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免疫アレルギー疾患等予防・治療研究事業

食餌性脂質を中心とした生理活性脂質による粘膜
免疫制御ならびにアレルギー疾患との関連解明

平成20年度 総括研究報告書

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I. 総括研究報告書

食餌性脂質を中心とした生理活性脂質による粘膜免疫制御ならびに
アレルギー疾患との関連解明

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研究要旨：本研究においてはこれまでの我々の研究から食物アレルギーに関わることを示している脂質メディエーターの一つであるスフィンゴシン1リン酸（S1P）に焦点を当て、S1PによるIgA産生制御機構を明らかにした。さらにS1Pの代謝に関わるビタミンB6と食物アレルギーとの関連について検討し、ビタミンB6の機能阻害によりマスト細胞の機能が抑制され食物アレルギーの発症が抑制されることが確認された。さらに脂肪酸組成の異なる餌を調整し、それを6週間与えた際の腸管IgAの産生について検討し、脂肪酸組成の違いにより自然免疫型分泌型腸管IgAの産生量が増加することを確認した。これらの結果はS1Pを始めとする脂質関連分子とその代謝制御を行うビタミン等の食餌性成分が腸管免疫の制御に密接に関与しており、その恒常性維持が食物アレルギーの発症と関連していることを強く示唆する結果であると考えられる。

本研究課題においては分担研究者を配していない。

A. 研究目的

アレルギー疾患は現在国民の約3人に1人が罹患していると言われている国民病であり、患者のQOLを著しく低下させること、またその治療に伴う医療費の増加などが問題となっており、その改善に向けた新規予防・治療法の開発が待望されている。国内外のグループによるこれまでの研究から、食餌性成分による免疫制御がアレルギー発症に関与していることが示唆されているが、その詳細な分子、細胞レベルでのメカニズムは不明である。本研究においては、我々

がこれまで行ってきた研究のうち、生理活性脂質の一つであるスフィンゴシン1リン酸（S1P）による腸管免疫制御と食物アレルギーの発症に関する研究結果を基盤に、食餌性脂質を含む脂質による粘膜免疫制御とアレルギー発症との関連について体系的な解析を行うことで、脂質を介した腸管免疫制御とアレルギー発症に関する基礎的情報を提供し、新規アレルギー予防・治療戦略を構築することを目的とする。

3年計画の初年度である本年度はS1Pを介した腸管免疫制御機構に関する検討をさらに発展させることを第一の目的とする。また、S1Pの代謝制御にビタミンB6が重要

であるという報告に基づき、ビタミンB6とS1P、粘膜免疫の三者間相互作用による粘膜免疫制御と食物アレルギーという観点からの検討を第二の目的とする。さらに脂肪酸組成の異なる食用油を含む餌を摂取した際の免疫制御に関する予備的検討を第三の目的とする。

B. 研究方法

1) 食物アレルギー発症におけるビタミンB6の関与

ビタミンB6の機能を抑制するために、ビタミンB6のアンタゴニストである4'-deoxypridoxine (DOP)を飲料水に加え自由摂取させた。DOPを2週間摂取した後の腸管組織における免疫担当細胞の分布をFACS法にて検討した。さらに同マウスにフロイントの完全アジュバントを用いニトリ卵白アルブミン(OVA)で全身感作を行った後、OVAを経口頻回投与することでアレルギー性下痢を誘導した。これらのマウスにおける下痢症状を観察すると共に、各種免疫担当細胞の分布をFACS法やELISA法等の免疫学的手法を用い検討した。

2) 分泌型腸管IgA産生におけるS1Pの関与

分泌型IgAの主要産生細胞であるパイエル板B細胞をFACS Ariaを用い分化段階に応じて分離、精製した。各B細胞のS1P受容体の発現を定量的PCRにて測定した。さらにS1P受容体の発現低下を引き起こす免疫抑制剤であるFTY720を用いS1Pシグナルを遮断した際のB細胞動態をFACS法、なら

びに組織学的解析にて検討した。また経口的に投与された抗原に対する分泌型IgA産生をELISA法にて測定し、FTY720投与時と非投与時と比較した。

もう一つの分泌型IgAの主要産生細胞である腹腔B細胞の遊走制御におけるNFκB-inducing kinase (NIK)の役割を検討する目的で、NIK変異マウスであるaly/alyマウスにおける腹腔B細胞のFTY720反応性をFACS法にて検討した。さらにaly/alyマウス由来腹腔B細胞に発現しているS1P受容体を定量的PCRで、aly/alyマウス由来腹腔B細胞をSCIDマウスに移入した際の腸管IgA産生と遊走制御機構についてELISA, ELISPOT, FACS法等の免疫学的手法を用い、それぞれ解析した。

3) 腸管免疫制御における食用油の関与

通常のマウス用食餌に用いられる大豆油の代わりに、脂肪酸組成の異なる食用油を用いた飼料を数種類作製し、マウスに6週間与えた。その後の糞便中IgA産生についてELISA法を用いて測定した。

(倫理面への配慮)

動物実験は東京大学医科学研究所のガイドラインに則り行った。

C. 研究結果

1) 食物アレルギー発症におけるビタミンB6の関与

ビタミンB6の機能を抑制する4'-deoxypridoxine (DOP)を投与したマウス

においては、アレルギー性下痢の発症が抑制された。これらのマウスにおいてはマスト細胞の浸潤抑制が観察されたが、興味深いことに活性化 T 細胞の浸潤には有意な差は認められなかった。また IgA 産生細胞においても DOP 処理マウスでは IgA 産生細胞に大きな違いは認められなかった。

