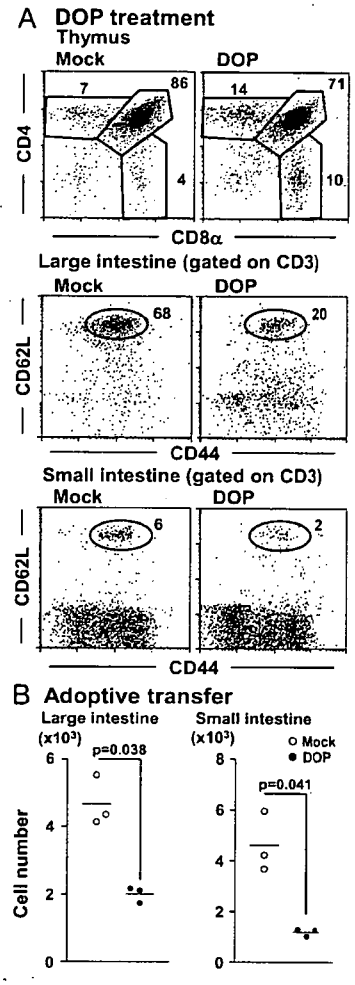


Figure 5. Disruption of S1P gradient halts naive IEL migration. (A) Lymphocytes were collected from the thymus (top) and large (middle) and small (bottom) intestines of mice treated with DOP (right) or water (left) for 3 d, and were analyzed for naive cells in the $CD3^+$ population. The data are representative of three independent experiments. (B) At 7 d after the adoptive transfer of CFSE-labeled T cells, cells were isolated from the large (left) or small (right) intestinal epithelia of mice treated with mock (open circle) or DOP (filled circle), and the total number of $CFSE^+$ cells was examined.

By 6 d after the single BrdU injection, BrdU-labeled cells were barely detected in the thymus (unpublished data), indicating that newly developed naive T cells were BrdU negative. The number of $BrdU^+$ naive CD4 IELs started to fall on day 8 in the large intestine and day 6 in the small intestine, after a linear regression (Fig. 4 F). The estimated 50% turnover rates of naive CD4 IELs were >3 d in both small and large intestines (Fig. 4 F). In contrast, the cell number of $BrdU^+$ cells in the MLN was maintained at a similar level during the experiment, excluding the possibility that the decay curves reflected the survival time of $BrdU^+$ cells. These findings led us to suggest that FTY720 affects naive IEL retention in the intestinal epithelium, which would lead to a reduction in the number of $CFSE^-$ IELs. To test this hypothesis, we examined $CFSE^-$ cells in the intestines 1 d after the transfer because few cells migrated into the intestinal epithelium after this time under this experimental condition (Fig. 4 E). We observed a significant decrease in naive ($CD62L^{high}CD44^{int}$), but not in activated ($CD62L^{neg}CD69^+$), CFSE-negative cells residing in the small and large intestines of FTY720-treated mice (Fig. 4 G). To examine the possibility that FTY720 treatment could lead to the disappearance of resident naive IELs by triggering either the activation of naive IELs or their programmed cell death, we next cultured naive lymphocytes with FTY720 for 2 d and examined their phenotype and viability and noted no changes in either cell activation or viability (Fig. S3 B and not depicted). These findings suggest that FTY720 inhibits not only naive lymphocyte migration into the intestine from the systemic immune compartments but also their retention in the intestinal epithelium.

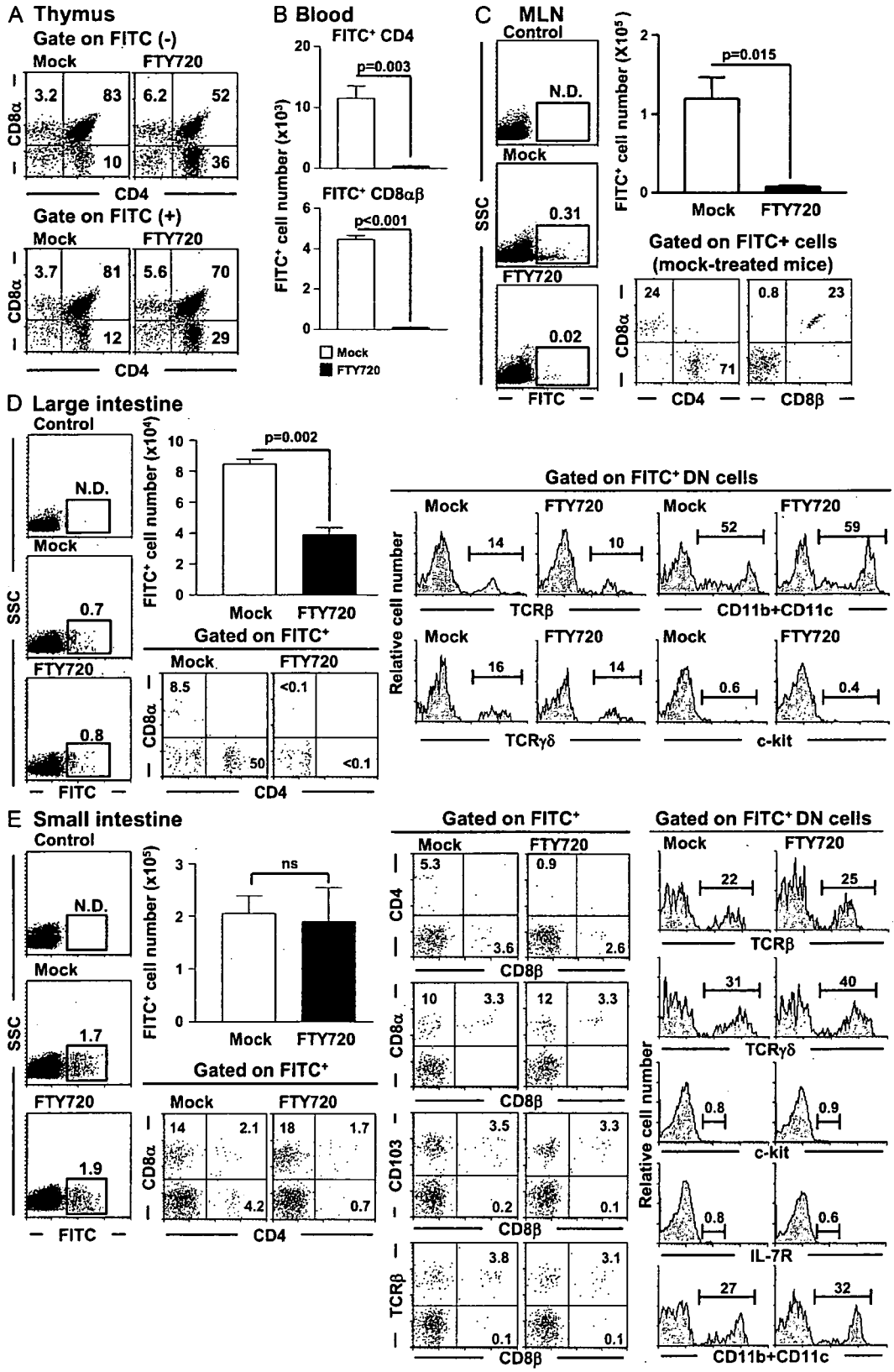
Inhibiting S1P lyase activity disrupts the S1P gradient and hampers cell trafficking into the intestine

To further confirm the involvement of S1P in the regulation of IEL trafficking, we next used DOP, which was reported to disrupt the S1P gradient by inhibiting S1P lyase (23). Our results confirmed this, and demonstrated that disruption of the S1P gradient by oral feeding with DOP for 3 d resulted in the accumulation of SP thymocytes (Fig. 5 A). DOP treatment also reduced the number of naive IELs in both the small and large intestines (Fig. 5 A). An adoptive-transfer experiment using CFSE-labeled naive T cells revealed that DOP treatment inhibited their trafficking into the intestines (Fig. 5 B). These findings convincingly demonstrated that an S1P-mediated pathway regulates the naive IEL trafficking.

FTY720-insensitive trafficking of unconventional thymic IEL precursors into the intestinal epithelium

Our focus was next shifted to FTY720-insensitive IEL populations, such as $CD8\alpha\alpha$ IELs. Recent studies have revealed several distinctive developmental pathways for IELs, including unconventional thymic IEL precursors (6–11). Thus, we examined whether S1P mediated the migration of these thymus-originated IEL precursors into the small and large intestines using the intrathymic FITC-labeling system. By 24 h

Figure 6. FTY720-insensitive migration of RTEs and thymic DN IEL precursors expressing TCRαβ or TCRγδ into the intestinal epithelium. By 24 h after intrathymic FITC injection, the cell populations in $FITC^+$ and $FITC^-$ thymocytes (A) in mock-treated (left) or FTY720-treated (right) mice were examined by flow cytometry. Cells were isolated from the blood (B), MLN (C), large intestine (D), and small intestine (E) and examined by flow cytometry. Absolute cell numbers of $FITC^+$ cells were calculated using the total cell numbers and flow cytometric data. Expression of CD4, CD8α, CD8β, CD11b, CD11c, CD103, c-kit, IL-7R, TCRαβ, and TCRγδ was examined in $FITC^+$ cells. The graph data depicts the means ' the SEM (*n* 4). Other data are representative of at least four independent experiments.



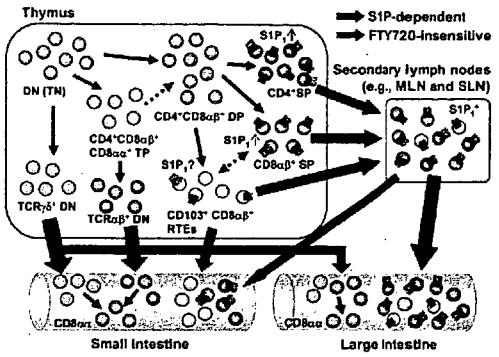


Figure 7. Dependency of lymphocyte migration into the small and large intestines on S1P. Conventional CD4 (green) and CD8 (orange) SP thymocytes emigrate from the thymus into secondary lymphoid organs, and, subsequently, into the small and large intestines. These SP cells express high levels of $S1P_1$ and pathways regulated by S1P (blue lines), and can be inhibited by FTY720. Unconventional thymic IEL precursors, including mature post-selected TCRαβ DN cells (red) derived from TP ($CD4^+CD8\alpha\beta^+CD8\alpha\alpha^+$) thymocytes (pink), TCRγδ DN thymocytes (purple), and $CD103^+$ $CD8\alpha\beta$ RTEs (yellow), migrate into the intestinal epithelium through FTY720-insensitive pathways (red lines).

after the intrathymic FITC injection, we found that each population of thymocytes was equally stained with FITC. Thus, comparable percentages of CD4 and CD8 SP, DP, and DN thymocytes were found in $FITC^+$ and $FITC^-$ thymocytes in mock-treated control mice (Fig. 6 A). Additionally, FTY720 treatment resulted in similar accumulation levels of CD4 and CD8 SP thymocytes in both $FITC^+$ and $FITC^-$ fractions, suggesting that FTY720 inhibited the emigration of SP thymocytes regardless of FITC labeling (Fig. 6 A). These effects coincided with the barely detectable levels of CD4 and CD8 SP $FITC^+$ cells in the blood circulation of FTY720-treated mice (Fig. 6 B).

Consistent with previous results (8), $FITC^+$ cells were detected in the secondary lymphoid organs (such as the MLN) in mock-treated control mice (Fig. 6 C). The positive fraction mainly consisted of cells expressing CD4 or CD8αβ, but not DN or DP cells (Fig. 6 C). The group of mice treated with FTY720 possessed barely detectable levels of $FITC^+$ cells (Fig. 6 C), which was plausibly caused by the inhibition of SP thymocyte emigration by FTY720. These findings led us to conclude that the pathway from the thymus into the MLN is regulated by S1P.

$FITC^+$ cells were found in the large intestine of mock-treated control mice (Fig. 6 D). In FTY720-treated mice, a similar percentage of $FITC^+$ cells was observed, but the absolute $FITC^+$ cell number was decreased because of the reduction in total cell numbers in large intestinal IELs (Figs. 1 A and 6 D). Flow cytometric analysis revealed that $FITC^+$ cells were comprised of DN cells in addition to cells expressing either CD8α or CD4, and that they did not contain any DP cells (Fig. 6 D). As with $FITC^+$ CD4 and CD8α cells in the MLN (Fig. 6 C), FTY720 treatment inhibited the trafficking of $FITC^+$ cells expressing CD4 or CD8α into the large intestine, but did not influence the migration of DN cells (Fig. 6 D). As recent studies have revealed unconventional CD8αα IEL precursors in the DN thymocytes (6, 7, 12), we analyzed TCR expression to reveal that $FITC^+$ DN cells expressed TCRαβ (10–20%) or TCRγδ (10–20%; Fig. 6 D). We also found that $FITC^+$ DN cells barely expressed c-kit, but 50–60% of $FITC^+$ DN cells expressed CD11b and/or CD11c, suggesting that they were phagocytic cells taking up leaked FITC or FITC-labeled apoptotic cells (Fig. 6 D). In addition, $FITC^+$ TCR^+ DN cells were not detected when the same amount of FITC was injected intravenously (unpublished data), excluding a possibility that the $FITC^+$ TCR^+ DN cells observed in this experiment were labeled in the extrathymic compartments. These findings indicate that some $FITC^+$ DN cells are derived from phagocytic cells, whereas others are composed exclusively of cells expressing TCRαβ or TCRγδ. In agreement with these findings, considerable numbers of DN cells expressing TCRαβ or TCRγδ were detected in the blood of FTY720-treated mice, although FTY720 significantly reduced CD4 and CD8 SP cells in the blood (Fig. S3 C). These data suggest that thymic CD4 and CD8 SP cells migrate into the large intestine via an S1P-mediated pathway, and DN cells expressing TCRαβ or TCRγδ migrate via FTY720-insensitive manner.

As one might expect based on the data summarized in Fig. 1, the number of $FITC^+$ cells in the small intestine remained similar after FTY720 treatment (Fig. 6 E), and the $FITC^+$ cells in the small intestine of mock-treated control mice were comprised of DP and DN cells, as well as cells expressing either CD4 or CD8α (Fig. 6 E). Because $CD4^+$ cells exclusively expressed TCRαβ and no cells expressed CD4 together with CD8β in the $FITC^+$ fraction, it seems that DP IELs are $TCR\alpha\beta^+CD8\alpha\alpha^+$ CD4 cells uniquely observed in the intestinal epithelium, and are not thymic DP cells (Fig. 6 E). Small intestinal $FITC^+$ DN IELs included cells expressing CD11b and/or CD11c (~30%), TCRαβ (20–30%), or TCRγδ (30–40%) and few cells expressing c-kit or IL-7R (Fig. 6 E). Like the large intestine, $FITC^+$ TCR^+ DN cells were not detected when the same amount of FITC was injected intravenously (unpublished data). These findings suggest that, similar to the large intestine, some small intestinal $FITC^+$ DN cells are derived from phagocytic cells, whereas others are composed exclusively of cells expressing TCRαβ or TCRγδ, and not cells derived from TCR-negative DN (triple-negative [TN]) cells.

Intriguingly, the ratio of CD8α to CD4 cells was higher in the small intestine than in MLN or the large intestine (Fig. 6, C and D). Those CD8α $FITC^+$ IELs were composed of CD8αα IELs (75%) and CD8αβ IELs (25%; Fig. 6 E), the latter of which expressed CD103, which is a recently reported identifying characteristic of RTEs (Fig. 6 E) (8, 31). FTY720 significantly inhibited the migration of CD4 cells into the small intestine (mock, 4.3 ± 0.35%; FTY720, 1.1 ± 0.23%; $P = 0.009$), whereas other populations including TCRαβ DN cells, TCRγδ DN cells, and $CD103^+$ CD8αβ RTEs were unaffected (Fig. 6 E).

