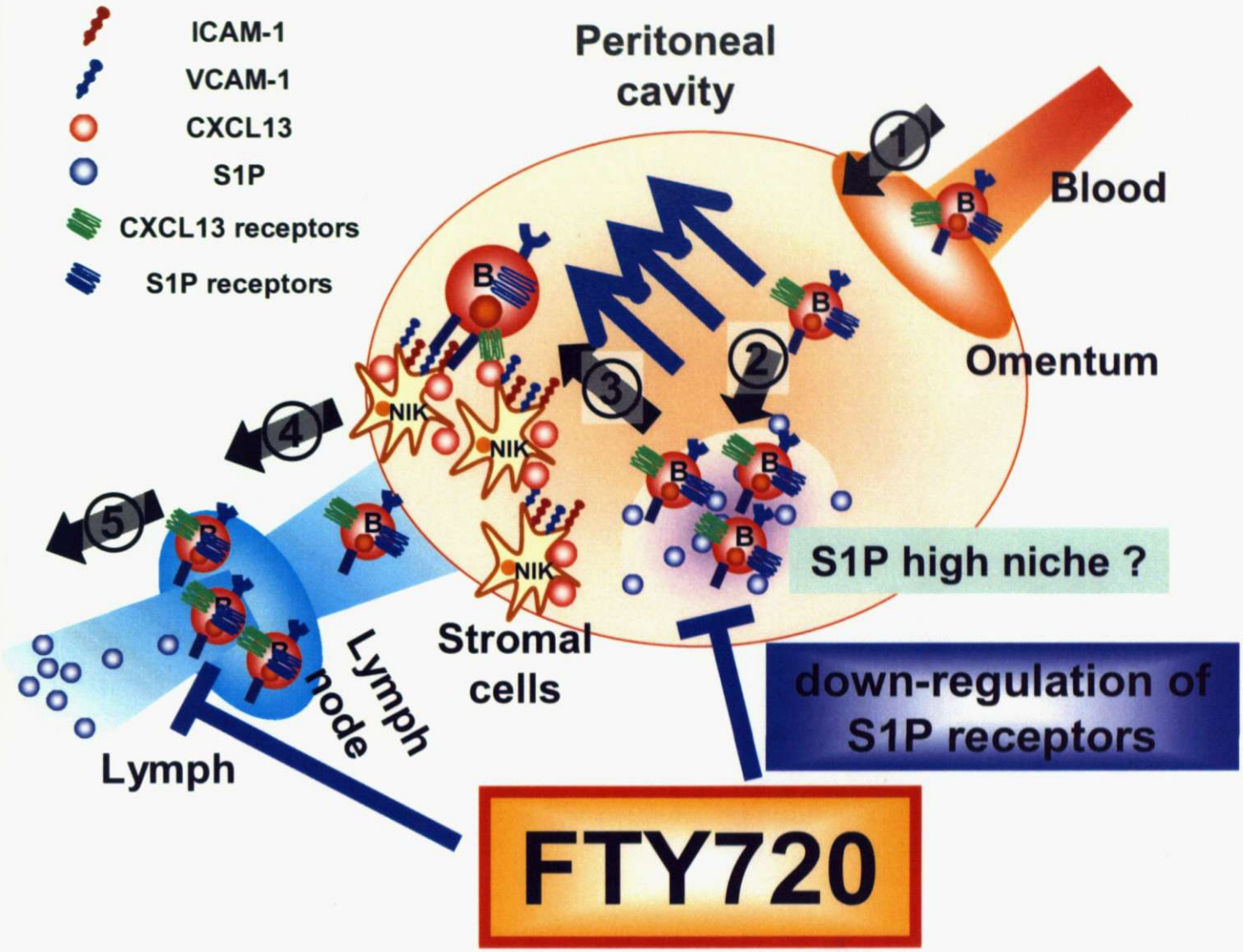

Figure 12 The putative role of FTY720 in peritoneal B cell egression from PerC.

It was considered that peritoneal B cells could recirculate via the bloodstream into PerC. B cells migrated from blood stream into PerC in NIK independent manner (arrow 1). The hypothesis was that there were niches with a relative large amount of S1P (S1P high niche). Peritoneal B cells usually accumulated around there in S1P dependent manner (arrow 2). At once the surface S1P receptor were down-regulated by various stimulants (e.g. FTY720 treatment, inflammatory cytokine and TLR ligand), the stimulated-B cells held ability to migrate toward the CXCL13, which formed gradient from the exist site of PerC (arrow 3). Further, B cells might egress from PerC via interaction with ICAM-1 and VCAM-1 on stromal cells, expressed in NIK-dependent manner (arrow 4). Finally, peritoneal B cells exited from regional lymph nodes in S1P dependent manner, and these steps were inhibited by FTY720 treatment (arrow 5).