2) 分泌型腸管 IgA 産生における SIP の関与

分泌型腸管 IgA の主要産生細胞であるパイエル板 B 細胞の各分化段階における SIP 受容体の発現レベルを検討したところ、ナイーブ B 細胞である IgM 陽性 B 細胞は SIP 受容体を高レベルで発現するが、刺激を受け IgA 陽性細胞へとクラススイッチすると共に SIP 受容体の発現は低下していた。その後、IgA 陽性形質芽細胞へ分化するに伴い発現が回復した。この結果と関連し、FTY720 を投与し SIP を介したシグナルを抑制したマウスにおいては、IgA 陽性形質芽細胞のパイエル板内への選択的集積が認められた。組織学的な解析から、IgA 陽性形質芽細胞の集積はパイエル板の管腔とは反対側に当たるリンパ管が密集している部位に観察されることが示された。さらには FTY720 の投与により SIP 依存的 IgA 陽性形質芽細胞のパイエル板からの移出を阻害したマウスにおいては、経口投与した抗原に対する分泌型 IgA の産生が著しく低下していた。

さらにもう一方の腸管分泌型 IgA 産生経路である腹腔 B 細胞の SIP 依存的遊走経路においては NF κ B-inducing kinase (NIK)

が必要であることを発見した。そのため、NIK 変異マウスである aly/aly マウスは FTY720 に対する感受性が低下していた。しかしながら、aly/aly マウスの腹腔 B 細胞の SIP 受容体の発現レベルは正常であり、aly/aly マウス由来腹腔 B 細胞を SCID マウスに移入した際には正常マウスと同様の FTY720 に対する感受性を示した。一方で、aly/aly マウスに正常ストローマ細胞を移入すると aly/aly マウスにおいても FTY720 に対する感受性が回復することから、aly/aly マウスの FTY720 に対する低感受性は、B 細胞レベルではなくストローマ細胞における機能欠損が一因であることが示唆された。さらにその機能欠損の一つが、腹腔 B 細胞の SIP 依存的遊走を協調的に制御するケモカインの発現異常によることを明らかにした。

3) 食物アレルギーの発症における食用油の関与

脂肪酸組成の異なる食用油を 4% 含む餌を作製し、6 週間摂食させた際の糞便中 IgA 量を測定した。その結果、コントロール群である大豆油を用いて作製した餌を摂取した場合に比べ、IgA の産生を増強する食用油と抑制する食用油が存在した。

D. 考察

我々のこれまでの研究から、ビタミン B6 のアンタゴニストである DOP の投与により食物アレルギーの発症が抑制されることが確認された。我々のこれまでの検討から、

アレルギー誘導期間に FTY720 処理を行うとアレルギー性下痢の発症が抑制されることが明らかになっている (Y Kurashima et al., J Immunol, 2007)。これらは食物アレルギーの抑制という観点では同様の結果である。また血清 IgE の産生には影響を与えない、マスト細胞の大腸への浸潤が抑制されているといった所見は DOP と FTY720 で共通した知見となっている。しかしながら FTY720 の処理では T 細胞や B 細胞の浸潤抑制が観察されているのに対し、DOP 処理では T 細胞や B 細胞に対しては大きな影響が観察されなかった。これまでの報告から、細胞種により S1P に対する感受性が異なることが示唆されているが、本結果よりマスト細胞は T 細胞、B 細胞と異なる S1P 依存経路を用いていることが示唆された。今後この依存性の違いを規定する分子メカニズムを同定することで、S1P 依存性の違いに基づく新たなアレルギー予防・治療法を開発できると期待される。

また、これまでの研究を進展させた S1P を介した腸管免疫制御に関する研究では、パイエル板と腹腔という異なる二つの経路において S1P が重要であることを見いだした。そのうち腹腔を介した経路ではストローマ細胞の NIK 依存的ケモカイン産生を介した協調的経路が重要であることを報告し、S1P とケモカインとの協調的相互作用が腸管 IgA の産生であることが示唆された。

さらに、食餌性脂質に関する予備的検討から、わずか 4%しか含まれない食餌性脂質であるが、その脂肪酸組成の違いにより、

腸管 IgA の産生が変化することが示された。これは食とアレルギーとの関連を脂質と腸管免疫という観点から検討する上で重要な知見であると考えられ、今後そのメカニズム等を重点的に検討したいと考える。

E. 結論

S1P を始めとする脂質関連分子とその供給源となる食餌性脂質、さらにはそれらの代謝を制御するビタミン等の食餌性成分が密接に関連して腸管免疫の制御を行っており、その制御機構が食物アレルギーの発症と関連していると考えられる。

F. 健康危機情報

なし

G. 研究発表

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H. 知的財産権の出願・登録状況 (予定を含む)

1. 特許取得
なし
2. 実用新案登録
特記事項なし。
3. その他
特記事項なし。

II. 研究成果の刊行に関する一覧表

研究成果の刊行に関する一覧表

書籍

著者氏名	論文タイトル名	書籍全体の編集者名	書籍名	出版社名	出版地	出版年	ページ
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III. 研究成果の刊行物・別冊
(主要なもの)