Collectively, our current findings demonstrate that thymic DN IEL precursors expressing TCRαβ or TCRγδ are likely to migrate into the large or small intestine, whereas RTEs preferentially migrate into the small intestine (Fig. 7). Unlike S1P-dependent SP thymocytes' migration into the intestine through secondary lymphoid organs, the migration pathway of unconventional IEL precursors is totally insensitive to FTY720 treatment (Fig. 7).

DISCUSSION

In this study, we demonstrate that the lipid mediator S1P determines whether a given type of IEL goes into the small or the large intestine, resulting in varying proportions of naive cells, RTEs, and thymic DN IEL precursors in the two compartments. Naive IELs, which primarily express $S1P_1$, are more abundant in the large intestine than in the small intestine (Fig. 3 A), explaining why large intestinal IELs are more sensitive to FTY720 or DOP (Figs. 1, 3, and 5). These findings confirm previous reports that identify $CD62L^+$ cells as the primary targets of FTY720 in systemic immunity, and show that $S1P_1$ is preferentially expressed on naive and central memory T cells rather than activated T cells for efficient S1P-mediated migration (18, 32, 33). In contrast, activated IELs, which were abundant in the small intestine but barely detectable in the large intestine, did not respond to FTY720 (Figs. 3 and 4). In this context, a previous study demonstrated that the activation marker CD69 itself induced down-regulation of $S1P_1$, thereby rendering activated cells unresponsive to S1P (34). These findings offer a plausible explanation for why activated IELs expressing CD69 in the intestinal compartments are less reactive to FTY720 (Fig. 3 B).

Our results imply that FTY720 inhibits not only the trafficking of naive IELs into the intestine, but also their retention in the intestinal epithelium (Fig. 4 and Fig. S3). This might explain why naive T cells accumulated in the SLN of FTY720-treated mice, despite their reduced migration from the blood into the SLN (Figs. 2 E and 4 C). A previous work has demonstrated that lymphocyte exit from the skin is not a random process, but is regulated by a chemokine-mediated pathway (35). Additionally, we recently reported that S1P regulates B cell retention in the peritoneal cavity (24). Our current study therefore not only confirms that lymphocyte trafficking in nonlymphoid tissues is regulated biologically, but also shows that S1P plays an important role in that pathway.

In contrast to the S1P-dependent trafficking of naive cells into the intestines through secondary lymphoid organs, thymic DN IEL precursors migrate into both the small and large intestines in an FTY720-insensitive manner (Fig. 6). Recently, several lines of evidence have proposed the presence of IEL precursors in DN thymocytes, including $TCR\alpha\beta^+$ DN thymocytes, $TCR\gamma\delta^+$ DN thymocytes, and $TCR\alpha\beta^-$ $CD25^+$ TN thymocytes (6, 7, 12). $TCR\alpha\beta^+$ DN thymocytes are known as mature post-selected DN thymocytes, as they arise from $CD8\alpha\alpha^+$ $CD4^+$ $CD8\alpha\beta^+$ triple-positive (TP) thymocytes after agonist selection (6). $TCR\alpha\beta^+$ DN thymocytes then migrate into the intestine, where they reinduce $CD8\alpha\alpha$ under the influence of IL-15 (6). It has been shown that TN thymocytes emigrate from the thymus into the intestinal epithelium, where they characteristically express c-kit and IL-7R on the cell surface and mRNA encoding CD3ε (7). This study demonstrates that $FITC^+$ thymic IEL precursors in the intestine of FTY720-treated mice express either $TCR\alpha\beta$ or $TCR\gamma\delta$, but not c-kit and IL-7R (Fig. 6, D and E). Thus, although it remains to be determined whether other $CD8\alpha\alpha$ IEL-generating pathways (such as the cryptopatch-dependent route) are dependent on S1P (11) and whether FTY720-resistant S1P-mediated pathways (e.g., $S1P_2$-mediated pathway) are involved in the trafficking of DN thymocytes into the intestines (21), our findings suggest that mature post-selected $TCR\alpha\beta^+$ DN thymocytes and $TCR\gamma\delta^+$ DN thymocytes, but not TN thymocytes, migrate into the gut in a FTY720-insensitive manner.

In addition to thymic DN IEL precursors, the migration of RTEs from the thymus into the small intestine was not inhibited by FTY720 treatment (Fig. 6 E). It has been shown that RTEs uniquely express $\alpha4\beta7$ integrin and CCR9 in the thymus, which enables them to migrate directly into the small intestinal epithelium without undergoing activation in the secondary lymphoid organs (8). Their preferential migration into the small intestine rather than the large intestine can be explained by their CCR9 expression in the thymus, as small intestinal ECs, unlike those of the large intestine, produce an abundance of the CCR9 ligand CCL25 (28). Our current data indicate that FTY720 does not affect the direct migration of RTEs from the thymus into the small intestine. Although FTY720 had no effect on RTEs, which were known to express CD62L (8), naive $CD62L^{high}$ cells were barely detected in the small intestine of FTY720-treated mice (<0.1%; Fig. 3 A). This discrepancy is caused by the fact that the RTEs are very minor population in the small intestinal IELs. In this issue, it was previously demonstrated that ~2% of cells were RTEs in $CD62L^{high}CD44^{int}$ $CD8\alpha\beta$ cells, which consisted of 5% small intestinal $CD8\alpha\beta$ IELs (8). Therefore, an estimated percentage of RTEs in the total small intestinal IELs, including CD4, $CD8\alpha\alpha$, $CD8\alpha\beta$, and DN cells, is <0.1%. Thus, it is likely that the RTEs are present, but difficult to detect, in the small intestine of FTY720-treated mice. Additionally, there is still a minor possibility of the involvement of other cells that express CD103, CD62L, and CCR9 (e.g., circulating naive CD8 T cells and SP CD8 thymocytes). However, together with well-established effects of FTY720 on circulating naive CD8 T cells and SP CD8 thymocytes on their retention in the secondary lymphoid organs and thymus (16, 17) and our data on FITC-labeling experiments, the most plausible interpretation is that RTEs use the unique FTY720-insensitive trafficking pathway from the thymus into the small intestinal epithelium.

In addition to activated platelets, mast cells, and monocytes, dietary sphingolipids are another source of S1P (36). Interestingly, enzymes involved in the generation of sphingosine, which is a precursor of S1P, are principally expressed in the intestinal tracts, where their expression patterns differ

according to the intestinal compartment (37, 38). For instance, alkaline sphingomyelinase and neutral ceramidase, which are both important enzymes in the sequential degradation of sphingomyelin and ceramide to generate sphingosine, were predominantly expressed at the luminal side of the brush border in the distal jejunum and ileum, indicating that the amount of sphingosine was much lower in the upper and middle sections than in the lower sections of the small and large intestines (37, 38). The varying expression patterns of these enzymes, which might determine to what degree IELs migrate into a given region of the small or large intestine, depend on S1P under natural conditions. Although FTY720 inhibits the naive cell trafficking into the intestinal epithelium by preventing their emigration from the secondary lymphoid organs in our experimental condition (Fig. 2 E), it is interesting to examine whether luminal sphingosine-derived S1P regulates intestinal immunity, including T cell trafficking. Studies are currently underway in our group to further investigate this issue.

As S1P mediates the migration of mucosal T cells, such as IELs, it might also regulate other groups of immunocompetent and/or pathological cells in the mucosal immune system. Most irritations of the gastrointestinal tract, including inflammatory bowel diseases (IBDs) and food allergies, are caused by the influx of pathological cells into the intestine from systemic compartments (39, 40). If the migration of these pathological cells to the gastrointestinal tract is indeed S1P-dependent, it should be susceptible to FTY720, perhaps opening a new avenue for the treatment of IBDs and intestinal allergic diseases. Indeed, our previous study demonstrated that FTY720 effectively inhibited the development of IBDs in IL-10–deficient mice (41). Moreover, our recent separate studies show that FTY720 is also effective at preventing the development of allergic diarrhea by inhibiting pathogenic cell migration into the large intestine (42). In addition to regulating IEL trafficking under natural conditions, these findings suggest that the S1P-mediated pathway is involved in the development of immunological diseases of the intestine, such as IBDs and food allergies.

MATERIALS AND METHODS

Mice and experimental treatment. Normal female BALB/c mice (7–9 wk of age) were purchased from Japan Clea or Japan SLC. All mice were provided with sterile food and water ad libitum. Mice were injected intraperitoneally with 1 mg/kg FTY720 (Novartis Pharma) to assess reactivity (18, 24). To inhibit S1P lyase activity, the mice received drinking water containing 10 g/liter glucose and 30 mg/liter 4-deoxypyridoxine-HCl (Sigma-Aldrich) for 3 d (23). All animals were maintained in the experimental animal facility at the University of Tokyo, and the experiments were conducted in accordance with the guidelines provided by the Animal Care and Use Committee of the University of Tokyo.

Lymphocyte isolation. Single IEL cells were isolated from the small and large intestinal epithelium as previously described (43). In brief, after removing the PPs, the small and large intestines were dissected into short segments and stirred at 37°C in prewarmed RPMI 1640 containing 2% FCS and 0.5 mM EDTA for 15 min, followed by vigorous shaking for 15 s. This process was repeated twice. A discontinuous Percoll density gradient centrifugation was performed to purify the lymphocytes. IELs were collected from the layer between the 40% and 75% fractions. The spleen, thymus, MLN, SLN, PPs, and CPs were removed, and single-cell suspensions were prepared by passing them through a 70-μm mesh filter, as previously described (24, 44). Lymphocytes were prepared from the liver according to a previously established method (45). In brief, the liver was passed through a 200-gauge stainless mesh to obtain single cells. These cells were treated with erythrocyte-lysing solution (155 mM NH_4Cl, 10 mM $KHCO_3$, 1 mM EDTA, and 170 mM Tris-HCl, pH 7.3) to remove the erythrocytes, and were then fractionated by centrifugation in 35% Percoll.

Flow cytometry and cell sorting. Flow cytometry and cell sorting were performed as previously described (24, 44). Cells were preincubated with anti-CD16/32 antibody, and then stained with fluorescent antibodies specific for CD4, CD8α, CD8β, CD11b, CD11c, CD44, CD62L, CD69, CD103, c-kit, IL-7R, TCRβ, TCRγδ, α4β7 integrin (BD PharMingen), and CCR9 (R&D Systems). A Viaprobe (BD PharMingen) was used to discriminate between dead and living cells. Flow-cytometric analysis and cell sorting were performed using FACSCalibur and FACSAria (BD Biosciences), respectively.

Histological analysis. Immunohistochemical analysis was performed as previously described (40). In brief, large intestines were fixed in 4% paraformaldehyde (Wako) and treated with a sucrose gradient after extensive washing. The tissue was embedded in Tissue-Tek OCT compound (Sakura Finetechnical). For confocal microscopy analysis, TSA-direct kit (Perkin Elmer) was used according to the manufacturer's instructions. In brief, 6-μm cryostat sections were treated with 3% H_2O_2 in PBS for 15 min to quench endogenous peroxidase activity. Sections were preblocked with anti-CD16/CD32 antibody in PBS containing 2% FCS for 15 min at room temperature, and stained with biotin-conjugated antibodies specific for CD4 or CD62L for 15 h at 4°C. After washing with TNT buffer (0.1 M Tris-HCl, pH 7.5, 0.15 M NaCl, and 0.05% Tween20), sections were treated with horseradish peroxidase–conjugated streptavidin in TNT buffer for 30 min at room temperature. After washing with TNT buffer, amplification of the fluorescent signal with FITC or Cy5-tyramide was performed. The specimens were analyzed using a confocal laser-scanning microscope (TCS SP2; Leica). Hematoxylin and eosin staining was used to confirm the dissociation of the epithelial region, as previously described (40)

Quantitative RT-PCR. To measure mRNA expression for S1P receptors, quantitative RT-PCR using LightCycler (Roche) was performed as previously described (24). Total RNA was prepared using TRIzol reagent (Invitrogen), and cDNA was synthesized using Powerscript reverse transcriptase (BD Biosciences). The oligonucleotide primers and probes specific for $S1P_1$ (forward primer, TACACTCTGACCAACAAGGA; reverse primer, ATAATGGTCTCTGGGTTGTC;FITC-probe,TGCTGGCAATTCAAGAGGCCCATCATC; and LCRed 640-probe, CAGGCATGGAATTTAGCCGCAGCAAATC) and GAPDH (forward primer, TGAACGGGAAGCTCACTGG; reverse primer, TCCACCACCCTGTTGCTGTA; FITC-probe, CTGAGGACCAGGTTGTCTCCTGCGA; and LCRed 640-probe, TTCAACAGCAACTCCCACTCTTCCACC) were designed and produced by Nihon Gene Research Laboratory.

Adoptive transfer of CFSE-labeled lymphocytes. $CD3^+$ T cells were isolated from the MLN and SLN using anti-mouse CD3-coupled microbeads and a MACS column (Miltenyi Biotec) as previously described (44). For CFSE labeling, 10^7 cells were incubated in the dark with 10 μM CFSE (Invitrogen) for 10 min at 37°C, before being washed twice with PBS (8, 24). Labeled cells (2×10^7) were adoptively transferred via the tail vein into naive mice, which were either treated with FTY720 5 min after the cell transfer or left untreated. Lymphocytes were isolated from the MLN, SLN, blood, PPs, and small and large intestinal epithelium 24 h and 7 d after the transfer for flow-cytometric analysis.

Intrathymic labeling of thymocytes with FITC. The intrathymic injection of FITC was performed as previously described (8, 22, 31). In brief, 10 μl FITC solution (1 mg/ml; Sigma-Aldrich) was injected into the thymus of anesthetized mice and the skin was closed with silk sutures. For FTY720 treatment, the mice were treated with FTY720 5 min before the FITC injection. Lymphocytes were isolated from the thymus, blood, MLN, and small and large intestinal epithelium 24 h after the FITC injection for flow-cytometric analysis.

BrdU incorporation and measurement. Mice were injected intraperitoneally with 1 mg BrdU (Sigma-Aldrich) in PBS as previously described (46). At the indicated times, the IELs were isolated from the small and large intestines and stained with fluorescent antibodies specific for CD4 and CD62L (BD PharMingen). BrdU incorporation was detected by flow cytometry with a BrdU Flow kit according to the manufacturer's instructions (BD Biosciences).

Statistics. Results were compared using the Student's or Welch's *t* test. Statistical significance was established at $P < 0.05$.

Online supplemental material. Fig. S1 shows the data on CD4 and CD8 cells in the thymus, spleen, liver, PPs, CPs, and lymphocyte aggregates of mice treated with FTY720. Fig. S2 provides the data on the CCR9 expression on small and large intestinal CD8 IELs. Fig. S3 shows data on FACS profile in the PPs of FTY720-treated mice, naive T cells cultured with FTY720, and cell numbers of blood lymphocytes of FTY720-treated mice. The online version of this article is available at http://www.jem.org/cgi/content/full/jem.20062446/DC1.

We thank Y. Takahama for helpful discussions and technical advice and K. McGhee for editorial help.

This work was supported by grants from Core Research for Evolutional Science and Technology of the Japan Science and Technology Corporation; the Ministry of Education, Science, Sports, and Culture; the Ministry of Health and Welfare in Japan; the Waksman Foundation of Japan; and Yakult Bio-Science Foundation.

The authors have no conflicting financial interests.