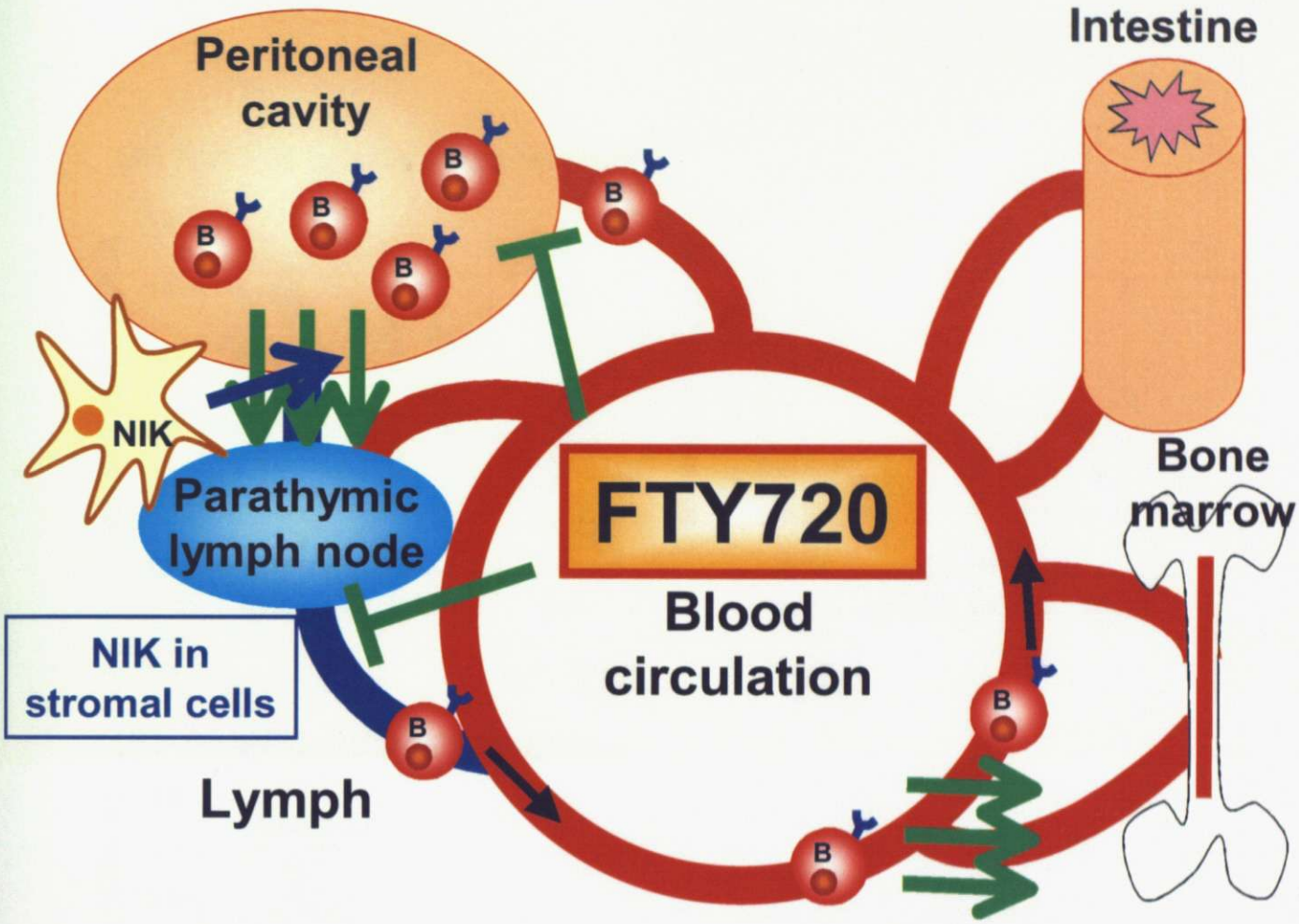

Figure 13 The mechanism of FTY720-induced peritoneal B cell disappearance.

FTY720 promotes the egress step of B cells from PerC and simultaneously inhibits the immigration step from blood into PerC. NFκB inducing kinase (NIK) in stromal cell is key molecule for FTY720 induced-peritoneal B cell egression form PerC. FTY720 also inhibits the egress from parathymic lymph node into blood circulation. In bood circulation, FTY720 enhances immigration step of peritoneal B cells to bone marrow, and consequently inhibits immigration into PerC in NIK independent manner.