Sphingosine 1-phosphate-dependent trafficking of peritoneal B cells requires functional NF κ B-inducing kinase in stromal cells

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We previously reported that sphingosine 1-phosphate (S1P) regulates peritoneal B-cell trafficking and subsequent intestinal IgA production, but the underlying mechanisms remain obscure. We demonstrate here that nuclear factor κ B-inducing kinase (NIK) is involved in the regulation of S1P-mediated trafficking of peritoneal B cells. Although peritoneal B cells from NIK-mutated alymphoplasia (*aly*) mice expressed type 1 S1P receptor (S1P₁) at comparable levels and demonstrated normal migration toward S1P, *aly* peritoneal B cells showed decreased sen-

sitivity to FTY720, an S1P₁ modulator. NIK-mutated stromal cells showed decreased levels of adhesion molecules (VCAM-1 and ICAM-1) and increased CXCL13 expressions, leading to impaired ability to support S1P-mediated emigration, but not immigration, of peritoneal B cells. Therefore, *aly* peritoneal B cells exhibited normal S1P-mediated peritoneal B-cell trafficking from peritoneum to intestine for IgA production when they were transferred into severe combined immunodeficient or wild-type mice. However, S1P-mediated emigration of wild-

type B cells from the *aly* peritoneal cavity was impaired without affecting their immigration from the blood. Further, transfer of wild-type stromal cells into the peritoneum restored S1P-mediated trafficking of *aly* peritoneal B cells. These findings suggest that NIK in stromal cells has a specific role in the regulation of S1P-mediated trafficking of peritoneal B cells. (Blood. 2008;111:4646-4652)

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Introduction

The peritoneal cavity contains numerous B cells, especially B1 cells, which play important roles in protective immunity in the peritoneal cavity and in the production of secretory IgA antibody (S-IgA) in the intestine.^{1,2} Accumulating evidence has demonstrated that the trafficking of peritoneal B cells is regulated by chemokines (eg, CCL19, CCL21, and CXCL13), cytokines (eg, interleukin [IL]-10), and adhesion molecules (eg, integrins) at various points in the immigration, retention, and emigration of these cells.³⁻⁸

Sphingosine 1-phosphate (S1P) is another key molecule in the regulation of lymphocyte trafficking.^{9,10} Among the 5 types of S1P receptors, type 1 S1P receptor (S1P₁) is preferentially expressed on lymphocytes and is required for their emigration from secondary lymphoid organs and the thymus.^{11,12} FTY720 is an agonist for S1P receptors, except type 2 S1P receptor (S1P₂), and blocks S1P-mediated signaling by inducing internalization of S1P receptors.¹²⁻¹⁶ Therefore, treatment with FTY720 decreases the number of circulating lymphocytes in both blood and lymph by inhibiting their emigration from secondary lymphoid organs and the thymus.¹²⁻¹⁶

In addition to its role in the systemic immune compartments, we recently reported that S1P is involved in the trafficking of mucosa-associated immunocompetent cells, including peritoneal B cells, intraepithelial T lymphocytes, and intestinal mast cells.¹⁷⁻¹⁹ In those studies, we showed that peritoneal B1 and B2 cells expressed comparable levels of S1P₁ and that FTY720

treatment impaired trafficking of peritoneal B cells into the intestine by enhancing their emigration from the peritoneal cavity and by inhibiting their immigration from blood into the peritoneal cavity. The FTY720-associated disruption of peritoneal B-cell trafficking into the intestine was associated with impaired intestinal immunoglobulin A (IgA) production by peritoneal B cells.¹⁷ These findings provide strong evidence that S1P plays an essential role in the regulation of peritoneal B-cell trafficking into the intestine and subsequent intestinal IgA production.

Alymphoplasia (*aly*) mice carry a point mutation in nuclear factor κ B-inducing kinase (NIK), leading to the inability to bind to I κ B kinase α , a molecule essential for nuclear factor κ B (NF κ B) activation.²⁰⁻²² *aly* mice lack lymph nodes and Peyer patches; they have impaired development of the spleen and thymus and showed accumulation of peritoneal B1 cells.^{20,23} Because the B1 and B2 cells that originate from the peritoneal cavity and Peyer patches are the primary sources of intestinal IgA,^{1,2} the immunologic defects in *aly* mice almost completely ablate intestinal IgA production.²³ A previous study revealed that the impaired peritoneal B-cell trafficking in *aly* mice was, at least in part, due to the defect of signaling pathway coupling with G-proteins, such as chemokine receptors, in lymphocytes.²³ A subsequent study revealed that the impaired function of stromal cells in *aly* mice was also attributable to the defective trafficking of bone marrow-derived naive IgM⁺ IgA⁻ B cells to the intestine.²⁴

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Given the defective G-protein signaling pathways in *aly* mice,²³ and the fact that S1P receptors couple to G proteins,²⁵ our findings led us to hypothesize that the defective peritoneal B-cell trafficking and consequent impaired intestinal IgA production in *aly* mice might be mediated by S1P. We therefore sought here to investigate the interaction between NIK- and S1P-mediated pathways in peritoneal B-cell trafficking and subsequent intestinal IgA production. Our findings provide new evidence that NIK-mediated signaling in stromal cells regulates S1P-mediated trafficking of peritoneal B cells, especially their emigration from the peritoneal cavity.