Submitted: 21 November 2006
Accepted: 15 August 2007

REFERENCES

1. Kiyono, H., and S. Fukuyama. 2004. NALT- versus Peyer's-patch-mediated mucosal immunity. *Nat. Rev. Immunol.* 4:699–710.
2. Kunisawa, J., and H. Kiyono. 2005. A marvel of mucosal T cells and secretory antibodies for the creation of first lines of defense. *Cell. Mol. Life Sci.* 62:1308–1321.
3. Kunisawa, J., S. Fukuyama, and H. Kiyono. 2005. Mucosa-associated lymphoid tissues in aerodigestive tract: their shared and divergent traits and their importance to the orchestration of mucosal immune system. *Curr. Mol. Med.* 5:557–572.
4. Kunisawa, J., I. Takahashi, and H. Kiyono. 2007. Intraepithelial lymphocytes: their shared and divergent immunological behaviors in the small and large intestine. *Immunol. Rev.* 215:136–153.
5. Cheroutre, H. 2004. Starting at the beginning: new perspectives on the biology of mucosal T cells. *Annu. Rev. Immunol.* 22:217–246.
6. Gangadharan, D., F. Lambolez, A. Attinger, Y. Wang-Zhu, B.A. Sullivan, and H. Cheroutre. 2006. Identification of pre- and post-selection TCRαβ$^+$ intraepithelial lymphocyte precursors in the thymus. *Immunity.* 25:631–641.
7. Lambolez, F., M.L. Arcangeli, A.M. Joret, V. Pasqualetto, C. Cordier, J.P. Di Santo, B. Rocha, and S. Ezine. 2006. The thymus exports long-lived fully committed T cell precursors that can colonize primary lymphoid organs. *Nat. Immunol.* 7:76–82.
8. Staton, T.L., A. Habtezion, M.M. Winslow, T. Sato, P.E. Love, and E.C. Butcher. 2006. CD8$^+$ recent thymic emigrants home to and efficiently repopulate the small intestine epithelium. *Nat. Immunol.* 7:482–488.
9. Eberl, G., and D.R. Littman. 2004. Thymic origin of intestinal αβ T cells revealed by fate mapping of RORγt+ cells. *Science.* 305:248–251.
10. Guy-Grand, D., O. Azogui, S. Celli, S. Darche, M.C. Nussenzweig, P. Kourilsky, and P. Vassalli. 2003. Extrathymic T cell lymphopoiesis: ontogeny and contribution to gut intraepithelial lymphocytes in athymic and euthymic mice. *J. Exp. Med.* 197:333–341.
11. Saito, H., Y. Kanamori, T. Takemori, H. Nariuchi, E. Kubota, H. Takahashi-Iwanaga, T. Iwanaga, and H. Ishikawa. 1998. Generation of intestinal T cells from progenitors residing in gut cryptopatches. *Science.* 280:275–278.
12. Pennington, D.J., B. Silva-Santos, and A.C. Hayday. 2005. γδ T cell development–having the strength to get there. *Curr. Opin. Immunol.* 17:108–115.
13. Beagley, K.W., K. Fujihashi, A.S. Lagoo, S. Lagoo-Deenadaylan, C.A. Black, A.M. Murray, A.T. Sharmanov, M. Yamamoto, J.R. McGhee, C.O. Elson, et al. 1995. Differences in intraepithelial lymphocyte T cell subsets isolated from murine small versus large intestine. *J. Immunol.* 154:5611–5619.
14. Camerini, V., C. Panwala, and M. Kronenberg. 1993. Regional specialization of the mucosal immune system. Intraepithelial lymphocytes of the large intestine have a different phenotype and function than those of the small intestine. *J. Immunol.* 151:1765–1776.
15. Ibraghimov, A.R., and R.G. Lynch. 1994. Heterogeneity and biased T cell receptor α/β repertoire of mucosal CD8$^+$ cells from murine large intestine: implications for functional state. *J. Exp. Med.* 180:433–444.
16. Rosen, H., and E.J. Goetzl. 2005. Sphingosine 1-phosphate and its receptors: an autocrine and paracrine network. *Nat. Rev. Immunol.* 5:560–570.
17. Cyster, J.G. 2005. Chemokines, sphingosine-1-phosphate, and cell migration in secondary lymphoid organs. *Annu. Rev. Immunol.* 23: 127–159.
18. Matloubian, M., C.G. Lo, G. Cinamon, M.J. Lesneski, Y. Xu, V. Brinkmann, M.L. Allende, R.L. Proia, and J.G. Cyster. 2004. Lymphocyte egress from thymus and peripheral lymphoid organs is dependent on S1P receptor 1. *Nature.* 427:355–360.
19. Allende, M.L., J.L. Dreier, S. Mandala, and R.L. Proia. 2004. Expression of the sphingosine 1-phosphate receptor, $S1P_1$, on T-cells controls thymic emigration. *J. Biol. Chem.* 279:15396–15401.
20. Graler, M.H., and E.J. Goetzl. 2004. The immunosuppressant FTY720 down-regulates sphingosine 1-phosphate G-protein-coupled receptors. *FASEB J.* 18:551–553.
21. Mandala, S., R. Hajdu, J. Bergstrom, E. Quackenbush, J. Xie, J. Milligan, R. Thornton, G.J. Shei, D. Card, C. Keohane, et al. 2002. Alteration of lymphocyte trafficking by sphingosine-1-phosphate receptor agonists. *Science.* 296:346–349.
22. Yagi, H., R. Kamba, K. Chiba, H. Soga, K. Yaguchi, M. Nakamura, and T. Itoh. 2000. Immunosuppressant FTY720 inhibits thymocyte emigration. *Eur. J. Immunol.* 30:1435–1444.
23. Schwab, S.R., J.P. Pereira, M. Matloubian, Y. Xu, Y. Huang, and J.G. Cyster. 2005. Lymphocyte sequestration through S1P lyase inhibition and disruption of S1P gradients. *Science.* 309:1735–1739.
24. Kunisawa, J., Y. Kurashima, M. Gohda, M. Higuchi, I. Ishikawa, F. Miura, I. Ogahara, and H. Kiyono. 2007. Sphingosine 1-phosphate regulates peritoneal B cell trafficking for subsequent intestinal IgA production. *Blood.* 109:3749–3756.
25. Chiba, K., Y. Yanagawa, Y. Masubuchi, H. Kataoka, T. Kawaguchi, M. Ohtsuki, and Y. Hoshino. 1998. FTY720, a novel immunosuppressant, induces sequestration of circulating mature lymphocytes by acceleration of lymphocyte homing in rats. I. FTY720 selectively decreases the number of circulating mature lymphocytes by acceleration of lymphocyte homing. *J. Immunol.* 160:5037–5044.
26. Wagner, N., J. Lohler, E.J. Kunkel, K. Ley, E. Leung, G. Krissansen, K. Rajewsky, and W. Muller. 1996. Critical role for β7 integrins in formation of the gut-associated lymphoid tissue. *Nature.* 382:366–370.
27. Cepek, K.L., S.K. Shaw, C.M. Parker, G.J. Russell, J.S. Morrow, D.L. Rimm, and M.B. Brenner. 1994. Adhesion between epithelial cells and T lymphocytes mediated by E-cadherin and the αEβ7 integrin. *Nature.* 372:190–193.

28. Kunkel, E.J., J.J. Campbell, G. Haraldsen, J. Pan, J. Boisvert, A.I. Roberts, E.C. Ebert, M.A. Vierra, S.B. Goodman, M.C. Genovese, et al. 2000. Lymphocyte CC chemokine receptor 9 and epithelial thymus-expressed chemokine (TECK) expression distinguish the small intestinal immune compartment: epithelial expression of tissue-specific chemokines as an organizing principle in regional immunity. *J. Exp. Med.* 192:761–768.
29. Halin, C., M.L. Scimone, R. Bonasio, J.M. Gauguet, T.R. Mempel, E. Quackenbush, R.L. Proia, S. Mandala, and U.H. von Andrian. 2005. The S1P-analog FTY720 differentially modulates T-cell homing via HEV: T-cell-expressed $S1P_1$ amplifies integrin activation in peripheral lymph nodes but not in Peyer patches. *Blood.* 106:1314–1322.
30. Lefrancois, L., C.M. Parker, S. Olson, W. Muller, N. Wagner, M.P. Schon, and L. Puddington. 1999. The role of β7 integrins in CD8 T cell trafficking during an antiviral immune response. *J. Exp. Med.* 189:1631–1638.
31. Staton, T.L., B. Johnston, E.C. Butcher, and D.J. Campbell. 2004. Murine $CD8^+$ recent thymic emigrants are αE integrin-positive and CC chemokine ligand 25 responsive. *J. Immunol.* 172:7282–7288.
32. Bohler, T., J. Waiser, M. Schuetz, H.H. Neumayer, and K. Budde. 2004. FTY720 exerts differential effects on $CD4^+$ and $CD8^+$ T-lymphocyte subpopulations expressing chemokine and adhesion receptors. *Nephrol. Dial. Transplant.* 19:702–713.
33. Graeler, M., and E.J. Goetzl. 2002. Activation-regulated expression and chemotactic function of sphingosine 1-phosphate receptors in mouse splenic T cells. *FASEB J.* 16:1874–1878.
34. Shiow, L.R., D.B. Rosen, N. Brdickova, Y. Xu, J. An, L.L. Lanier, J.G. Cyster, and M. Matloubian. 2006. CD69 acts downstream of interferon-α/β to inhibit $S1P_1$ and lymphocyte egress from lymphoid organs. *Nature.* 440:540–544.
35. Debes, G.F., C.N. Arnold, A.J. Young, S. Krautwald, M. Lipp, J.B. Hay, and E.C. Butcher. 2005. Chemokine receptor CCR7 required for T lymphocyte exit from peripheral tissues. *Nat. Immunol.* 6:889–894.
36. Vesper, H., E.M. Schmelz, M.N. Nikolova-Karakashian, D.L. Dillehay, D.V. Lynch, and A.H. Merrill Jr. 1999. Sphingolipids in food and the emerging importance of sphingolipids to nutrition. *J. Nutr.* 129:1239–1250.
37. Duan, R.D., L. Nyberg, and A. Nilsson. 1995. Alkaline sphingomyelinase activity in rat gastrointestinal tract: distribution and characteristics. *Biochim. Biophys. Acta.* 1259:49–55.
38. Kono, M., J.L. Dreier, J.M. Ellis, M.L. Allende, D.N. Kalkofen, K.M. Sanders, J. Bielawski, A. Bielawska, Y.A. Hannun, and R.L. Proia. 2006. Neutral ceramidase encoded by the Asah2 gene is essential for the intestinal degradation of sphingolipids. *J. Biol. Chem.* 281:7324–7331.
39. Powrie, F., M.W. Leach, S. Mauze, S. Menon, L.B. Caddle, and R.L. Coffman. 1994. Inhibition of Th1 responses prevents inflammatory bowel disease in scid mice reconstituted with $CD45RB^{hi}$ $CD4^+$ T cells. *Immunity.* 1:553–562.
40. Kweon, M.N., M. Yamamoto, M. Kajiki, I. Takahashi, and H. Kiyono. 2000. Systemically derived large intestinal $CD4^+$ Th2 cells play a central role in STAT6-mediated allergic diarrhea. *J. Clin. Invest.* 106:199–206.
41. Mizushima, T., T. Ito, D. Kishi, Y. Kai, H. Tamagawa, R. Nezu, H. Kiyono, and H. Matsuda. 2004. Therapeutic effects of a new lymphocyte homing reagent FTY720 in interleukin-10 gene-deficient mice with colitis. *Inflamm. Bowel Dis.* 10:182–192.
42. Kurashima, Y., J. Kunisawa, M. Higuchi, M. Gohda, I. Ishikawa, N. Takayama, M. Shimizu, and H. Kiyono. 2007. Sphingosine 1-phosphate-mediated trafficking of pathogenic Th2 and mast cells for the control of food allergy. *J. Immunol.* 179:1577–1585.
43. Kinoshita, N., T. Hiroi, N. Ohta, S. Fukuyama, E.J. Park, and H. Kiyono. 2002. Autocrine IL-15 mediates intestinal epithelial cell death via the activation of neighboring intraepithelial NK cells. *J. Immunol.* 169:6187–6192.
44. Kunisawa, J., T. Nakanishi, I. Takahashi, A. Okudaira, Y. Tsutsumi, K. Katayama, S. Nakagawa, H. Kiyono, and T. Mayumi. 2001. Sendai virus fusion protein mediates simultaneous induction of MHC class I/II-dependent mucosal and systemic immune responses via the nasopharyngeal-associated lymphoreticular tissue immune system. *J. Immunol.* 167:1406–1412.
45. Kato, T., Y. Sato, S. Takahashi, H. Kawamura, K. Hatakeyama, and T. Abo. 2004. Involvement of natural killer T cells and granulocytes in the inflammation induced by partial hepatectomy. *J. Hepatol.* 40:285–290.
46. Penney, L., P.J. Kilshaw, and T.T. MacDonald. 1995. Regional variation in the proliferative rate and lifespan of $\alpha\beta$ TCR^+ and $\gamma\delta$ TCR^+ intraepithelial lymphocytes in the murine small intestine. *Immunology.* 86:212–218.

tapraid4/zh8-bloo/zh8-bloo/zh800907/zh80062d07g | sweigarl | S=9 | 3/22/07 | 11:53 | Art: 2006/041582 | Input-DCT-???