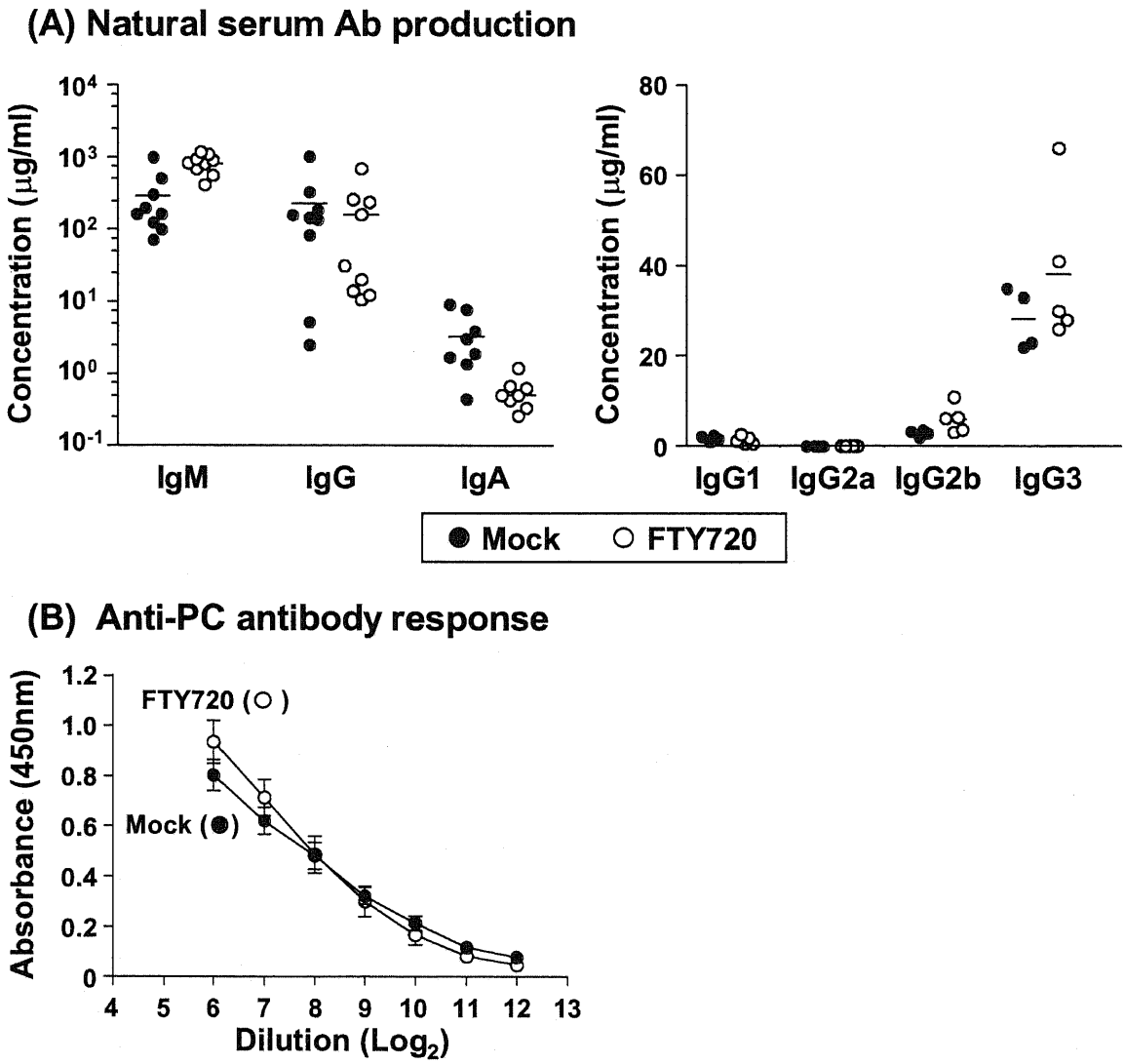

Figure 14 Effects of FTY720 on serum antibody production.

(A) SCID mice were adoptively transferred with 5 x 10^6 normal Peritoneal B cells and were treated with mock (closed circle) or FTY720 (open circle) every 2 days. Two weeks after the transfer, serum was collected for the measurement of total Ig levels by ELISA. (B) Mice pretreated with FTY720 and intraperitoneally immunized with heat-killed, pepsin-treated *S. Pneumoniae* strain R36A, received daily treatment with FTY720. After 5 days, serum anti-PC IgM was measured by ELISA. The error bars are ± SEM (n=4) from 2 separate experiments.