Methods

Mice, FTY720 treatment, and cell isolation

Female C57BL/6, *aly*, and severe combined immunodeficient (SCID) mice (7–9 weeks) were purchased from Japan Clea (Tokyo, Japan). All mice were maintained in horizontal laminar flow cabinets and provided with sterile food and water ad libitum. For FTY720 treatment, mice were injected intraperitoneally with FTY720 (1 mg/kg; Novartis Pharma, Basel, Switzerland).^{17–19} Peritoneal cells were obtained by flushing the peritoneum with 8 mL ice-cold phosphate-buffered saline (PBS).¹⁷ All experiments were approved by the Animal Care and Use Committee of the University of Tokyo and conducted in accordance with its guidelines.

Flow cytometry and cell sorting

A standard protocol was used for flow cytometric analysis and cell sorting.^{17–19} Cells were incubated with anti-CD16/32 antibody (Ab; BD Biosciences, San Diego, CA) and then stained with the appropriate fluorescent-conjugated Abs specific for B220, CD11b, ICAM-1, and VCAM-1 (BD Biosciences). Viaprobe (BD Biosciences) was used to discriminate between dead and live cells. Flow cytometric analysis and cell sorting were performed with FACScalibur (BD Biosciences) and FACSaria (BD Biosciences), respectively.

In vitro migration assay

In vitro migration assays using purified B1 and B2 cells were performed according to a previously established method.¹² Briefly, peritoneal B cells were applied to the upper chambers (pore diameter, 5 μ m; Invitrogen, Carlsbad, CA) and 0, 20, 200, or 2000 nM S1P was added to the lower wells. After a 6-hour incubation, the B cells that had migrated into the lower wells were counted with the aid of trypan blue staining.

RT-PCR

To measure mRNA expression for S1P₁, quantitative and conventional reverse-transcription-polymerase chain reaction (RT-PCR) using LightCycler (Roche Diagnostics, Mannheim, Germany) were performed.^{17–19} Briefly, total RNA was isolated using TRIzol reagent (Invitrogen), and cDNA was synthesized using Powerscript reverse transcriptase (BD Biosciences). The oligonucleotide primers and probes specific for S1P₁ (forward primer, TACTCTGACCAACAAGGA; reverse primer, ATAATGGTCTCTGGGTTGTC; FITC-probe, TGCTGGCAATTCAGAGGCCCATCATC; LCRed 640-probe, CAGGCATGGAATTTAGCCGACGAAATC), glyceraldehyde-3-phosphate dehydrogenase (GAPDH; forward primer, TGAACGGGAAGCTCACTGG; reverse primer, TCCACCACCTGTGCTGTA; FITC-probe, CTGAGGACCAGGTTGTCTCTGCGA; LCRed 640-probe, TTCAACAGCAACTCCACTCTTCCACC), CCL19 (forward primer, GCAAGCAACAAGGCAACA; reverse primer, CACTC-CACATCGACTCTTA), CCL21 (forward primer, ACAGACACAGCCCTCAA; reverse primer, CATGAGGTGGCTGTCTT), and CXCL13 (forward primer, GAACAGGCATTAGTGACAAC; reverse primer, TTTTGGAAGCTGCGTTT) were designed and synthesized by Nihon Gene Research Laboratory (Sendai, Japan).²⁶

Adoptive cell transfer

For tracing cells in vivo, peritoneal B cells (10^7 cells) were incubated with 0.25 μ M 5-(and-6)-carboxyfluorescein diacetate, succinimidyl ester (CFSE; Molecular Probes, Eugene, OR) in the dark for 10 minutes at 37°C and then were washed twice with PBS according to a previously described method.^{17,18} Labeled B cells (5×10^6 cells) were transferred into recipient mice intraperitoneally or intravenously; FTY720 was administered intraperitoneally simultaneously. After 12 hours, peritoneal cells were collected for flow cytometric analysis.

For experiments involving stromal cell transfer, stromal cells were isolated from the small intestines of wild-type (WT) mice as previously described.²⁷ Briefly, cells were isolated from intestinal lamina propria and cultured on 10-cm culture plates in complete RPMI1640 medium. After 1 hour, nonadherent cells were removed by washing with PBS, and remaining adherent cells were cultured overnight in complete RPMI1640 medium. After overnight culture, the plates were washed with PBS, and remaining adherent cells were cultured in complete RPMI1640 medium. After 2 rounds of subculture, confluent cells were used as stromal cells and transferred into the peritoneal cavities of *aly* mice (10^7 cells per mouse). Two weeks after transfer, mice were treated with FTY720 for analysis of peritoneal B-cell trafficking. To analyze Ab production from peritoneal B cells, SCID mice were adoptively transferred with peritoneal B cells (5×10^6 cells per mouse) via the intraperitoneal route and treated with FTY720 every 2 days for 2 weeks. Two weeks after adoptive transfer, fecal extracts were collected for the measurement of total IgA levels by enzyme-linked immunosorbent assay (ELISA).

Measurement of fecal IgA by ELISA

The concentration of fecal IgA was determined by ELISA as previously described.¹⁷ Purified murine IgA Ab (BD Pharmingen, San Diego, CA) was used as a standard for the quantification. After blocking of coated anti-mouse Ig Ab (Southern Biotechnology Associates, Birmingham, AL) with 5% bovine serum albumin in PBS, diluted fecal extract was added and incubated in the coated wells for 2 hours at room temperature. Bound Ab was quantified using HRP-conjugated anti-mouse IgA (Southern Biotechnology Associates) and 3,3',5,5'-tetramethylbenzidine (Moss, Pasadena, CA), as previously described.¹⁷

Statistics

The results were compared using the Student *t* test or Welch *t* test. *P* value of less than .05 was considered statistically significant.