DOI: 10.1182/blood-2006-08-041582 Collection Codes: 12,23

IMMUNOBIOLOGY

Sphingosine 1-phosphate regulates peritoneal B-cell trafficking for subsequent intestinal IgA production

Jun Kunisawa,[1] Yosuke Kurashima,[1] Masashi Gohda,[1] Morio Higuchi,[1] Izumi Ishikawa,[1] Fumi Miura,[1] Ikuko Ogahara,[1] and Hiroshi Kiyono[1]

[1]Division of Mucosal Immunology, Department of Microbiology and Immunology, Institute of Medical Science, University of Tokyo, and Core Research for Evolutional Science and Technology (CREST), Japan Science and Technology Corporation (JST), Tokyo, Japan

Sphingosine 1-phosphate (S1P) is known to play a pivotal role in the regulation of lymphocyte emigration from organized lymphoid tissues such as the peripheral lymph nodes and thymus, but its immunologic role in unorganized and diffused tissues remains to be elucidated. Here we show that the trafficking of peritoneal B cells is principally regulated by S1P. All peritoneal B cells including B1a, B1b, and B2 B cells express comparable levels of the type 1 S1P receptor. Thus, treatment with FTY720, an S1P receptor modulator, caused the rapid disappearance of peritoneal B cells by inhibiting both their emigration from parathymic lymph nodes and their recirculation from the blood into the peritoneal cavity without affecting their progenitor populations. These changes did not affect natural plasma antibody production or phosphorylcholine (PC)–specific antibody production in serum after peritoneal immunization with heat-killed *Streptococcal pneumoniae* (R36A). However, FTY720 dramatically reduced peritoneal B cell-derived natural intestinal secretory IgA production without affecting the expression of J-chain and polyimmunoglobulin receptors. Additionally, FTY720 impaired the generation of PC-specific fecal IgA responses after oral immunization with R36A. These findings point to a pivotal role for S1P in connecting peritoneal B cells with intestinal B-cell immunity. (Blood. 2007;109:3749-3756)

Introduction

Sphingosine 1-phosphate (S1P) has been identified as an important molecule in the regulation of lymphocyte egress from the organized lymphoid structures including the thymus and secondary lymphoid organs.[1,2] At present, 5 kinds of S1P receptors have been identified, each sharing S1P as its ligand but associating with a different type of G protein, resulting in a distinct signal transduction.[3] Accumulating evidence has demonstrated that type 1 S1P receptor (S1P1) is preferentially expressed on lymphocytes, and their expression is closely regulated by lymphocyte activation or development, which determines lymphocyte emigration from secondary lymph organs as well as the thymus.[4,5] FTY720, 2-amino-2-[2-(4-octylphenyl)-ethyl] propane-1,3-diol hydrochloride, acts as an agonist for S1P receptors, except the type 2 S1P receptor (S1P2).[6-8] FTY720 blocks S1P-mediated signaling by inducing internalization of receptors.[4,9] Therefore, treatment with FTY720 decreased the number of circulating lymphocytes in both blood and lymph, by inhibiting their emigration from the secondary lymphoid organs and thymus and by modulating integrin-dependent lymphocyte homing into peripheral lymph nodes.[10-12]

Several lines of evidence have revealed that S1P also regulates B-cell distribution in the spleen, suggesting that FTY720 can impair plasma antibody production, especially against T-dependent (TD) antigen due to the abolishment of germinal center formation.[13-15] In addition, a recent study revealed that S1P plays an important role in the determination of plasma cell tropism to bone marrow.[16] Despite the substantial evidence pointing to the role of S1P in the regulation of lymphocyte trafficking at the systemic immune compartments, it still remains unclear whether S1P is also involved in lymphocyte trafficking and immune responses in the mucosa-associated unorganized and diffused tissues, such as the intestinal lamina propria and the peritoneal cavity (PerC). The PerC contains numerous B cells, especially B1 B cells that can be distinguished by cell surface markers (eg, B220, IgM, IgD, CD5, and Mac-1) from conventional B2 B cells.[17-19] B1 B cells are thought to play an important role in the protective immunity in PerC by producing antibodies in response to T-independent (TI) antigen, such as phosphorylcholine (PC), a haptenlike antigen associated with many pathogenic bacteria.[20,21] In addition to playing a role in peritoneal immunity, B cells have been shown to be a source of IgA for the formation of secretory IgA antibody (S-IgA) in the intestine.[22-24] S-IgA, a hallmark antibody principally produced at mucosal sites, plays an important role in the creation of immunologic surveillance and homeostasis at mucosa by abolishing pathogenic microbial infections and establishing symbiosis with commensal flora.[25,26] One major source of S-IgA is B2 B cells derived from the common mucosal immune system (CMIS) that links inductive (eg, Peyer patches and isolated lymphoid follicles) and effector tissues (eg, lamina propria region). These B2 B cells have been shown to play a key role in the induction of TD antigen-specific S-IgA.[27,28] In addition to the B cells belonging to the CMIS, peritoneal B cells have also been identified as another source of S-IgA, especially specific for the TI antigen.[23,24] Accumulating evidence suggests that peritoneal B-cell trafficking is biologically regulated, in some cases by chemokines (eg, CXCL12 and CXCL13) and cytokines (eg, IL-10),[29-32] but there is not currently

Submitted August 22, 2006; accepted January 2, 2007. Prepublished online as *Blood* First Edition Paper, January 18, 2007; DOI 10.1182/blood-2006-08-041582.

The publication costs of this article were defrayed in part by page charge payment. Therefore, and solely to indicate this fact, this article is hereby marked "advertisement" in accordance with 18 USC section 1734.

enough information to fully understand the involvement of obvious cell trafficking molecules such as S1P in the pathway.

This study first sought to investigate the role of S1P in the regulation of peritoneal B-cell trafficking and then to assess the influence of the S1P-mediated pathway on the production of serum and intestinal antibody production from peritoneal B cells. Our current findings provide new evidence that S1P regulates peritoneal B-cell trafficking and subsequent S-IgA production in the intestine.

Materials and methods

Mice and FTY720 treatment

Female Balb/c and ICR SCID mice (7-9 weeks) were purchased from Japan Clea (Tokyo, Japan). All mice were maintained in horizontal lamina flow cabinets and provided with sterile food and water ad libitum. Mice were injected intraperitoneally with 1 mg/kg/time of FTY720 (Novartis Pharma, Basel, Switzerland).[12] All animals were maintained and experiments were conducted in accordance with the guidelines of the Animal Care and Use Committee of the University of Tokyo.

Cell isolation

PerC cells were obtained by flushing the peritoneum with 8 mL ice-cold PBS.[33] Lymphocytes and epithelial cells were isolated from the small intestine by the enzymatic dissociation procedure using collagenase IV (Nitta Gelatin, Osaka, Japan), as previously described.[34] Lymphocytes were collected from the omentum, parathymic lymph nodes, and blood in accordance with a previously established protocol.[29,35,36]

Flow cytometry and cell sorting

A standard protocol, previously described, was used for flow cytometric analysis and cell sorting.[37,38] Cells were first incubated with anti-CD16/32 antibody and then stained with the appropriate fluorescent-conjugated antibodies specific for CD5, CD11b, B220, IgA, and IgM (BD PharMingen, San Diego, CA). Viaprobe (BD PharMingen) was used to discriminate between dead and live cells. Flow cytometric analysis and cell sorting were performed using FACSCalibur and FACSAria (BD Biosciences, Franklin Lakes, NJ), respectively.

Quantitative and conventional RT-PCR

To measure mRNA expression for S1P receptors, quantitative reverse transcription-polymerase chain reaction (RT-PCR) using LightCycler (Roche Diagnostics, Mannheim, Germany) was performed.[37] Briefly, total RNA was collected using a TRIzol reagent (Invitrogen, Carlsbad, CA) and cDNA was synthesized using Powerscript reverse transcriptase (BD Biosciences). The oligonucleotide primers and probes specific for S1P1 (forward primer, TACACTCTGACCAACAAGGA; reverse primer, ATAATGGTCTCTGGGTTGTC; FITC-probe, TGCTGGCAATTCAAGAGGCCCATCATC; LCRed 640-probe, CAGGCATGGAATTTAGCCGCAGCAAATC), S1P2 (forward primer, CATCGTACTGGGTGTTTTC; reverse primer, CCACGTATAGATGACAGGA; FITC-probe, AATAGTGGGCTTTGTAGAGGACAGGGCAGG; LCRed 640-probe, CCGAACGGGACAGGTGGAGTCTAAGAGAAG), S1P3 (forward primer, TCCTCTTCCTCATCGACGTG; reverse primer, CCTTGCCCTTGACTAGACAG; FITC-probe, TTCATCATGCTGGCTGTCCTCAACTCGG; LCRed 640-probe, CATGAACCCTGTCATCTACACGCTGGCC), S1P4 (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, TTCAACAGCAACTCCCACTCTTCCACC) were designed and synthesized by Nihon Gene Research Laboratory (Sendai, Japan). Conventional RT-PCR was performed to measure pIgR and J-chain, using specific primers (pIgR forward, AGTATTCAGGCAGAGCCAAC; pIgR reverse, ATTCATCCGGCACAGATATT; J-chain forward, ATGAAGACCCACCTGCTTCTCTGG; J-chain reverse, AGGGTAGCAAGAATCGGGGGTCAA).

Adoptive cell transfer

For tracing cells in vivo, peritoneal cells (1×10^7 cells) were incubated with 0.25 μM CFSE (Molecular Probes, Eugene, OR) in the dark for 10 minutes at 37°C, and then washed with PBS twice in accordance with a previously described method.[39] The labeled cells were transferred into severe combined immunodeficient (SCID) mice intraperitoneally (4×10^6 cells) or intravenously (1×10^7 cells) and FTY720 was simultaneously administered intraperitoneally. After 12 hours, peritoneal cells were collected for fluorescence-activated cell sorting (FACS) analysis.

For the analysis of antibody production from peritoneal B cells, SCID mice were adoptively transferred with normal peritoneal B cells (5×10^6 cells) via intraperitoneal route and treated with FTY720 every 2 days. As described under "Detection of total immunoglobulin and PC-specific antibody levels in serum and fecal extract by ELISA," 2 weeks after the adoptive transfer, we simultaneously collected serum and fecal extracts for the measurement of total immunoglobulin levels by enzyme-linked immunosorbent assay (ELISA) and isolated mononuclear cells from the intestinal lamina propria for enumeration of antibody-forming cells (AFCs) by enzyme-linked immunospot assay (ELISPOT).

Immunization

Mice were immunized intraperitoneally with 10^7 heat-killed pepsin-treated *Streptococcal pneumoniae* strain R36A (gift of Dr John Kearney, University of Alabama, Birmingham, AL).[20,29] For immunization, mice were orally immunized with 2×10^8 heat-killed pepsin-treated *S pneumoniae* strain R36A together with 10 μg mucosal adjuvant cholera toxin (List Biological Laboratories, Campbell, CA).[40] In the FTY720-treated group, mice were injected intraperitoneally with FTY720 6 hours before the immunization and then again once per day during the experiment. Serum and fecal extracts were prepared for the analysis of PC-specific IgM and IgA production by ELISA, as described in the next section.[34,38]

Detection of total immunoglobulin and PC-specific antibody levels in serum and fecal extract by ELISA

Total immunoglobulin levels in serum and fecal extracts were determined by ELISA as previously described.[37] To measure antibody concentration, purified murine isotype-specific antibodies (BD PharMingen) were used as standards for the quantification. For the detection of PC-specific antibodies, microtiter plates were coated with 5 μg/mL of PC-BSA (Biosearch Technologies, Novato, CA) in bicarbonate buffer (pH 9.6).[29] Following blocking with 5% BSA in PBS, diluted serum or fecal extracts were added and incubated in the coated wells for 2 hours at room temperature. Bound antibodies were then determined using HRP-conjugated anti-mouse IgM or IgA (Southern Biotechnology, Birmingham, AL) and 3,3′,5,5′-tetramethylbenzidine (Moss, Pasadena, CA), as previously described.[29,38]

Enumeration of AFCs by ELISPOT

To measure IgM- or IgA-producing AFCs in the intestinal lamina propria, an ELISPOT assay was used as previously described.[37] Briefly, various concentrations of mononuclear cells were cultured in 96-well nitrocellulose membrane plates (Millititer HA; Millipore, Bedford, MA) coated with 5 μg/mL affinity-purified goat anti-immunoglobulin (Southern Biotechnology) at 37°C for 4 hours. After vigorous washing with PBS and PBS containing 0.05% Tween 20, HRP-conjugated antibodies specific for mouse IgM or IgA (Southern Biotechnology) were added and incubated overnight. The spots of AFCs were developed using 2-amino-9-ethylcarbazole (Polysciences, Warrington, PA) containing hydrogen peroxide.

Statistics

The results were compared using a Student t test or a Welch t test. $P < .05$ was considered statistically significant.

tapraid4/zh8-bloo/zh8-bloo/zh800907/zh80062d07g | sweigarl | S=9 | 3/22/07 | 11:53 | Art: 2006/041582 | Input-DCT-???

Results

Rapid and reversible disappearance of peritoneal B cell by FTY720 treatment

The initial aim of this study was to examine the involvement of S1P in the regulation of peritoneal B-cell trafficking. To accomplish this, we intraperitoneally administered FTY720, a modulator for S1P receptors, and examined cellular population by flow cytometry. The analysis based on forward (FSC) and side scatter (SSC) revealed that FTY720 treatment resulted in the dramatic changes in cellular population with different size and intracellular structure (Figure 1A, top panels). The profile indicated that intraperitoneal administration of FTY720 simultaneously reduced the number of lymphoid cells and induced a remarkable accumulation of granulocytes or monocytes or both (Figure 1A, top panels). Because PerC cells are known to contain both B1 and B2 B cells, we next used the expression of B220 and CD11b to confirm that numbers of both $B220^+CD11b^+$ B1 and $B220^+CD11b^-$ B2 B cells were dramatically reduced (Figure 1A, bottom panels). Because the total PerC cell number remained unchanged even after FTY720 treatment (data not shown), the changes in the cellular percentage can be assumed to directly reflect the absolute cell number of each population (Figure 1B). In short, FTY720 treatment significantly decreased B1 and B2 B-cell numbers (Figure 1B).

We next sought to analyze the kinetics of the change and recovery of peritoneal B cells after treatment with FTY720. Marked reductions in both B1 and B2 B-cell levels were found only 3 hours after FTY720 treatment (Figure 1C), and a partial recovery was detected 24 hours after the injection, with full recovery observed 7 days after the administration (Figure 1D). These data suggest that the effect of FTY720 on peritoneal B cells is both rapid and reversible.

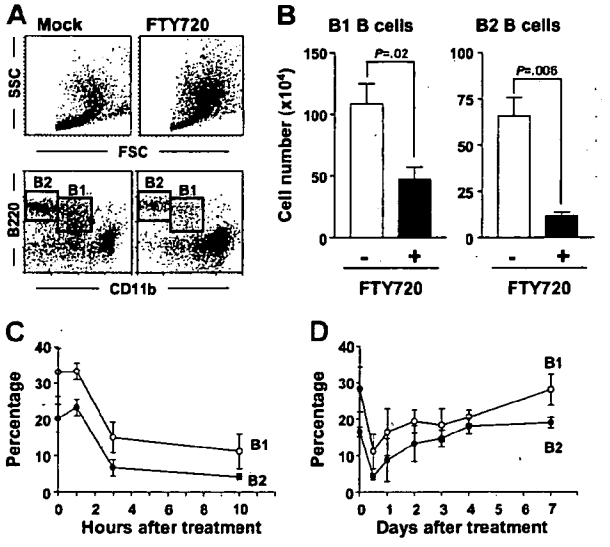


Figure 1. Rapid but reversible disappearance of peritoneal B cells induced by FTY720. (A) PerC cells were isolated 10 hours after injection of FTY720 (right) or mock (left), and cell populations were analyzed using flow cytometry. The data are representative of 5 independent experiments. (B) Cell numbers 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-D) At each time point after FTY720 injection, PerC cells were analyzed by flow cytometry (○, $B220^+CD11b^+$ B1 B cells; ●, $B220^+CD11b^-$ B2 B cells). The data represent the mean ± SD (n = 4).

Specific and equal expression of S1P1-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 S1P1 with no or only a dim expression of the other subtype of S1P receptors (S1P2-S1P4) (Figure 2A). Because B1 B cells are further divided into B1a and B1b B cells based on CD5 expression,[17-19] we then compared the S1P1 expression of B1a and B1b B cells, finding that the level of S1P1-specific mRNA expression was similar both cell groups (Figure 2B). Consistent with these observations, flow cytometric analysis revealed that FTY720 treatment resulted in roughly equivalent reductions in $B220^+CD11b^+CD5^+$ B1a, $B220^+CD11b^+CD5^-$B1b, and $B220^+CD11b^-CD5^-$B2 B cells, indicating that the S1P-mediated pathway exerted the same degree of influence on the various types of peritoneal B cells (Figure 2C).

No influence of FTY720 on the differentiation and viability of peritoneal B cells

Because it has been previously reported that peritoneal B1 B cells might differentiate into monocytes such as macrophage-like cells,[41] and that high concentrations of FTY720 induced lymphocyte apoptosis,[42] we tested whether FTY720 induced differentiation or apoptosis of PerC cells. To address this issue, we performed in vitro culture of PerC cells with various concentrations of FTY720 or S1P. The cellular population of PerC cells such as B1 B cells, B2 B cells, and $B220^-CD11b^+$ cells (eg, macrophages) remained unaltered after 3 days of culture with biologic concentrations (1-1000 nM) of FTY720 and S1P (Figure 3A; results at 100 nM were shown). In addition, flow cytometric analysis using annexin V revealed comparable numbers of apoptotic cells in untreated and treated groups (data not shown). Together, these results indicate that FTY720 affected neither differentiation nor apoptosis of PerC cells under these experimental conditions.