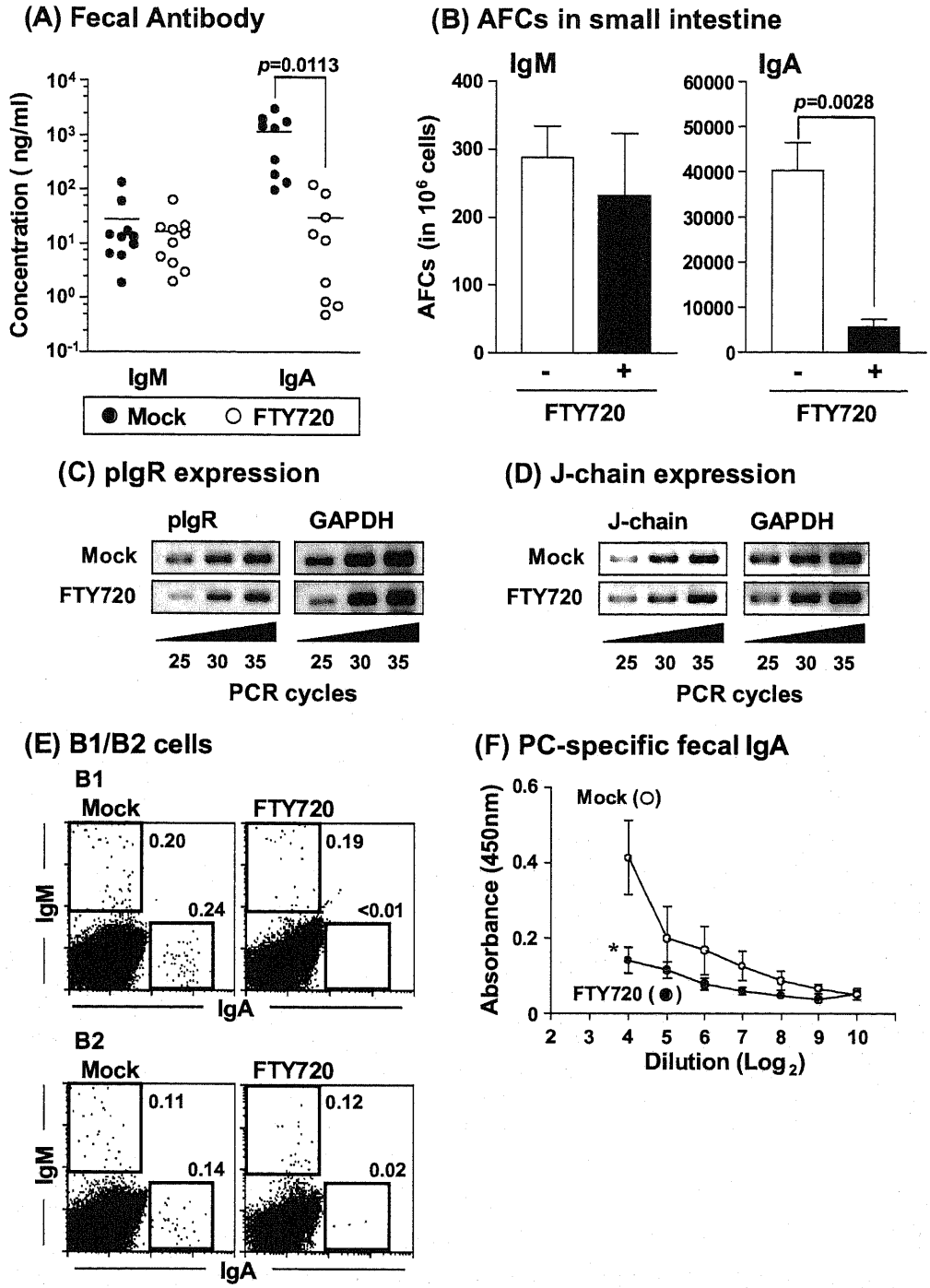

Figure 15 Impaired fecal IgA production after treatment with FTY720.

(A) Fecal extracts were collected from the reconstituted SCID mice and analyzed for Ig production by ELISA as described in Fig. 10A. (B) mononuclear cells were isolated from iLP and used for the ELISPOT assay. The error bars are ± SEM (n=5). (C and D) Poly immunogloblin receptor (pIgR) expression in epithelial cells (C) and J-chain expression iLP lymphocytes (D) were examined by RT-PCR. Data are representative of three independent experiments. (E) SCID mice were adoptively transferred with purified peritoneal B1 or B2 cells and treated with FTY720. FACS analysis was performed to detect IgA^+ and IgM^+ cells in the iLP of mice treated with (right) or without (left) FTY720. Data are representative of three independent experiments. (F) Mice were orally immunized with R36A together with cholera toxin and treated with (closed circle) or without (open circle) FTY720. After 3 days, fecal PC-specific IgA levels were measured by ELISA. The error bars are ± SEM (n=4). *, $p<0.05$.