Results

Decreased sensitivity to FTY720 in *aly* mice

To test whether the defective trafficking of peritoneal B cells in *aly* mice was attributable to a dysfunctional S1P-mediated pathway, we compared the effect of FTY720 on peritoneal B cells in *aly* mice with those in WT mice. Consistent with our previous results,¹⁷ a single injection of FTY720 induced rapid reductions in the percentages and absolute cell numbers of peritoneal B1 and B2 cells in WT mice (Figure 1A). In contrast, *aly* mice showed scant reduction in peritoneal B cells after a single injection of FTY720 (Figure 1B). To elucidate whether the FTY720 reactivity of *aly* mice was complete or partial, mice were injected repeatedly with FTY720 and their peritoneal B cells were examined. In agreement with our previous results,¹⁷ treating WT mice with multiple injections of FTY720 did not increase its effect on peritoneal B cells, such that B1- and B2-cell counts and percentages were similar to those of the single-treatment group (Figure 1A). In contrast, repeated, but not single, FTY720 treatment significantly (*P* < .05) reduced the peritoneal B1- and B2-cell populations of *aly* mice (Figure 1B). These findings suggest that the peritoneal B cells

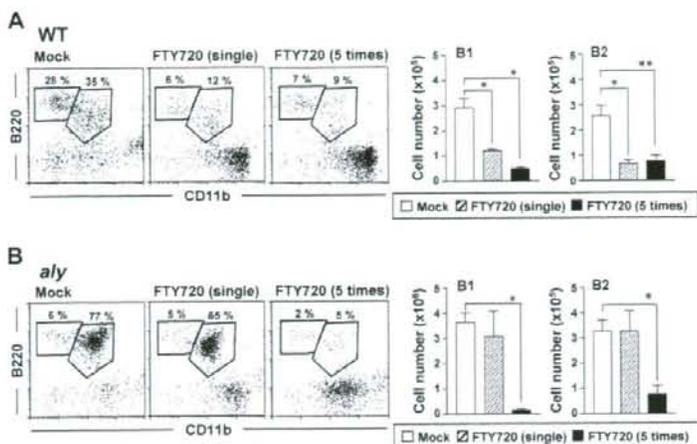


Figure 1. Decreased reactivity of peritoneal B cells to FTY720 in *aly* mice. Cells were isolated from the peritoneal cavities of WT (A) or *aly* (B) mice 12 hours after single or multiple (that is 5) injections of FTY720 (right) or vehicle only (mock, left), and cell populations were analyzed by flow cytometry. The data are representative of at least 4 independent experiments. The numbers of B220⁺CD11b⁺ B1 cells and B220⁺CD11b⁺ B2 cells were calculated from the total cell number and flow cytometric data. Data are presented as mean plus or minus SEM (n = 4). *P < .01; **P < .05.

of *aly* mice showed reduced sensitivity but are still reactive to FTY720.

Normal S1P₁ expression and migration to S1P in *aly* peritoneal B cells

We hypothesized that the decreased reactivity of *aly* peritoneal B cells to FTY720 was due to their minimal expression of S1P receptors, especially S1P₁, whose type was exclusively expressed on peritoneal B cells.¹⁷ To test this hypothesis, we performed quantitative RT-PCR analysis and found that the levels of S1P₁ in peritoneal B1 and B2 cells and splenic B cells were comparable between *aly* and WT mice (Figure 2A). Together with a previous report indicating that NIK-mediated signaling is linked to the same G-coupled protein that S1P receptors use,²⁵ our results suggested that NIK mutation abolished S1P₁-mediated signaling. To test whether S1P₁-mediated

signaling in *aly* peritoneal B cells was functional, we investigated the *in vitro* migration of peritoneal B cells toward S1P. We found that, like the peritoneal B cells isolated from WT mice, both peritoneal B1 and B2 cells from *aly* mice migrated to the gradient of S1P (Figure 2B). These data indicated that the reduced reactivity to FTY720 in *aly* mice was not due to a defect in S1P₁ expression or S1P₁-mediated signaling.

To further examine whether *aly* peritoneal B cells show normal reactivity to S1P *in vivo*, we isolated *aly* peritoneal B cells, labeled them with CFSE, and adoptively transferred them into SCID mice that were treated concurrently with FTY720 to disrupt S1P-mediated signaling. Because our recent study demonstrated that FTY720 inhibited B-cell immigration into the peritoneal cavity and enhanced their emigration from it,¹⁷ we transferred the labeled B cells through 2 different routes, intraperitoneal and intravenous injection. When *aly* peritoneal