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, or by inhibiting their migration into the PerC, or both, we isolated peritoneal B cells from normal mice, labeled them with 5- (and -6)–carboxyfluorescein diacetate succinimidyl ester (CFSE), and adoptively transferred them into the PerC of SCID mice and compared their emigration in animals receiving FTY720 treatment and in those that did not. We found that FTY720 treatment significantly decreased the numbers of peritoneal $CFSE^+$ cells in the PerC of treated mice (Figure 4A). We then addressed whether the transferred B cells were present in the circulation or migrated to other tissues. We barely detected $CFSE^+$ cells in the blood of FTY720-treated mice (Figure 4A). Because it has been considered that lymphocytes pass through the omentum and parathymic lymph nodes on their way to the blood from the PerC,[35,36] we 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 (Figure 4A). 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.

tapraid4/zh8-bloo/zh8-bloo/zh800907/zh80062d07g | sweigarl | S=9 | 3/22/07 | 11:53 | Art: 2006/041582 | Input-DCT-???

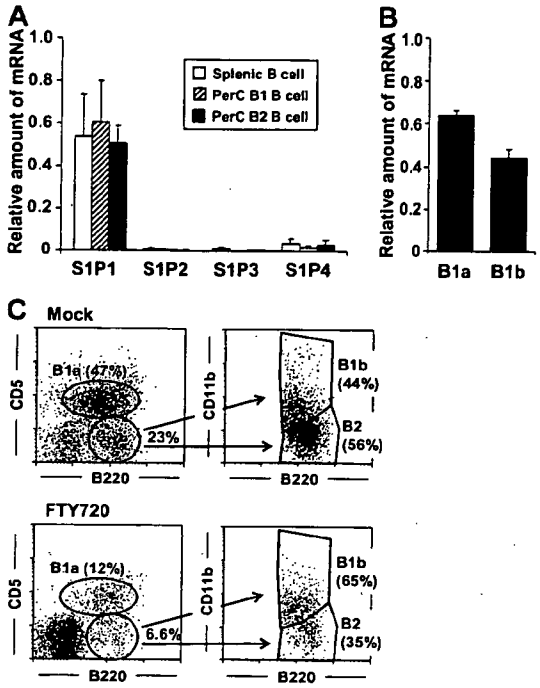


Figure 2. Equal expression of S1P1 by peritoneal B1 and B2 B cells. (A) Quantitative RT-PCR analysis for S1P receptors was performed using RNA isolated from sorted splenic B (□), peritoneal B1 (▨), and B2 (■) B cells. The relative quantity of specific mRNA was expressed as a ratio to GAPDH. The data are expressed as mean ± SD from 4 mice. (B) S1P1 expression in B1a and B1b was determined by quantitative RT-PCR analysis. (C) Flow cytometric analysis was performed to characterize B1a, B1b, and B2 B cells in the PerC of mice treated with FTY720. The data are representative of 3 independent experiments.

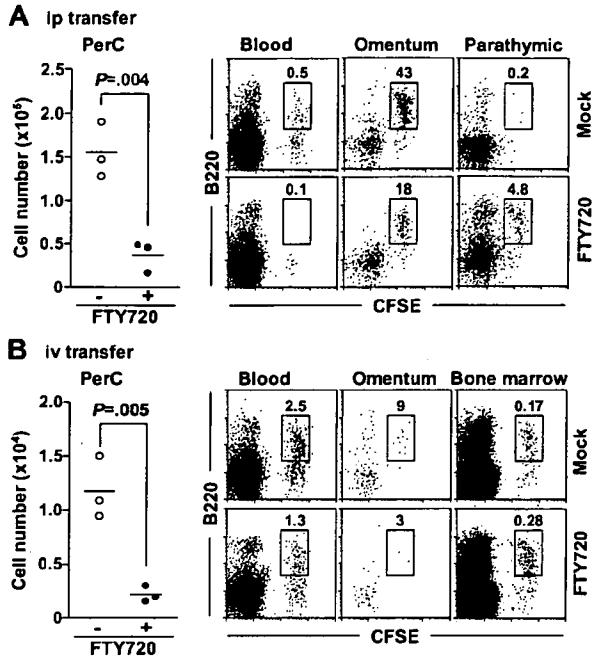


Figure 4. FTY720 simultaneously inhibits their emigration from the parathymic lymph nodes and their entrance from the blood into the PerC. SCID mice were adoptively transferred with CFSE-labeled normal PerC B cells via the intraperitoneal (A) or the intravenous (B) route, and simultaneously treated with (● or bottom panels) or without (○ or top panels) FTY720. After 12 hours, cells were isolated from the PerC, blood, omentum, parathymic lymph nodes, and bone marrow for the analysis of CFSE⁺ cells. Horizontal bars represent the mean.

Under similar experiments, we 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.[29] We 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 (Figure 4B). In these mice, $CFSE^+$ cell numbers were reduced in the blood, omentum, and parathymic lymph nodes but increased in the bone marrow (Figure 4B 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 inhibiting their emigration from the parathymic lymph nodes and by changing the tropism of circulating B cells to bone marrow.

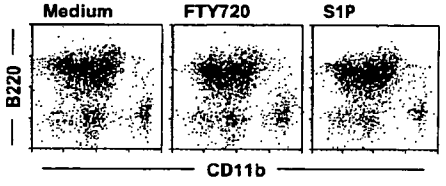


Figure 3. FTY720 does not affect peritoneal B-cell differentiation and viability. PerC cells were cultured with 100 nM FTY720 or S1P for 3 days. Cell populations were examined by flow cytometry using antibodies specific for B220 and CD11b. Results were reproducible, with very similar data obtained from each of 3 independent experiments.

No influence of FTY720 treatment on B1 B-cell progenitors in the PerC

Having established that the effects of FTY720 on peritoneal B cells were rapid and reversible (Figure 1), we next sought to determine whether FTY720 influenced peritoneal B-cell development. To address this issue, we investigated the B1 B-cell progenitor population ($CD11b^-IgM^-B220^{low/neg}CD19^+$) recently identified in the bone marrow.[43] In this study, we found the presence of $CD11b^-CD11c^-IgM^-B220^{low/neg}CD19^+$ cells in the PerC at rates of 2.5×10^3 cells/mouse (0.1% in total peritoneal cells; Figure 5A-B). Treatment with FTY720 did not appreciably affect progenitor numbers in either the PerC (Figure 5A-B) or the bone marrow (data not shown). F5

Comparable serum antibody production under natural conditions and after immunization with bacterial antigen

In the next experiment, we set out to determine whether the FTY720-induced disappearance of peritoneal B cells exerted any influence on systemic and mucosal antibody production. Because peritoneal B cells are well characterized as a source of natural antibody production,[17-19] we 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, whereas serum IgA production decreased partially in mice treated with FTY720 (Figure 6A). F6 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 (Figure 6A). These data indicate that FTY720 induces B1 B-cell alteration in the PerC but does not affect the generation of natural serum antibody production.

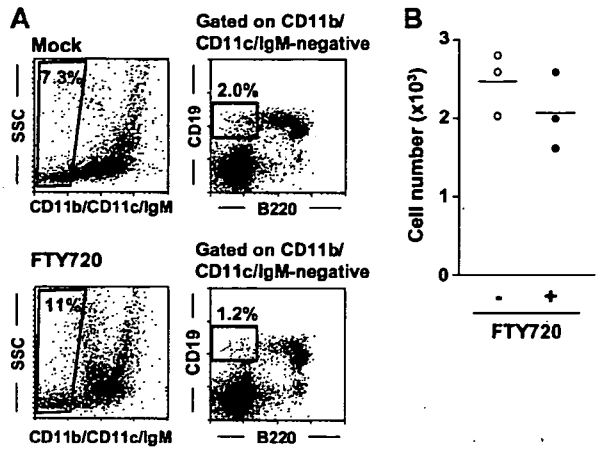


Figure 5. No influence of FTY720 treatment on peritoneal B1 B-cell progenitor. (A) B1 B-cell progenitors were determined as $CD11b^{-}CD11c^{-}IgM^{-}B220^{int\text{-}neg}CD19^{+}$ cells. These cells were isolated from the PerC 6 hours after mock (top panels) or FTY720 (bottom panels) treatment. Data are representative of 3 independent experiments. (B) The number of peritoneal $CD11b^{-}CD11c^{-}$ $IgM^{-}B220^{int\text{-}neg}CD19^{+}$ cells was calculated by using the total cell number and the flow cytometric data. Horizontal bars represent the mean.

To investigate the effects of FTY720 on the induction of bacterial antigen-specific antibody production, we used 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.[20,29] Accordingly, we intraperitoneally immunized mock- or FTY720-pretreated mice with R36A, a heat-killed, pepsin-treated *S pneumoniae* strain[20,29] and continued to treat every day for 5 days with mock or FTY720, respectively. Although repeated treatment with FTY720 was 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 (Figure 6B). 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.

Reduction of intestinal IgA production by treatment with FTY720

Finally, we investigated whether the alteration of peritoneal B-cell trafficking affected intestinal antibody production since peritoneal B cells are thought to migrate into intestinal lamina propria and contribute to subsequent IgA production.[22-24] When peritoneal B cells were adoptively transferred into SCID mice, considerable IgA and IgM production was noted in the feces (Figure 7A) and consistent with such antibody production, IgA or IgM AFCs were detected in the lamina propria region (Figure 7B). 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, whereas fecal IgM production was comparable to that seen in the mock-treated mice (Figure 7A). ELISPOT analysis confirmed this finding, demonstrating that IgA AFCs levels were significantly reduced in the intestinal lamina propria of mice treated with FTY720, whereas IgM AFCs levels were to mock-treated group (Figure 7B). Additionally, RT-PCR analysis revealed that mock- and FTY720-treated mice expressed identical polyimmunoglobulin receptors (pIgRs) in intestinal epithelial cells and J-chain in intestinal B cells. 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 (Figures 7C-D). F7

We next examined the contribution, if any, made to S1P-dependent intestinal IgA production by B1 and B2 B cells. To address this issue, we 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 (Figure 7E). As might be expected from the observed selective reduction in intestinal IgA production after FTY720 treatment (Figure 7A-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 (Figure 7E). These findings were further confirmed by showing that FTY720 treatment reduced both B1- and B2-derived fecal IgA production (data not shown). Additionally, PC-specific fecal IgA production was impaired when mice received FTY720 after oral immunization with R36A (Figure 7F). 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.

Discussion

S1P is principally produced by platelets during platelet activation and thrombotic processes.[44] S1P concentrations in serum are stably maintained (100-300 nM concentration) by binding with serum protein (eg, albumin) and enzymatic degradation.[45-47] However,

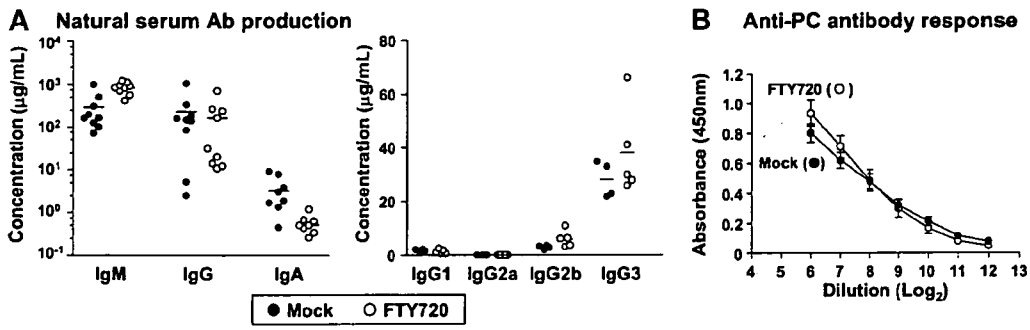


Figure 6. Effects of FTY720 on serum antibody production. (A) SCID mice were adoptively transferred with 5×10^6 normal PerC B cells and were treated with mock (●) or FTY720 (○) every 2 days. Two weeks after the transfer, serum was collected for the measurement of total immunoglobulin levels by ELISA. Horizontal bars represent the mean. (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.

tapraid4/zh8-bloo/zh8-bloo/zh800907/zh80062d07g | sweigarl | S=9 | 3/22/07 | 11:53 | Art: 2006/041582 | Input-DCT-???

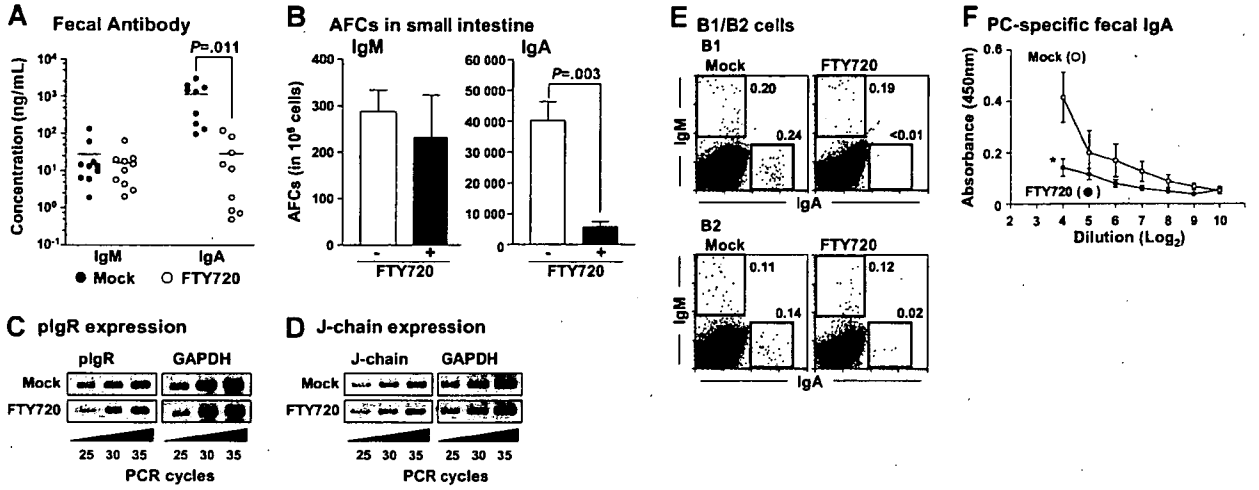


Figure 7. Impaired fecal IgA production after treatment with FTY720. (A) Fecal extracts were collected from the reconstituted SCID mice and analyzed for immunoglobulin production by ELISA as described in Figure 6A. Horizontal bars represent the mean. (B) Similarly, mononuclear cells were isolated from small intestinal lamina propria and used for the ELISPOT assay. The error bars are ± SEM (n = 5). (C-D) pIgR expression in epithelial cells (C) and J-chain expression in lamina propria lymphocytes (D) were examined by RT-PCR. Data are representative of 3 independent experiments. (E) SCID mice were adoptively transferred with purified peritoneal B1 or B2 cells and treated with FTY720 as described in Figure 6A. FACS analysis was performed to detect IgA^+ and IgM^+ cells in the intestinal lamina propria of mice treated with (right) or without (left) FTY720. Data are representative of 3 independent experiments. (F) Mice were orally immunized with R36A together with cholera toxin and treated with (●) or without (○) FTY720. After 3 days, fecal PC-specific IgA levels were measured by ELISA. The error bars are ± SEM (n = 4). $^*P < .05$.