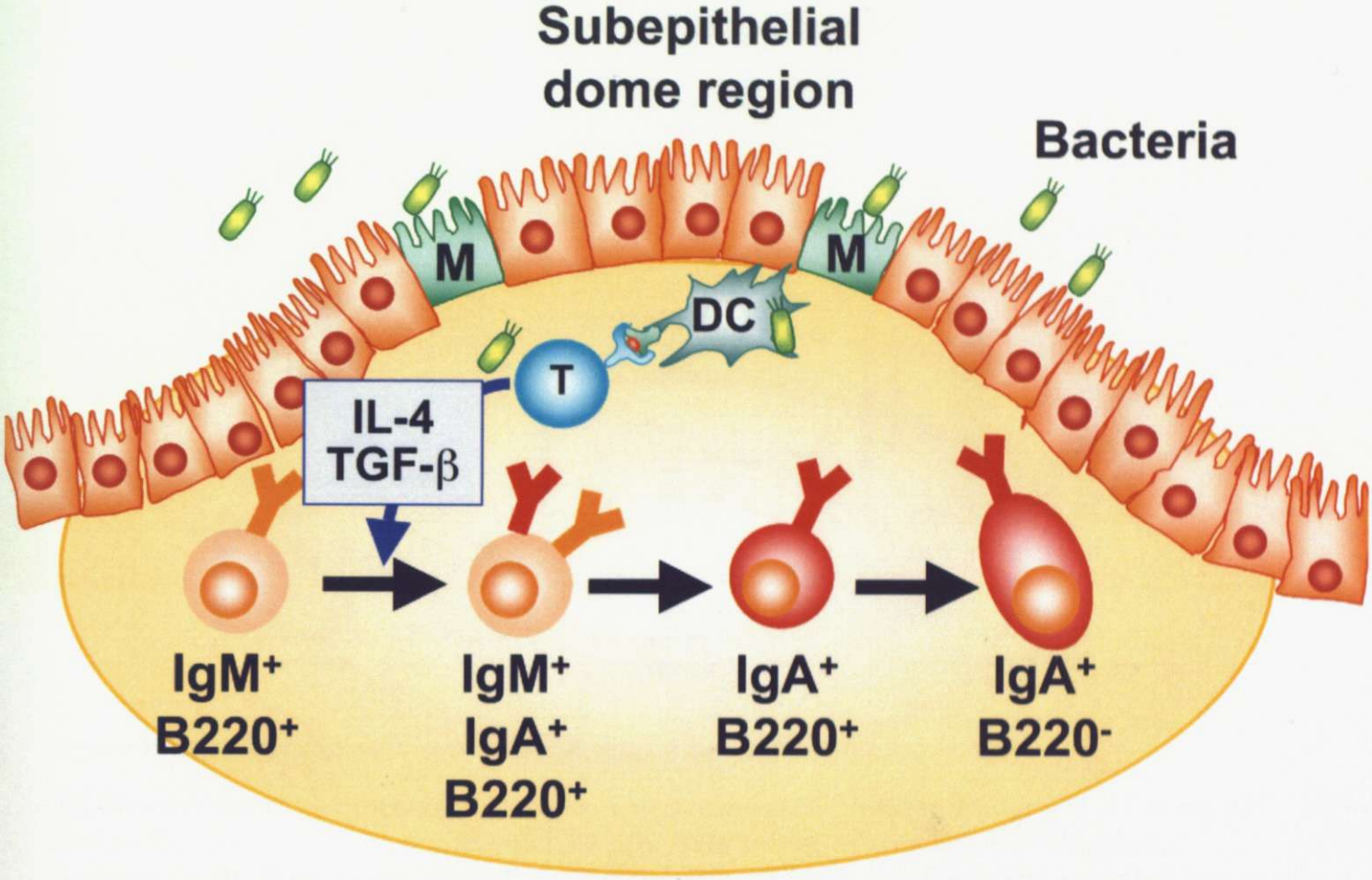

Figure 16 Sequential differentiation of B cells in the PPs for the intestinal IgA production.

PPs are major organized lymphoid tissues in the small intestine. Upon the stimulation through the antigen-uptaking dendritic cells (DC), T cells produce IL-4 and TGF-β, rending IgM^+B220^+ B cells to undergo $\mu \rightarrow \alpha$ class switching for the differentiation into $IgM^+IgA^+B220^+$ B cells. The $IgM^+IgA^+B220^+$ B cells additionally differentiate to $IgM^-IgA^+B220^+$ B cells, and finally differentiate into $IgM^-IgA^+B220^-$ B cells.

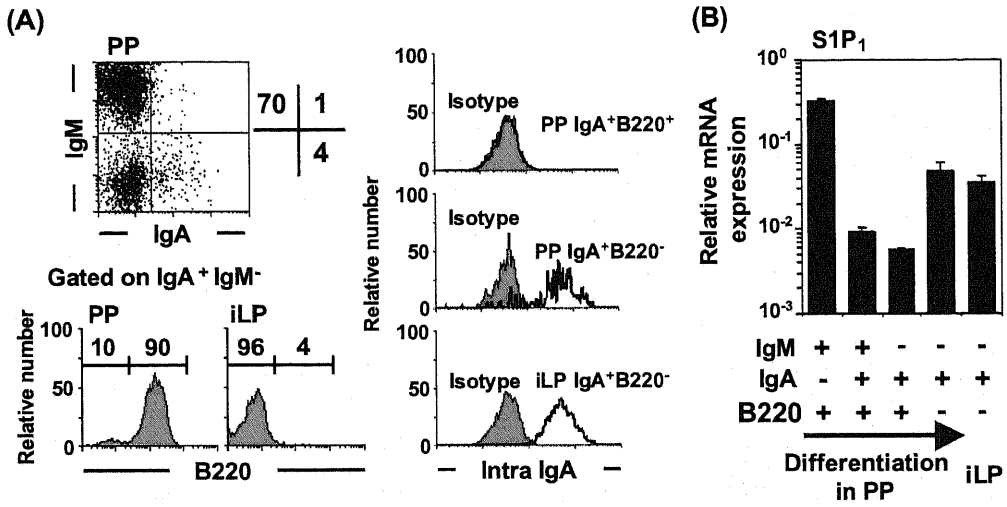

Figure 17 Alteration of $S1P_1$ expression during B cell differentiation in PPs.

(A) Flow cytometry was performed to characterize the cell populations in the PPs and iLP using Abs specific for IgM, IgA, B220, and intracellular IgA. Data are representative of five independent experiments. (B) 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.