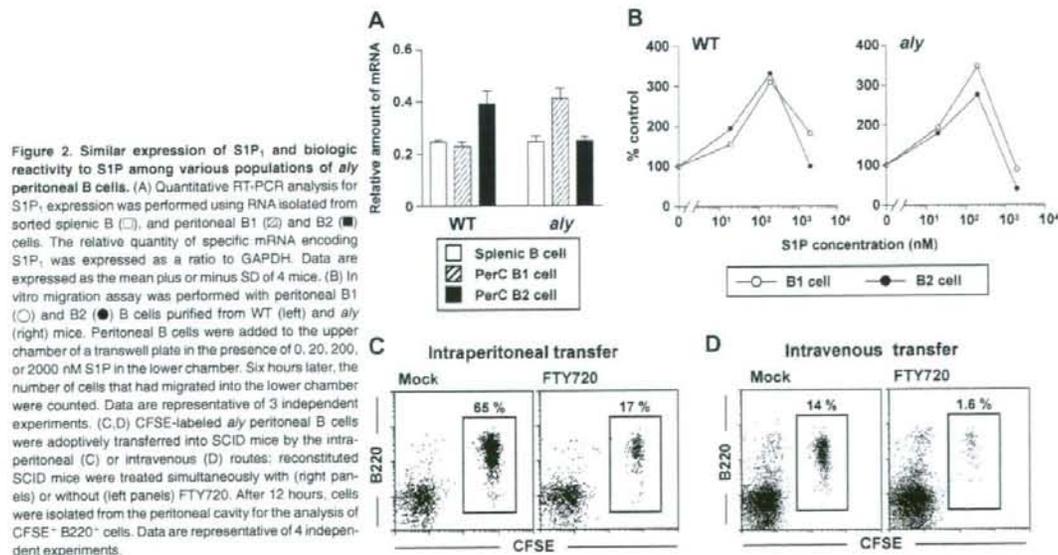


Figure 2. Similar expression of S1P₁ and biologic reactivity to S1P among various populations of *aly* peritoneal B cells. (A) Quantitative RT-PCR analysis for S1P₁ expression was performed using RNA isolated from sorted splenic B (□), and peritoneal B1 (▨) and B2 (■) cells. The relative quantity of specific mRNA encoding S1P₁ was expressed as a ratio to GAPDH. Data are expressed as the mean plus or minus SD of 4 mice. (B) *In vitro* migration assay was performed with peritoneal B1 (○) and B2 (●) B cells purified from WT (left) and *aly* (right) mice. Peritoneal B cells were added to the upper chamber of a transwell plate in the presence of 0, 20, 200, or 2000 nM S1P in the lower chamber. Six hours later, the number of cells that had migrated into the lower chamber were counted. Data are representative of 3 independent experiments. (C, D) CFSE-labeled *aly* peritoneal B cells were adoptively transferred into SCID mice by the intraperitoneal (C) or intravenous (D) routes: reconstituted SCID mice were treated simultaneously with (right panels) or without (left panels) FTY720. After 12 hours, cells were isolated from the peritoneal cavity for the analysis of CFSE⁺ B220⁺ cells. Data are representative of 4 independent experiments.

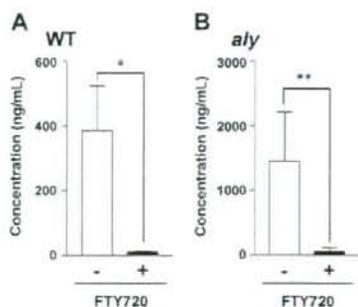


Figure 3. *aly* peritoneal B cells show a comparable dependence on S1P for intestinal IgA production. WT (A) or *aly* (B) peritoneal B cells (5×10^6 cells) were adoptively transferred into SCID mice, which were treated with vehicle only (□) or FTY720 (■) every 2 days. Two weeks after cell transfer, fecal extracts were collected for measurement of IgA levels by ELISA. Data are presented as means plus or minus SEM ($n = 4$). * $P < .01$; ** $P < .05$.

B cells were adoptively transferred into the peritoneal cavities of SCID mice. FTY720 treatment resulted in a marked reduction in B-cell numbers, suggesting that, as with WT B cells, FTY720 enhanced the emigration of *aly* B cells from the peritoneal cavity (Figure 2C). Further, immigration of *aly* B cells from the blood into the peritoneal cavity was impaired when SCID mice were treated with FTY720 after the intravenous transfer of *aly* peritoneal B cells (Figure 2D). Taken together with the new *in vitro* and *in vivo* data, our findings convincingly show that *aly* peritoneal B cells can react to S1P and FTY720. However, the sensitivity to FTY720 is lower in *aly* mice than in WT mice.

A previous study demonstrated that S1P lyase, which degrades S1P to phosphoethanolamine, is abundant in secondary lymphoid organs, thus establishing a S1P gradient with lower concentrations in the secondary lymphoid organs than in the blood and lymph.²⁸ These findings suggested to us that the lack of secondary lymphoid organs in *aly* mice might contribute to their decreased sensitivity to FTY720 owing to the presence of an impaired S1P gradient. But some evidence obtained in our study disproved this hypothesis. We found that $Id2^{-/-}$ mice, which lacked secondary lymphoid organs due to deficiency of a negative regulator of basic helix-loop-helix transcription factors, showed normal sensitivity to FTY720, and that disruption of the S1P gradient by oral feeding of deoxypridoxine, an inhibitor of S1P lyase, did not affect the peritoneal B-cell trafficking in WT mice (J.K., unpublished data, January 2007).

Taken together with a previous report that lymphoid organs were not required for S1P-mediated trafficking of peripheral lymphocytes,²⁹ these findings suggest that the impaired reactivity of *aly* mice to FTY720 is not attributable to their defective secondary lymphoid organ structure.