S1P receptors are biologically regulated and so more given to fluctuation.[2,48] It has been well demonstrated that S1P1 is preferentially expressed on the lymphocytes and that its expression is altered during lymphocyte development and activation, a process that plays an important role in the regulation of lymphocyte egress from the organized lymphoid structures including the thymus and secondary lymphoid organs.[4,5] However, its immunologic role in the regulation of lymphocyte trafficking at nonorganized tissues such as the PerC and the intestinal lamina propria region remains less than fully understood. In this study, we demonstrated that the interaction between S1P and its receptor (S1P1) also regulated peritoneal B-cell trafficking, both inbound and outbound. Despite their immunologic differences,[18,21,49] B1a, B1b, and B2 B cells in the PerC expressed similar levels of S1P1 expression and exhibited a comparable dependency on S1P (Figures 1-2). These comparable involvements of S1P-mediated pathway were different from chemokine-mediate pathway. For instance, CXCL13/CXCR5- or CCR7-mediated pathways showed different regulation ability against peritoneal B1 and B2 B cell.[29,30] The difference might instead be explained by the previously demonstrated hierarchy that exists between S1P and chemokines.[13] The study showed that S1P signaling overcame the recruiting activity of CXCL13 in the regulation of marginal zone B-cell localization.[13] Because CXCL13 has been reported to play the major role in the B-cell retention in the PerC,[29] it is likely that S1P simultaneously overcomes the CXCL13-mediated retention of peritoneal B cells and enhances the emigration of B cells out of the peritoneal cavity.

In addition, adoptive transfer experiments also revealed that FTY720 removed peritoneal B cells via at least 2 distinct pathways (Figure 4): (1) inhibition of their egress out of the parathymic lymph nodes on their way to the blood circulation from the PerC and (2) their migration from the blood into the PerC. The first observation accords well with current thinking that lymphocytes can traffic from the peritoneal cavity to the blood through omentum and parathymic lymph nodes[35,36] and that cells accumulate in the lymph nodes after FTY720 treatment.[10-12] Although it has been demonstrated that FTY720 treatment induces an accumulation of naïve B cell in the bone marrow,[10] we demonstrate here for the first time that S1P also regulates the immigration of mature B cells from the blood circulation into the PerC. These results lend support to the recent contention by Butcher's group that lymphocyte egress from structurally nonlymphoid tissues (eg, skin) is not random, but biologically regulated and that CCR7-mediated signaling plays an important role in that regulation.[39] Our current findings point to S1P as another key molecule regulating lymphocyte trafficking in unorganized and diffused tissues (eg, the PerC).

Although the precise developmental pathway for peritoneal B cells remains controversial, it is generally thought that peritoneal B1 B cells originate from fetal liver,[50] fetal omentum,[51] and para-aortic splanchnopleura,[52] whereas B2 cells are preferentially generated from bone marrow.[53] In addition, a recent study has identified B1 B progenitor cells in bone marrow.[43] These progenitor cells are characterized as Lin^- $CD19^+$ $B220^{low\text{-}neg}$ and have the ability to differentiate into either peritoneal B1a or B1b cells.[43] In this study, we found similar $CD11b^-CD11c^-IgM^-CD19^+$-$B220^{low\text{-}neg}$ cells in the PerC (Figure 5). Although it is still unclear whether peritoneal $CD11b^-CD11c^-IgM^-$ $CD19^+B220^{low\text{-}neg}$ cells are derived from the bone marrow or from other sites (eg, fetal liver), it is interesting to note that FTY720 removed a marked number of peritoneal $B220^+$ B cells, but did not affect $CD11b^-CD11c^-IgM^-CD19^+B220^{low\text{-}neg}$ cell numbers (Figure 5). Together with a previous study demonstrating that S1P1 expression is up-regulated during T-cell development in the thymus,[4] our current results raise the possibility that S1P1 expression is similarly up-regulated during B-cell development in PerC, perhaps accounting for the fact that FTY720 affects $B220^+$ PerC cells, but not $CD11b^-CD11c^-$ $IgM^-CD19^+B220^{low\text{-}neg}$ cells (Figures 1-2 and 5).

FTY720 treatment diminishes germinal center formation and so drastically curtails antibody production against the TD antigen, but it does not affect antibody production against TI antigen.[14,15] Consistent with these reports, FTY720 treatment of SCID mice adoptively transferred with normal PerC cells did not influence the natural antibody production in serum. Thus, comparable levels of total plasma IgG production, mainly IgG2b and IgG3, were detected in mock- and FTY720-treated mice (Figure 6A). Since we transferred PerC cells into SCID mice lacking functional T cells,

the preferential production of IgG2b and IgG3, well-known subclasses dominantly reactive to TI antigen, was expected. We also found that FTY720 did not affect anti-PC IgM production after immunization of normal mice with heat-killed, pepsin-treated *S pneumoniae* strain, R36A (Figure 6B). This observation was consistent with previous reports demonstrating that FTY720 did not influence antibody production against soluble TI antigen (eg, TNP-Ficoll and NP-Ficoll)[14,15] and further suggested that FTY720-mediated alteration of peritoneal B-cell distribution did not affect antibody production against TI antigen regardless of antigen form (eg, soluble or particulate). However, this observation contradicted a previous report demonstrating that peritoneal B-cell paucity in CXCL13-deficient mice resulted in the impaired antibody responses against intraperitoneal immunization of *S pneumoniae*.[29] A variety of scenarios could account for this discrepancy. Our findings showed that the peritoneal B1 and B2 B cells never completely disappeared, even after repeated administration of FTY720 (Figures 1-2 and data not shown). Those remaining cells are nonreactive to FTY720 and might contribute to antibody production against *S pneumoniae*. Using chemokine receptor expression (CXCR5 and CCR7) to distinguish among B-cell populations and to identify the nonreactive population in the PerC, we compared the effect of FTY720 on the cells types and did not find any differences (data not shown). In addition, based on the recent report that CD69 induced the internalization of S1P1 and negatively regulated S1P-mediated signaling,[54] we sought to examine the CD69 expression of peritoneal B cells, demonstrating that peritoneal B cells were exclusively CD69 negative (data not shown). Similarly, no difference was detected in surface immunoglobulin expression (data not shown). Alternatively, though a small dose of bacterial antigen (10^7) was intraperitoneally administered, intact or processed bacterial antigen could be carried into other immunologic sites (eg, spleen, intestine, and bone marrow) by peritoneal macrophages or dendritic cells and activated B cells for the production of PC-specific IgM.

Although the serum antibody production originating peritoneal B cells was unaltered in FTY720-treated mice, peritoneal B1 and B2 B cell–derived intestinal S-IgA production and IgA AFCs in the intestinal lamina propria were markedly reduced after FTY720 treatment and so shown to be under the regulation of S1P. S-IgA production was significantly reduced (150 times less than mock-treated mice), but IgM levels remained largely unaffected (Figure 7A-B). However, FTY720 treatment did not affect pIgR and J-chain expression (Figure 7C-D). Again, several scenarios could account for these findings. First, since previous studies proposed the mutual interaction between S1P- and chemokine-mediated pathway in the lymphocyte trafficking,[13,16,55-57] the cooperative pathway mediated by both S1P and chemokine may determine the selective effects of FTY720 on IgA^+ B cells. These selective effects could be mediated by the differing degree of dependency by IgM^+ B cells and IgA^+ B cells on S1P for the migration of peritoneal B cells into the intestine. In this regard, it was reported that CCR10 expression was prevalent on IgA^+ B cells with a plasmablast and plasma cell phenotype in the blood and the intestine but negligible on IgA^- B cells.[58] We found that intraperitoneally transferred $CFSE^+$ B cells were barely detected in the blood but some $CFSE^+$ B cells were still present in the omentum, which adjoins the gastrointestinal compartment (Figure 4A).[35,36] This finding led us to consider a second scenario, namely, that peritoneal B cells have a unique S1P-independent trafficking pathway from the PerC to the intestine through the omentum. Yet a third possibility is that FTY720 inhibited class switch recombination from IgM to IgA and thereby the subsequent differentiation into IgA plasma cells. Similar phenomena were observed in mice lacking activation-induced cytidine deaminase (AID), an essential molecule for class switch recombination.[59] A previous study demonstrating that sphingosine inhibited IL-5–induced IgA synthesis in LPS-stimulated murine B cells lends credibility to a fourth possibility: that FTY720 induces down-regulation of IgA production.[60] These possibilities may similarly account for the discrepancy that FTY720 reduced PC-specific fecal IgA production but not PC-specific serum IgM after immunization with R36A (Figures 5B and 7F). We are currently engaged in studies to clarify this issue.

In summary, we have demonstrated that S1P plays an important role in the regulation of lymphocyte trafficking not only in the organized lymphoid tissues, but also in the unorganized and diffused tissues. It also plays a pivotal role in linking peritoneal B cells to intestinal IgA production. Collectively, our findings point to a novel immunologic significance for S1P in CMIS-independent mucosal immunity.

Acknowledgments

This work was supported by grants from the Core Research for Evolutional Science and Technology (CREST) of the Japan Science and Technology Corporation (JST); the Ministry of Education, Science, Sports, and Culture; and the Ministry of Health and Welfare in Japan.

We thank Dr J. Kearney (University of Alabama, Birmingham) for reagents and helpful discussion. We also appreciate Dr K. McGhee for editorial help and Novartis Pharma for providing FTY720.

Authorship

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

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

Correspondence: Hiroshi Kiyono, Division of Mucosal Immunology, Department of Microbiology and Immunology, Institute of Medical Science, University of Tokyo, 4-6-1 Shirokanedai, Minato-ku, Tokyo 108-8639, Japan; e-mail: kiyono@ims.u-tokyo.ac.jp.

References

1. Cyster JG. Chemokines, sphingosine-1-phosphate, and cell migration in secondary lymphoid organs. Annu Rev Immunol. 2005;23:127-159.
2. Rosen H, Goetzl EJ. Sphingosine 1-phosphate and its receptors: an autocrine and paracrine network. Nat Rev Immunol. 2005;5:560-570.
3. Sanchez T, Hla T. Structural and functional characteristics of S1P receptors. J Cell Biochem. 2004;92:913-922.
4. Matloubian M, Lo CG, Cinamon G, et al. Lymphocyte egress from thymus and peripheral lymphoid organs is dependent on S1P receptor 1. Nature. 2004;427:355-360.
5. Graeler M, Goetzl EJ. Activation-regulated expression and chemotactic function of sphingosine 1-phosphate receptors in mouse splenic T cells. FASEB J. 2002;16:1874-1878.
6. Chiba K. FTY720, a new class of immunomodulator, inhibits lymphocyte egress from secondary lymphoid tissues and thymus by agonistic activity at sphingosine 1-phosphate receptors. Pharmacol Ther. 2005;108:308-319.
7. Mandala S, Hajdu R, Bergstrom J, et al. Alteration

of lymphocyte trafficking by sphingosine-1-phosphate receptor agonists. Science. 2002;296:346-349.