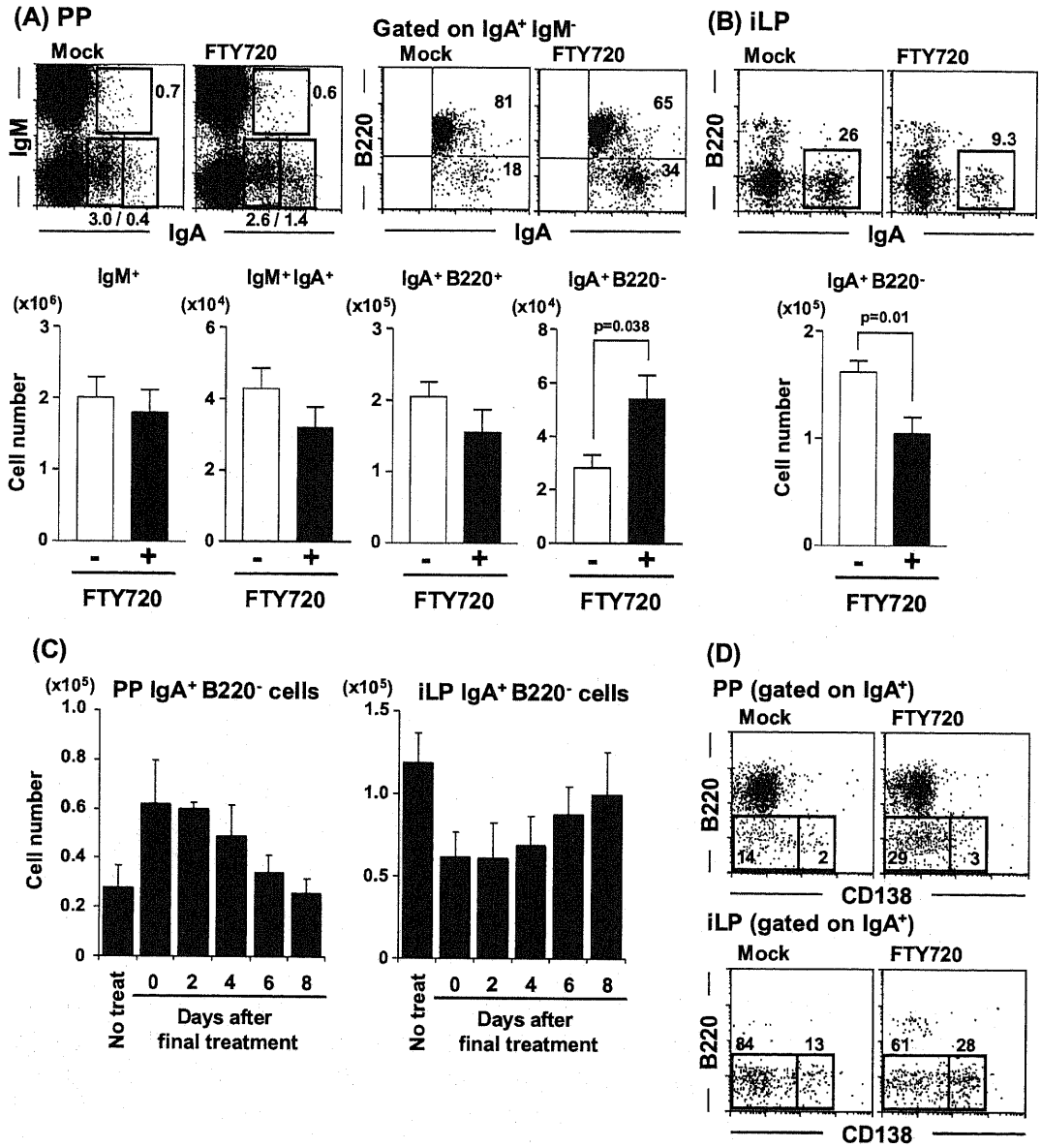

Figure 18 FTY720 induces the accumulation of IgA$^+$ B220$^-$ plasmablasts in the PPs and their reduction in iLP.

(A, 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) 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). (D) Cell population was examined by flow cytometry for analysis of plasmablasts (IgA$^+$ B220$^-$ CD138$^{-/low}$) and PCs (IgA$^+$ B220$^-$ CD138^{hi}). Data are representative of five independent experiments.

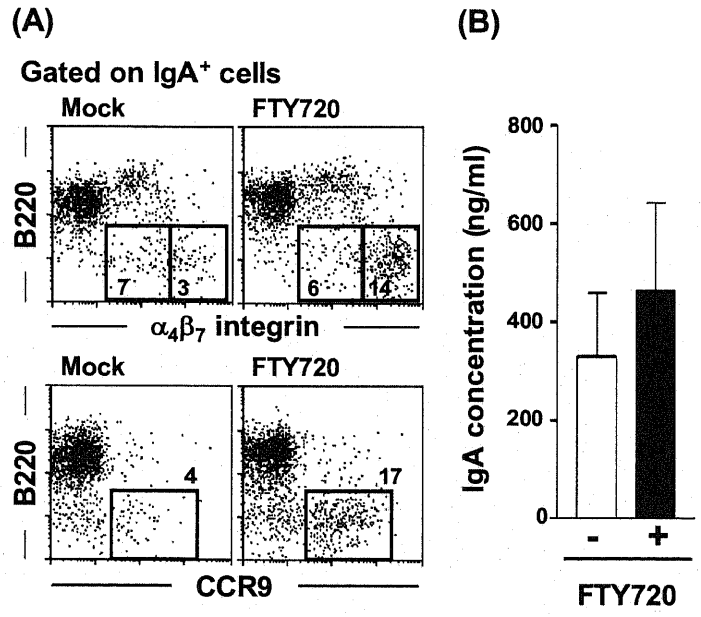

Figure 19 FTY720 does not affect expression of gut-homing molecules and IgA- producing ability of PP B cells.

(A) The expression of $\alpha_4\beta_7$ integrin and CCR9 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- (open) or FTY720-treated (closed) mice and their IgA production in the culture supernatant was examined after 72 h culture with 500 pg/ml of IL-6. Error bars are SEM (n=3).