Normal S1P-mediated trafficking of *aly* peritoneal B cells for the intestinal IgA production

Peritoneal B cells are primarily sources of intestinal IgA production,^{1,2} and we previously demonstrated that S1P mediates the production of intestinal IgA by peritoneal B cells.¹⁷ Therefore, we next examined whether *aly* peritoneal B cells could produce intestinal IgA in an FTY720-sensitive manner. We addressed this issue by investigating intestinal IgA production in SCID mice adoptively transferred with *aly* peritoneal B cells. Consistent with our previous findings,¹⁷ SCID mice that received WT peritoneal B cells produced considerable amounts of intestinal IgA, and FTY720 treatment inhibited this production (Figure 3A). Similar induction of intestinal IgA production occurred when SCID mice were reconstituted with *aly* peritoneal B cells (Figure 3B), and, as seen after transfer of WT peritoneal B cells, FTY720 abolished intestinal IgA production in the mice that received *aly* peritoneal B cells (Figure 3B). These data indicate that *aly* B cells migrate normally into the intestine and subsequently produce intestinal IgA in an FTY720-sensitive manner in SCID mice. In light of these data, NIK in B cells seems to be redundant in the S1P-mediated trafficking of peritoneal B cells and subsequent intestinal IgA production. Therefore, the impaired sensitivity of *aly* mice to FTY720 is not due to defective S1P-mediated signaling in B cells.

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

In light of our current findings that *aly* peritoneal B cells react to FTY720 and S1P, we hypothesized that their decreased sensitivity to FTY720 was due to the NIK mutation in the non-B cells. We therefore examined the FTY720 reactivity of WT peritoneal B cells adoptively transferred into SCID and *aly* mice. Regardless of the injection route, WT peritoneal B cells showed normal reactivity to FTY720 and thus were decreased after treatment with FTY720 when they were adoptively transferred into SCID mice (Figure 4A,B). In contrast, WT peritoneal B cells transferred intraperitoneally into *aly* mice lacked reactivity to FTY720. Indeed, the numbers

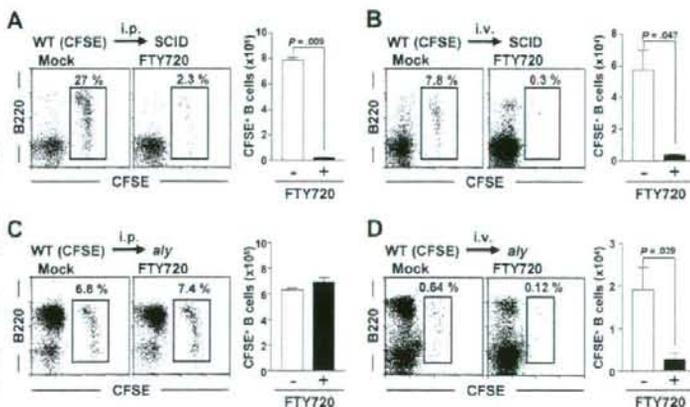


Figure 4. NIK-mediated signaling in non-B cells controls S1P-mediated peritoneal B-cell emigration of peritoneal B cells, but not their immigration. (A,B) Peritoneal B cells were isolated from WT mice, labeled with CFSE, and adoptively transferred via the intraperitoneal (i.p.) (A) or intravenous (i.v.) (B) routes into SCID mice. (C,D) Similarly, CFSE-labeled peritoneal WT B cells were transferred into *aly* mice from which peritoneal cells were removed 8 hours before transfer. The reconstituted mice were treated simultaneously with (right panels) or without (left panels) FTY720. After 12 hours, cells were isolated from the peritoneal cavity for the analysis of CFSE⁺ B220⁺ cells. Flow cytometric data are representative of 3 independent experiments and are presented as means plus or minus SEM ($n = 3$).

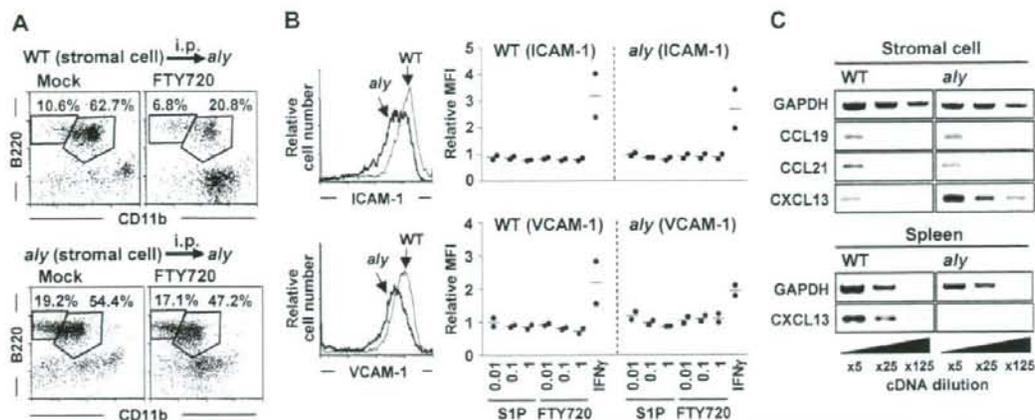


Figure 5. Requirement of NIK-mediated signaling in stromal cells for the emigration of peritoneal B cells. (A) *aly* mice were intraperitoneally (i.p.) transferred with WT (top panels) or *aly* (bottom panels) stromal cells. Two weeks after cell transfer, mice were treated with FTY720 for the analysis of peritoneal B-cell populations. Flow cytometric data are representative of 3 independent experiments and are presented as means plus or minus SEM ($n = 3$). (B) Expression of ICAM-1 (top panels) and VCAM-1 (bottom panels) on WT (thin lines) and *aly* (thick lines) stromal cells was determined by flow cytometry (left). Twenty-four hours after treatment of stromal cells with various concentrations of S1P, FTY720, or IFN γ (50 units/mL), expression of ICAM-1 and VCAM-1 was determined by flow cytometry. Relative mean fluorescence intensity (MFI) was expressed as a ratio to MFI of untreated cells. Data are representative of 2 independent experiments, and bars indicate mean values. (C) Expression of chemokines (CCL19, CCL21, and CXCL13) in stromal and spleen cells was examined by RT-PCR. Data are representative of 3 independent experiments.