8. Brinkmann V, Davis MD, Heise CE, et al. The immune modulator FTY720 targets sphingosine 1-phosphate receptors. J Biol Chem. 2002;277:21453-21457.
9. Graler MH, Goetzl EJ. The immunosuppressant FTY720 down-regulates sphingosine 1-phosphate G-protein-coupled receptors. FASEB J. 2004;18:551-553.
10. Halin C, Scimone ML, Bonasio R, et al. The S1P-analog FTY720 differentially modulates T-cell homing via HEV: T-cell-expressed S1P1 amplifies integrin activation in peripheral lymph nodes but not in Peyer patches. Blood. 2005;106:1314-1322.
11. Yagi H, Kamba R, Chiba K, et al. Immunosuppressant FTY720 inhibits thymocyte emigration. Eur J Immunol. 2000;30:1435-1444.
12. Chiba K, Yanagawa Y, Masubuchi Y, et al. FTY720, a novel immunosuppressant, induces sequestration of circulating mature lymphocytes by acceleration of lymphocyte homing in rats, I: FTY720 selectively decreases the number of circulating mature lymphocytes by acceleration of lymphocyte homing. J Immunol. 1998;160:5037-5044.
13. Cinamon G, Matloubian M, Lesneski MJ, et al. Sphingosine 1-phosphate receptor 1 promotes B cell localization in the splenic marginal zone. Nat Immunol. 2004;5:713-720.
14. Han S, Zhang X, Wang G, et al. FTY720 suppresses humoral immunity by inhibiting germinal center reaction. Blood. 2004;104:4129-4133.
15. Vora KA, Nichols E, Porter G, et al. Sphingosine 1-phosphate receptor agonist FTY720-phosphate causes marginal zone B cell displacement. J Leukoc Biol. 2005;78:471-480.
16. Kabashima K, Haynes NM, Xu Y, et al. Plasma cell S1P1 expression determines secondary lymphoid organ retention versus bone marrow tropism. J Exp Med. 2006;203:2683-2690.
17. Hardy RR, Hayakawa K. B cell development pathways. Annu Rev Immunol. 2001;19:595-621.
18. Martin F, Kearney JF. B1 cells: similarities and differences with other B cell subsets. Curr Opin Immunol. 2001;13:195-201.
19. Fagarasan S, Watanabe N, Honjo T. Generation, expansion, migration and activation of mouse B1 cells. Immunol Rev. 2000;176:205-215.
20. Martin F, Oliver AM, Kearney JF. Marginal zone and B1 B cells unite in the early response against T-independent blood-borne particulate antigens. Immunity. 2001;14:617-629.
21. Alugupalli KR, Leong JM, Woodland RT, Muramatsu M, Honjo T, Gerstein RM. B1b lymphocytes confer T cell-independent long-lasting immunity. Immunity. 2004;21:379-390.
22. Husband AJ, Gowans JL. The origin and antigen-dependent distribution of IgA-containing cells in the intestine. J Exp Med. 1978;148:1146-1160.
23. Kroese FG, Butcher EC, Stall AM, Lalor PA, Adams S, Herzenberg LA. Many of the IgA producing plasma cells in murine gut are derived from self-replenishing precursors in the peritoneal cavity. Int Immunol. 1989;1:75-84.
24. Fagarasan S, Honjo T. Intestinal IgA synthesis: regulation of front-line body defences. Nat Rev Immunol. 2003;3:63-72.
25. Kunisawa J, McGhee J, Kiyono H. Mucosal S-IgA enhancement: development of safe and effective mucosal adjuvants and mucosal antigen delivery vehicles. In: Kaetzel, CS, ed. Mucosal Immune Defense. New York, NY: Kluwer Academic/Plenum Publishers. In press.
26. Kunisawa J, Kiyono H. A marvel of mucosal T cells and secretory antibodies for the creation of first lines of defense. Cell Mol Life Sci. 2005;62:1308-1321.
27. Kunisawa J, Fukuyama S, Kiyono H. Mucosa-associated lymphoid tissues in aerodigestive tract: their shared and divergent traits and their importance to the orchestration of mucosal immune system. Curr Mol Med. 2005;5:557-572.
28. Kiyono H, Fukuyama S. NALT- versus Peyer's-patch-mediated mucosal immunity. Nat Rev Immunol. 2004;4:699-710.
29. Ansel KM, Harris RB, Cyster JG. CXCL13 is required for B1 cell homing, natural antibody production, and body cavity immunity. Immunity. 2002;16:67-76.
30. Hopken UE, Achtman AH, Kruger K, Lipp M. Distinct and overlapping roles of CXCR5 and CCR7 in B-1 cell homing and early immunity against bacterial pathogens. J Leukoc Biol. 2004;76:709-718.
31. Balabanian K, Foussat A, Bouchet-Delbos L, et al. Interleukin-10 modulates the sensitivity of peritoneal B lymphocytes to chemokines with opposite effects on stromal cell-derived factor-1 and B-lymphocyte chemoattractant. Blood. 2002;99:427-436.
32. Nie Y, Waite J, Brewer F, Sunshine MJ, Littman DR, Zou YR. The role of CXCR4 in maintaining peripheral B cell compartments and humoral immunity. J Exp Med. 2004;200:1145-1156.
33. Hiroi T, Yanagita M, Iijima H, et al. Deficiency of IL-5 receptor α-chain selectively influences the development of the common mucosal immune system independent IgA-producing B-1 cell in mucosa-associated tissues. J Immunol. 1999;162:821-828.
34. Kunisawa J, Takahashi I, Okudaira A, et al. Lack of antigen-specific immune responses in anti-IL-7 receptor α chain antibody-treated Peyer's patch-null mice following intestinal immunization with microencapsulated antigen. Eur J Immunol. 2002;32:2347-2355.
35. Van Vugt E, Van Rijthoven EA, Kamperdijk EW, Beelen RH. Omental milky spots in the local immune response in the peritoneal cavity of rats. Anat Rec. 1996;244:235-245.
36. Lopes Cardozo AM, Gupta A, Koppe MJ, et al. Metastatic pattern of CC531 colon carcinoma cells in the abdominal cavity: an experimental model of peritoneal carcinomatosis in rats. Eur J Surg Oncol. 2001;27:359-363.
37. Hiroi T, Yanagita M, Ohta N, Sakaue G, Kiyono H. IL-15 and IL-15 receptor selectively regulate differentiation of common mucosal immune system-independent B-1 cells for IgA responses. J Immunol. 2000;165:4329-4337.
38. Kunisawa J, Nakanishi T, Takahashi I, et al. Sendai virus fusion protein mediates simultaneous induction of MHC class I/II-dependent mucosal and systemic immune responses via the nasopharyngeal-associated lymphoreticular tissue immune system. J Immunol. 2001;167:1406-1412.
39. Debes GF, Arnold CN, Young AJ, et al. Chemokine receptor CCR7 required for T lymphocyte exit from peripheral tissues. Nat Immunol. 2005;6:889-894.
40. Hvalbye BK, Aaberge IS, Lovik M, Haneberg B. Intranasal immunization with heat-inactivated Streptococcus pneumoniae protects mice against systemic pneumococcal infection. Infect Immun. 1999;67:4320-4325.
41. Borrello MA, Phipps RP. The B/macrophage cell: an elusive link between CD5+ B lymphocytes and macrophages. Immunol Today. 1996;17:471-475.
42. Suzuki S, Li XK, Enosawa S, Shinomiya T. A new immunosuppressant, FTY720, induces bcl-2-associated apoptotic cell death in human lymphocytes. Immunology. 1996;89:518-523.
43. Montecino-Rodriguez E, Leathers H, Dorshkind K. Identification of a B-1 B cell-specified progenitor. Nat Immunol. 2006;7:293-301.
44. English D, Welch Z, Kovala AT, et al. Sphingosine 1-phosphate released from platelets during clotting accounts for the potent endothelial cell chemotactic activity of blood serum and provides a novel link between hemostasis and angiogenesis. FASEB J. 2000;14:2255-2265.
45. Murata N, Sato K, Kon J, et al. Interaction of sphingosine 1-phosphate with plasma components, including lipoproteins, regulates the lipid receptor-mediated actions. Biochem J. 2000;352(pt 3):809-815.
46. Mandala SM, Thornton R, Galve-Roperh I, et al. Molecular cloning and characterization of a lipid phosphohydrolase that degrades sphingosine-1-phosphate and induces cell death. Proc Natl Acad Sci U S A. 2000;97:7859-7864.
47. Goetzl EJ, Rosen H. Regulation of immunity by lysosphingolipids and their G protein-coupled receptors. J Clin Invest. 2004;114:1531-1537.
48. Brinkmann V, Cyster JG, Hla T. FTY720: sphingosine 1-phosphate receptor-1 in the control of lymphocyte egress and endothelial barrier function. Am J Transplant. 2004;4:1019-1025.
49. Haas KM, Poe JC, Steeber DA, Tedder TF. B-1a and B-1b cells exhibit distinct developmental requirements and have unique functional roles in innate and adaptive immunity to *S. pneumoniae*. Immunity. 2005;23:7-18.
50. Hayakawa K, Hardy RR, Herzenberg LA. Progenitors for Ly-1 B cells are distinct from progenitors for other B cells. J Exp Med. 1985;161:1554-1568.
51. Solvason N, Lehuen A, Kearney JF. An embryonic source of Ly1 but not conventional B cells. Int Immunol. 1991;3:543-550.
52. Godin IE, Garcia-Porrero JA, Coutinho A, Dieterlen-Lievre F, Marcos MA. Para-aortic splanchnopleura from early mouse embryos contains B1a cell progenitors. Nature. 1993;364:67-70.
53. Hao Z, Rajewsky K. Homeostasis of peripheral B cells in the absence of B cell influx from the bone marrow. J Exp Med. 2001;194:1151-1164.
54. Shiow LR, Rosen DB, Brdickova N, et al. CD69 acts downstream of interferon-α/β to inhibit S1P1 and lymphocyte egress from lymphoid organs. Nature. 2006;440:540-544.
55. Henning G, Ohl L, Junt T, et al. CC chemokine receptor 7-dependent and -independent pathways for lymphocyte homing: modulation by FTY720. J Exp Med. 2001;194:1875-1881.
56. Kimura T, Boehmler AM, Seitz G, et al. The sphingosine 1-phosphate receptor agonist FTY720 supports CXCR4-dependent migration and bone marrow homing of human CD34+ progenitor cells. Blood. 2004;103:4478-4486.
57. Yopp AC, Fu S, Honig SM, et al. FTY720-enhanced T cell homing is dependent on CCR2, CCR5, CCR7, and CXCR4: evidence for distinct chemokine compartments. J Immunol. 2004;173:855-865.
58. Kunkel EJ, Kim CH, Lazarus NH, et al. CCR10 expression is a common feature of circulating and mucosal epithelial tissue IgA Ab-secreting cells. J Clin Invest. 2003;111:1001-1010.
59. Muramatsu M, Kinoshita K, Fagarasan S, Yamada S, Shinkai Y, Honjo T. Class switch recombination and hypermutation require activation-induced cytidine deaminase (AID), a potential RNA editing enzyme. Cell. 2000;102:553-563.
60. Li YS, Gimond C, Revillard JP. IL-5-induced IgA synthesis by LPS-stimulated mouse B cells is prevented by protein kinase C inhibitors. Eur Cytokine Netw. 1992;3:103-107.

doi: 10.1111/j.1744-313X.2007.00658.x

A three-base-deletion polymorphism in the upstream non-coding region of human interleukin 7 (IL-7) gene could enhance levels of IL-7 expression

H. Song,* E. E. Nakayama,* S. Likanonsakul,† C. Wasi,‡ A. Iwamoto§ & T. Shioda*

Summary

Interleukin 7 (IL-7) is a key factor in the survival, development and proliferation of B and T lymphocytes. Elevation of plasma IL-7 has been reported in several lymphopenia cases such as HIV-1 patients. After patients started to receive antiretroviral drugs and their $CD4^+$ cell counts had recovered, IL-7 in plasma decreased to normal levels. There are considerable variations in the levels of plasma IL-7 as well as the rate of $CD4^+$ T-cell restoration. Although pre-treatment plasma IL-7 levels have been shown to be prognostic for the rate of post-treatment $CD4^+$ T-cell restoration, the mechanisms responsible for the variations in plasma IL-7 and rate of $CD4^+$ T-cell restoration are still completely unknown. In the study here, we searched for genetic polymorphisms that might affect levels of IL-7 gene expression. For this purpose, we used 1658-bp PCR-amplified fragments of the IL-7 gene containing 1470 bp of the upstream non-coding region obtained from 151 Japanese and 234 Thai subjects. We found two novel human genetic polymorphisms in the upstream non-coding region of the IL-7 gene. The luciferase reporter assay demonstrated that one of those polymorphisms could increase the gene expression of IL-7. We speculate that this polymorphism, a three base ATC deletion just upstream of an out-of-frame ATG codon in the upstream non-coding region of the IL-7 gene, reduces the efficiency of translation from the upstream, out-of-frame ATG, resulting in increased translation efficiency from the authentic ATG of IL-7. Although the frequency of this allele is very low, it would be interesting to analyse this polymorphism in HIV-1-infected individuals with different rates of immune reconstitution after treatment with a highly active antiretroviral therapy.

* Department of Viral Infections, Research Institute for Microbial Diseases, Osaka University, Osaka, Japan, † Bamrasnaradura Institute, Nonthaburi, Thailand, ‡ Department of Microbiology, Faculty of Medicine Siriraj Hospital, Mahidol University, Bangkok, Thailand and § Division of Infectious Diseases, Institute of Medical Science, University of Tokyo, Tokyo, Japan

Received 4 July 2006; revised 17 October 2006; accepted 26 November 2006

Correspondence: Tatsuo Shioda, Department of Viral Infections, Research Institute for Microbial Diseases, Osaka University, 3–1 Yamada-Oka, Suita, Osaka 565-0871, Japan. Tel: +81 6 6879 8346; Fax: +81 6 6879 8347; E-mail: shioda@biken.osaka-u.ac.jp

This work was supported by grants from the Human Health Foundation, the Ministry of Education, Culture, Sports, Science, and Technology, and the Ministry of Health, Labour and Welfare, Japan.
There is no conflict of interest.

Haihan Song, E-mail: hhsong@biken.osaka-u.ac.jp
Emi E. Nakayama, E-mail: emien@biken.osaka-u.ac.jp
Sirirat Likanonsakul, E-mail: siratlik@health.moph.go.th
Chantapong Wasi, E-mail: sicws@mahidol.ac.th
Aikichi Iwamoto, E-mail: aikichi@ims.u-tokyo.ac.jp

Introduction

Human interleukin 7 (IL-7) is a cytokine produced by stromal cells of the thymus and bone marrow (Wolf & Cohen, 1992; Heufler *et al.*, 1993; Sudo *et al.*, 1993) and has the capacity to induce growth of immature B lymphocytes (Namen *et al.*, 1988). Similarly, IL-7 contributes to the development, proliferation and homeostatic maintenance of T cells (Grabstein *et al.*, 1990; Plum *et al.*, 1996; Schluns *et al.*, 2000; Fry *et al.*, 2001). Human IL-7 gene located on chromosome 8q12–13, has six exons that distributed to more than 33-Kb of genomic DNA (Lupton *et al.*, 1990; Fry & Mackall, 2002). It is known that the IL-7 gene has no canonical core promoter sequence in the 5′ upstream region (Lupton *et al.*, 1990; Oshima *et al.*, 2004). Recently, it has been reported, however, that transcription start sites of the IL-7 gene are clustered within two distinct regions that are approximately 515 bp to 600 bp and 130 bp to 217 bp upstream from the translation initiation ATG codon (Oshima *et al.*, 2004). Moreover, the region −282 to −251 upstream from the initiation ATG codon contains an interferon regulatory factor element (IRF-E) and could thus up-regulate the transcription of the IL-7 gene upon stimulation with gamma interferon (IFN-γ) in human intestinal epithelial cells (Oshima *et al.*, 2004). This study also revealed the presence of several out-of-frame ATG codons with unknown function in the upstream non-coding region of the IL-7 gene (Oshima *et al.*, 2004).

With respect to HIV-1 infection, there is a reverse correlation between $CD4^+$ T-cell numbers and IL-7 plasma levels in HIV-1-infected patients (Llano *et al.*, 2001; Beq *et al.*, 2004; Kopka *et al.*, 2005). After these patients started to receive antiretroviral drugs and their $CD4^+$ T-cell counts had recovered, the elevated IL-7 in the plasma decreased to normal levels (Llano *et al.*, 2001). Furthermore, it is well known that there are considerable variations in the levels of plasma IL-7 as well as the rate of CD4 T-cell restoration after HIV-1 patients started to

receive antiretroviral drugs, and pre-treatment plasma IL-7 levels have been shown to be prognostic for the rate of post-treatment CD4 T-cell restoration (Beq *et al.*, 2004). However, knowledge of the molecular mechanisms controlling IL-7 gene expression remains very limited, and the mechanisms responsible for the variations in plasma IL-7 levels and rate of CD4 T-cell restoration among individuals are still completely unknown.

Human genetic polymorphisms have recently been shown to affect expression of the corresponding genes and to consequently modify the clinical course of several human diseases such as HIV-1 infection (Dean *et al.*, 1996; Michael *et al.*, 1997; Kostrikis *et al.*, 1998; Liu *et al.*, 1999; Nakayama *et al.*, 2000). We aimed to know the molecular mechanisms controlling variations in IL-7 gene expression among individuals. For this purpose, we searched for genetic polymorphisms that might affect levels of IL-7 gene expression in 1658-bp PCR-amplified fragments of the IL-7 gene containing 1470 bp of the upstream non-coding region, 9 bp of the first coding exon and 179 bp of the downstream intron, although there was no previous report on human genetic polymorphisms that alter the levels of IL-7 gene expression. We found two novel human genetic polymorphisms in the upstream non-coding region of the IL-7 gene, one of which could enhance IL-7 expression probably by reducing the efficiency of translation from an upstream, out-of-frame ATG that would result in diminished efficiency of translation from the downstream initiation ATG.

Materials and methods

Genotyping of IL-7 gene

Human genomic DNA was obtained from peripheral blood mononuclear cells of 52 unrelated non-HIV-1-infected and 99 HIV-1-infected Japanese, as well as 122 non-HIV-1-infected and 112 HIV-infected Thais, who provided written informed consent. Genomic regions of 1658 nucleotides containing 1470 nucleotides of the upstream non-coding region and the first exon and part of the intron of IL-7 were amplified by using the primer pair P1: 5′-TCCCTCCTCTTCCTTGTTC-3′ and P2: 5′-GGTTCAAGTGGCTATGTGC-3′. Polymerase chain reaction (PCR) was run for 40 cycles of denaturation at 94 °C for 30 s, annealing at 53 °C for 30 s and extension at 72 °C for 2 min. Fluorescence-based automated cycle sequencing of the PCR products was then carried out by an ABI 3100 using P1, P2 (mentioned previously), P3: 5′-TGCTGCATTTGGGCTGTAGA-3′, P4: 5′-TGGTTTTTCCTGCGGTGAT-3′ and P5: 5′-GGTCTGCAGGTTCAATCT-3′ as sequencing primers.

Luciferase reporter gene assays

NheI and NcoI-tagged DNA fragments, corresponding to the sequences spanning positions −632 to +3, −632 to −67 and −297 to +3 from the initiation ATG of the IL-7 gene, were inserted into the corresponding restriction enzyme cleavage sites of the pGL3-Basic Vector in order to fuse ATGs in the IL-7 gene directly to the firefly luciferase open reading frame (Promega, Madison, WI). Constructs carrying an ATC deletion at position −29 to −27 from the initiation ATG of IL-7 were generated by PCR-based in vitro mutagenesis using P6: 5′-GGCTAGCAGACGACTTGGCATCGTCC-3′ and P8: 5′-TGGACCATGGTCTGCGGGAGGCGGGCGTAGTCATGACCGC-3′ or P7: 5′-GGCTAGCAGATTGAACCTGCAGACCA-3′ and P8 (mentioned previously) as the respective primer pairs for the −632 to +3 or −297 to +3 upstream region of the IL-7 gene with ATC deletion. All constructs were verified for sequence authenticity. Four micrograms of the resultant constructs was transfected with DMRIE-C (Gibco/BRL, Gaithersburg, MD) into Jurkat ($CD4^+$ T-lymphocyte cell line) and U937 cells (monocytic cell line). Transfection efficiency was normalized by cotransfection with 0.2 μg of pRL-CMV vector, which expresses *Renilla* luciferase under the control of the cytomegalovirus immediate early promoter. When necessary, INF-γ (Peprotech, Rocky Hill, NJ) was added to the transfected cell culture at a final concentration of 50 ng mL^{-1} 5 h after transfection. The cells were harvested 40 h after transfection, and firefly and *Renilla* luciferase activities were determined according to the manufacturer's instructions (Dual-Luciferase Reporter Assay System, Promega) with a Luminometer Centro LB960 (Berthold, Bad Wildbad, Germany). Relative luciferase expression (fold increase) was calculated with the following equation: fold increase = (firefly luciferase activity of upstream region of IL-7 gene construct/*Renilla* luciferase activity) / (firefly luciferase activity of promoterless vector pGL3-Basic/*Renilla* luiferase activity).