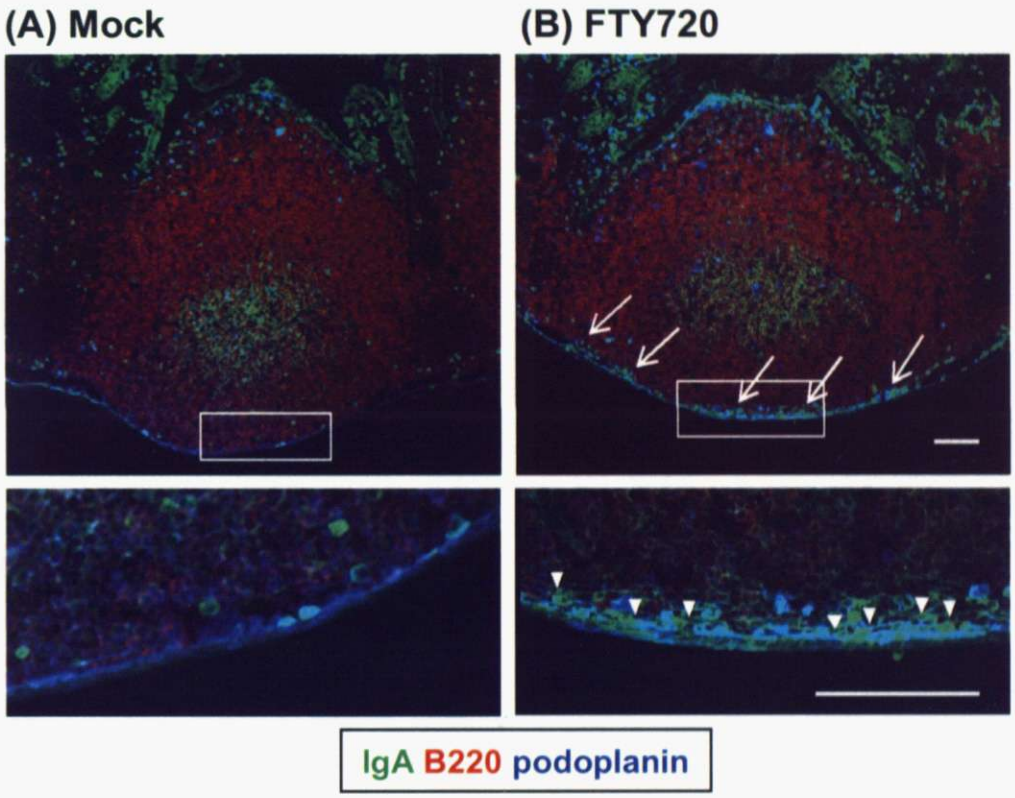

Figure 20 FTY720 causes accumulation of IgA^{+} B220^{-} plasmablasts on basal side of PPs.
Confocal microscopic analysis was performed to examine the distribution of cells expressing IgA (green), B220 (red), and podoplanin (blue) in the PPs of mock-treated (A) or FTY720-treated mice (B). Data are representative of five independent experiments. Bars indicate 100 μm.

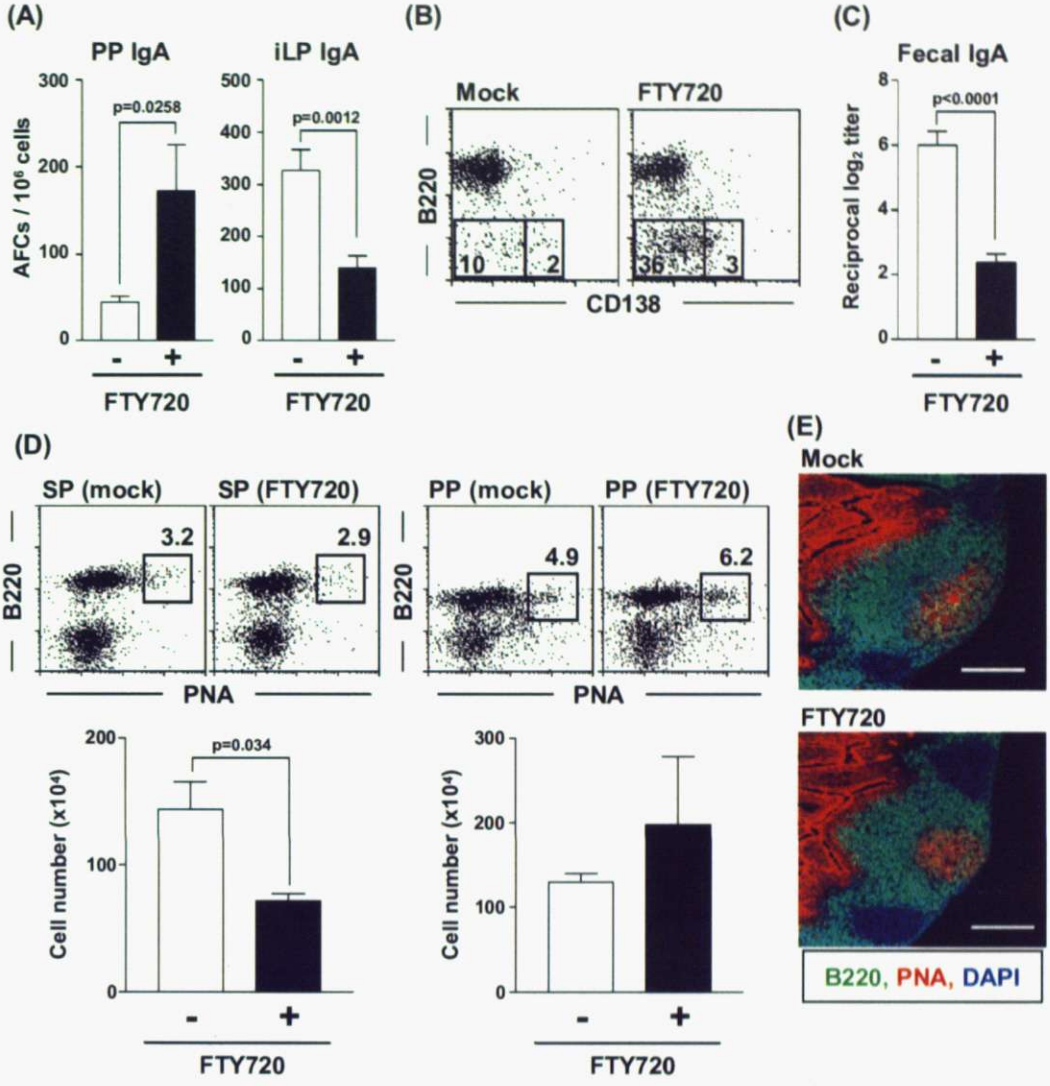

Figure 21 FTY720 reduces Ag-specific intestinal S-IgA production against orally administered OVA without affecting GC formation.