Statistical analysis

The unpaired *t*-test was used.

Results

Polymorphisms in the upstream non-coding region of the IL-7 gene

We sequenced a 1658-bp PCR-amplified fragment of the IL-7 gene containing 1470 bp of the upstream non-coding region, 9 bp of the first coding exon and 179 bp of the downstream intron. Samples were obtained from 52 unrelated non-HIV-1-infected and 99 HIV-1-infected Japanese, as well as from 122 non-HIV-1-infected and 112 HIV-infected Thais. Polymorphisms were identified at two positions: an A to G substitution at position −485 and an ATC deletion at a position from −29 to −27 upstream from the open frame ATG codon of the IL-7 gene (Fig. 1). Frequencies of these two polymorphisms are summarized in Table 1. As for the A to G mutation at position −485, there was no difference in frequency of the G allele between HIV-1-infected and non-HIV-1-infected individuals. For the allele of the ATC deletion, two of the 99 HIV-1-infected Japanese carried this allele, but none of the Thais. There was no linkage disequilibrium between these two mutations.

Figure 1. Fragment containing 650-bp of the upstream non-coding region and a part of the coding region of the IL-7 gene. Two polymorphisms, A to G at −485 and ATC deletion at −29 to −27 are underlined. The sequence with the ATC deletion is shown below the sequence without the ATC deletion. Asterisks denote sequence identity. Numbers denote positions from the initiation ATG of IL-7. An open arrow at −632 and a closed arrow at −297 denote the 5′ ends of the IL-7 upstream non-coding region inserted into reporter plasmids (see Figs 3 and 4). Triangles denote multiple transcription start sites that are clustered within the two distinct regions reported by Oshima *et al.* (2004). Open triangles denote transcription start sites specifically activated by IFN-γ. An open square denotes IRF-E (Oshima *et al.*, 2004).

In addition, calculation of nucleotide diversity in the 1470-bp fragment of the upstream non-coding region of IL-7 genes in all Japanese and Thai subjects showed 1.4×10^{-5} in Japanese and 0.9×10^{-5} in Thais. These results suggested that the human IL-7 gene has a highly conserved upstream non-coding region.

Roles of ATGs in the upstream non-coding region of IL-7 gene

In the upstream non-coding region, there are several out-of-frame ATGs (Fig. 1), with even the shortest transcript starting from position −130 containing two out-of-frame ATGs in the upstream non-coding region (Fig. 1). Because one of those out-of-frame ATGs occurred just downstream of the ATC deletion described previously, we then investigated roles of these upstream ATGs in IL-7 gene expression. For this purpose, we constructed a reporter plasmid in which the luciferase open reading frame was fused with the upstream ATG under the control of the upstream region of the IL-7 gene. As shown in Fig. 2, when a reporter plasmid carrying the region from position −632 to the authentic translation initiation ATG codon was transiently transfected into Jurkat or U937 cells, a significant increase in luciferase activity was observed, compared with the pGL3-basic vector employed as a control reporter plasmid in either cell, confirming a previous observation (Oshima *et al.*, 2004). When ATG at position −69 to −67 was fused with the luciferase open reading frame, luiferase activity became greatly enhanced (Fig. 2). These results indicated that the upstream AUG in IL-7 mRNA was more efficiently used for expression than the authentic AUG, and suggested that presence of upstream AUGs in IL-7 mRNA can be expected to reduce IL-7 translation levels.

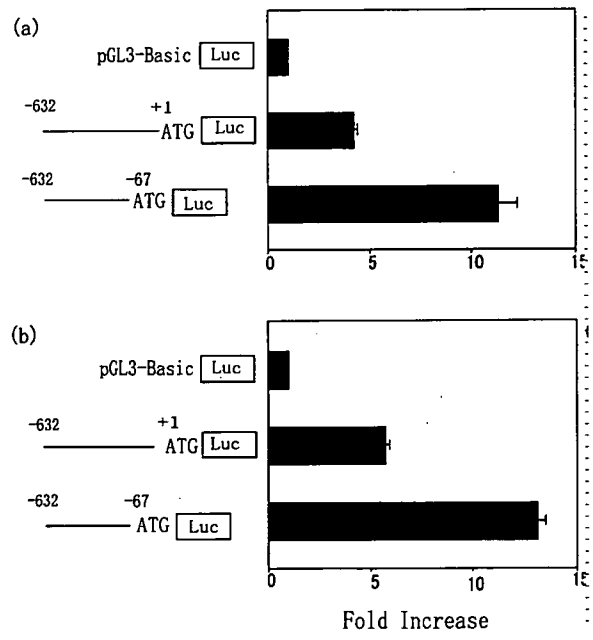


Figure 2. Luciferase activity mediated by the upstream non-coding region of the IL-7 gene. Jurkat (a) or U937 cells (b) were transfected with the plasmids indicated. The fold increase of each construct is represented by a bar. Data represent three independent experiments with similar results. Error bars show actual fluctuations among measurements of fold increase in four clones of each construct.

ATC deletion could affect the gene expression of IL-7

As mentioned previously, the ATC deletion occurred just upstream of the ATG located at position −26 to −24 (Fig. 1). Kozak previously reported that ACCATGG is

Table 1. Allele and genotype frequencies of A to G mutation at –485 and deletion mutation at –29 to –27 in HIV-1-infected and non-HIV-1-infected Japanese and Thai people

	Japan					Thailand			
	HIV-1-Infected *n* (%)	Non-HIV-1-Infected *n* (%)		HIV-1-Infected *n* (%)	Non-HIV-1-Infected *n* (%)		HIV-1-Infected *n* (%)	Non-HIV-1-Infected *n* (%)	
Allele			Genotype			Allele			Genotype
A	196 (99.0)	102 (98.1)	AA	97 (98.0)	50 (96.2)	A	221 (98.7)	241 (98.8)	AA	109 (97.3)	119 (97.5)
G	2 (1.0)	2 (1.9)	AG	2 (2.0)	2 (3.8)	G	3 (1.3)	3 (1.2)	AG	3 (2.7)	3 (2.5)
			GG	0 (0)	0 (0)				GG	0 (0)	0 (0)
Total	198	104		99	52	Total	224	244		112	122
Allele			Genotype			Allele			Genotype		
W[a]	196 (99.0)	104 (100)	WW	97 (98.0)	52 (100)	W[a]	224 (100)	244 (100)	WW	112 (100)	122 (100)
D[b]	2 (1.0)	0 (0)	WD	2 (2.0)	0 (0)	D[b]	0 (0)	0 (0)	WD	0 (0)	0 (0)
			DD	0 (0)	0 (0)				DD	0 (0)	0 (0)
Total	198	104		99	52	Total	224	244		112	122

[a] W denotes the wild type at –29 to –27.

[b] D denotes deletion at –29 to –27.

the optimal sequence for translation initiation of pre-proinsuline by eukaryotic ribosomes and that substitution of G for A at position –3 (3-bp upstream from the ATG codon) reduced translation efficiency (Kozak, 1986). The A to G substitution at position –3 of an upstream, out-of-frame ATG condon also reportedly diminished translation from the corresponding upstream ATG and consequently increased translation from the authentic downstream ATG (Kozak, 1986). In the case of the human IL-7 gene, the sequence surrounding the ATG at position –26 to –24 is ATCATG but the ATC deletion observed in our study converted it into GTCATG (Fig. 1). These data indicate that the ATC deletion altered the A at position –3 into G (Fig. 1), thus hypothetically reducing translation efficiency from the upstream ATG at position –26 to –24 and increasing translation from the authentic IL-7 ATG. We therefore decided to test experimentally whether the ATC deletion polymorphism actually affected levels of expression from the authentic IL-7 ATG.

We constructed a reporter plasmid containing the upstream non-coding region from –632 to +3 with the ATC deletion and compared its luciferase activity with that of the wild-type version. As shown in Fig. 3(a), the reporter activity of the deletion mutant was approximately 30% higher than that of the wild-type plasmid. We also generated shorter versions of the wild type as well as mutant constructs carrying the upstream non-coding region from –297 to +3, which spans the minimal promoter region containing IRF-E (–268 to –257) (Oshima *et al.*, 2004). Again, the reporter activity of the deletion mutant was approximately 30% higher than that of the wild-type plasmid (Fig. 3b). We repeated the same experiments by using monocytic U937 cells. Here too, luciferase activity in the deletion mutant was approximately 25% higher than that in the corresponding wild-type plasmid when the upstream non-coding region of –632 to +3 was used (Fig. 4a). An approximately 20% increase in luciferase activity was observed in the deletion mutant when the upstream non-coding region –297 to +3 was used (Fig. 4c). It is known that INF-γ is capable of up-regulating the gene expression of IL-7 in intestinal epithelial cells through the IRF-E in the region –268 to –257 from the initiation ATG codon (Oshima *et al.*, 2004). As shown in Fig. 4(b,d), the addition of INF-γ to the transfected cells in fact did augment luciferase activity in U937 cells. Moreover, the deletion mutant exhibited significantly higher luciferase activity than the wild-type constructs (Fig. 4b,d). These results clearly indicate that ATC deletion in the upstream non-coding region resulted in higher expression from the authentic IL-7 ATG.

Discussion

In the study reported here, we demonstrated that an out-of-frame ATG in the upstream non-coding exon of IL-7 gene was more efficiently used for expression than the authentic ATG of IL-7 gene. We also found a naturally occurring ATC deletion polymorphism at position –29 to

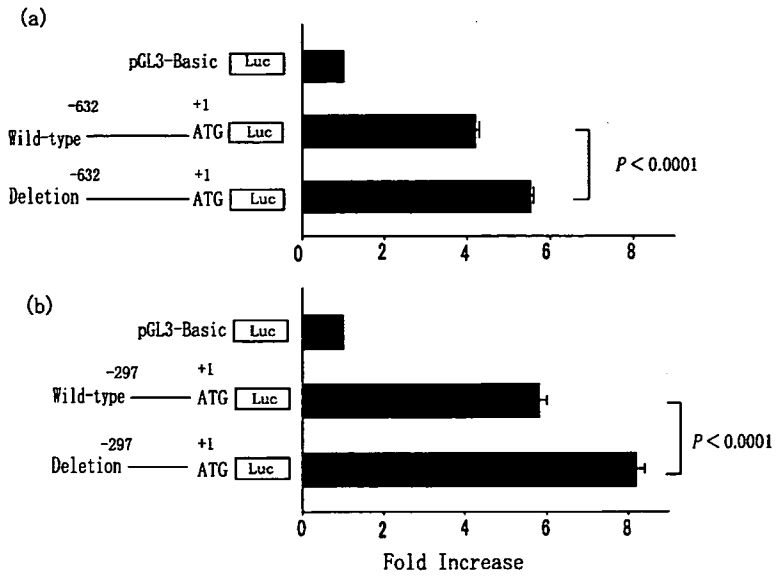


Figure 3. Luciferase activity mediated by the upstream non-coding region of the wild-type and ATC deletion in Jurkat cells. (a) A region from position −632 to +3 of the upstream non-coding region of the wild-type and ATC deletion. (b) A region from position −297 to +3 of the upstream non-coding region of the wild-type and ATC deletion. Data represent three independent experiments with similar results. Error bars show actual fluctuations among measurements of fold increase in four clones of each construct. *P* values for differences in fold increase are shown.

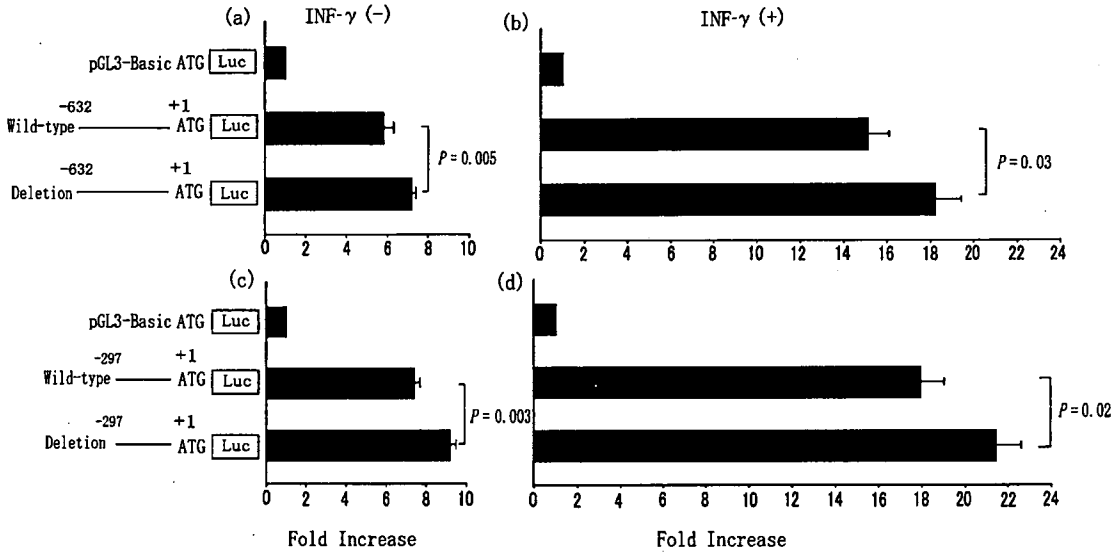


Figure 4. Luciferase activity mediated by the upstream non-coding region of the wild-type and ATC deletion in U937 cells. (a) A region from position −632 to +3 of the upstream non-coding region of the wild-type and ATC deletion without INF-γ. (b) A region from position −632 to +3 of the upstream non-coding region of the wild-type and ATC deletion with INF-γ. (c) A region from position −297 to +3 of the upstream non-coding region of the wild-type and ATC deletion without INF-γ. (d) A region from position −297 to +3 of the upstream non-coding region of the wild-type and ATC deletion with INF-γ. Data represent three independent experiments with similar results. Error bars show actual fluctuations among measurements of fold increase in four clones of each construct. *P* values for differences in fold increase are shown.

−27 in the upstream non-coding exon next to one of the upstream ATGs. This polymorphism was found to be capable of increasing the expression from the authentic IL-7 ATG in Jurkat T cell and U937 monocytic cell lines. This is the first time human genetic polymorphism has been identified that is supposed to affect expression of a protein by changing the translation efficiency from the out-of-frame AUG in the upstream non-coding region of mRNA.

There are a few precedents for a human genetic polymorphism near the initiation ATG codon affecting translation efficiency. A single nucleotide polymorphism (SNP) that switches C to T at position −1 upstream from the open frame ATG codon in the human annexin V gene has been found to increases translation efficiency and plasma levels of annexin V, and to decreases the risk of early myocardial infarction (Gonzalez-Conejero *et al.*, 2002). Also, an SNP that switches G to T at position −3 upstream from the open frame ATG codon of the BRCA1 gene in sporadic breast cancer causes down-modulation of translation efficiency (Signori *et al.*, 2001). Moreover, a mutation of G into A at +4 downstream from the open frame ATG codon of the human androgen receptor gene observed in