(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- (open) and FTY720-treated mice (closed). 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- (open) and FTY720-treated mice (closed) was determined by ELISA. Error bars are SEM (n=5). (D) 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). (E) Confocal microscopy analysis was performed to detect the GC formation in the PP of mice treated with mock (left) or FTY720 (right). Data are representative of three independent experiments. Bar indicates 200 μm.

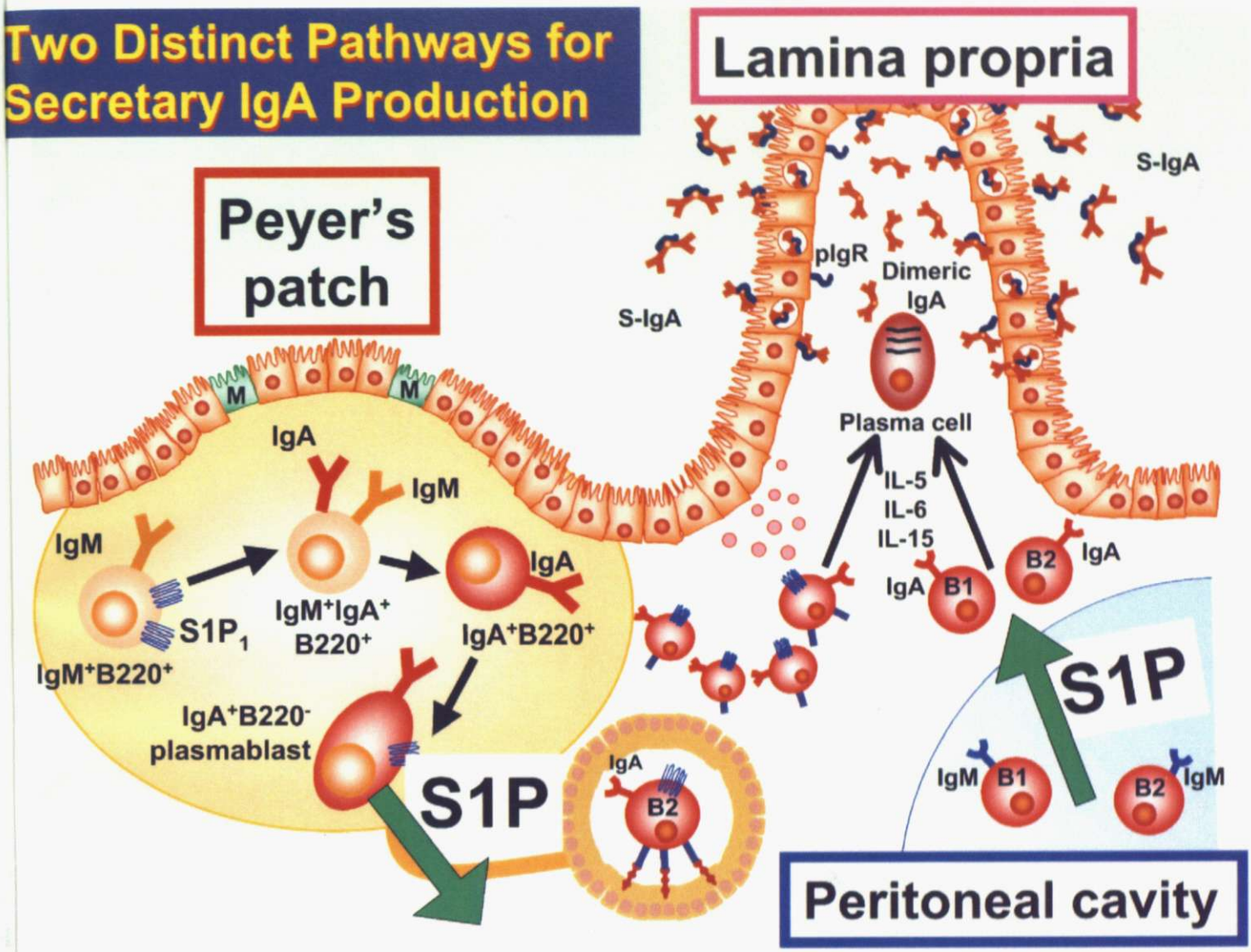

Figure 22 S1P regulates two distinct pathways for intestinal IgA production from PerC and PPs.
In the intestinal IgA production from PerC, S1P regulates both immigration and emigration steps of peritoneal B cells. In PP-dependent intestinal IgA production pathway, S1P plays a important role in emigration of IgA^+ plasmablasts from PPs. Each pathway is essential for the induction of TI- and TD-dependent intestinal IgA production, respectively.