of peritoneal B cells were similar with or without FTY720 treatment (Figure 4C).

We next addressed whether NIK mutation affected B-cell immigration from the blood into the peritoneal cavity. In this experiment, we removed the peritoneal cells from recipient *aly* mice 8 hours before adoptive transfer, because the *aly* peritoneal B cells were too numerous to allow detection of intravenously transferred B cells. Twenty-four hours after depletion of the peritoneal B cells, the peritoneal cavities of *aly* mice contained more B2 cells than untreated *aly* peritoneal B cells (data not shown), demonstrating that peritoneal B cells were removed and that many cells were derived from the blood. Removing peritoneal cells from recipient mice before adoptive transfer enabled us to detect intravenously injected WT peritoneal B cells in *aly* mice (Figure 4D). Unlike the case with intraperitoneally transferred WT B cells (Figure 4C), FTY720 prevented the immigration of intravenously transferred WT peritoneal B cells from the blood into the peritoneal cavity (Figure 4D). The number of adoptively transferred B cells was decreased consistently and significantly ($P < .05$) in the peritoneal cavities of FTY720-treated *aly* mice (Figure 4D). These findings suggest that NIK-mediated signaling in non-B cells participates in the regulation of S1P-mediated emigration of B cells from the peritoneal cavity but not in their immigration from the blood.

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

Because both *aly* and WT B cells showed normal emigration from the peritoneal cavities of SCID mice (Figures 2C and 4A, respectively), T cells likely do not play a role in this pathway. We therefore speculated that NIK-mediated signaling in stromal cells was involved in the emigration of peritoneal B cells. To test this hypothesis, we transferred WT stromal cells into the peritoneal cavities of *aly* mice treated with FTY720 and noted a subsequent reduction in the number of peritoneal B cells (Figure 5A top

panels). In contrast, FTY720 had no discernible effect when *aly* stromal cells were transferred into *aly* mice (Figure 5A bottom panels). These data suggest that NIK-mediated signaling in stromal cells participates in the regulation of S1P-mediated peritoneal B-cell emigration.

To investigate the mechanisms of peritoneal B-cell trafficking mediated by NIK in stromal cells, we compared the expression of vascular cell adhesion molecule (VCAM)-1 and intercellular adhesion molecule (ICAM)-1, adhesion molecules regulating peritoneal B-cell trafficking, between WT and *aly* stromal cells. The expression of these adhesion molecules was lower on *aly* stromal cells than on WT stromal cells (Figure 5B). A previous report that S1P regulated the expression of VCAM-1 on endothelial cells³⁰ led us to hypothesize that S1P affects the expression of VCAM-1 and ICAM-1 on stromal cells. However, unlike endothelial cells, WT and *aly* stromal cells showed negligible expression of all types of S1P receptors (data not shown). Therefore, treatment of stromal cells with S1P or FTY720 influenced neither VCAM-1 nor ICAM-1 expression, although IFN γ increased the expression of both of these molecules in both WT and *aly* stromal cells (Figure 5B).

We then examined the expression of chemokines that were reported to be involved in peritoneal B-cell trafficking (CCL19, CCL21, and CXCL13).^{3,4} Our results showed that expression of CCL19 and CCL21 was comparable between WT and *aly* stromal cells (Figure 5C). In contrast, CXCL13 expression was increased in *aly* stromal cells compared with WT stromal cells (Figure 5C), although CXCL13 expression in the *aly* spleen was lower than in the WT spleen (Figure 5C), as previously reported.²³ These findings collectively indicate that, in S1P-mediated peritoneal B-cell trafficking, S1P directly affects peritoneal B cells, not stromal cells, but stromal cells are involved in the S1P-mediated pathway through the expression of adhesion molecules and chemokines. Decreased expression of VCAM-1 and ICAM-1 on stromal cells, or unbalanced CXCL13 expression between the peritoneal cavity and other sites (eg, spleen) in *aly* mice (or both mechanisms), may explain the impaired S1P-mediated trafficking of peritoneal B cells in *aly* mice.

Discussion

Lymphocyte trafficking into and from lymph nodes and nonlymphoid organs is regulated through several bioactive molecules (eg, chemokines and adhesion molecules).⁹ We previously demonstrated that a lipid mediator, S1P, regulated mucosa-associated lymphocyte trafficking of peritoneal B cells, intraepithelial T lymphocytes, and mast cells into the intestine.¹⁷⁻¹⁹ To this end, our recent study¹⁷ showed that S1P plays important roles in both the immigration and emigration of B cells into and from the peritoneal cavity. Our current study extends this observation by showing that NIK-mutated *aly* mice were less sensitive (~5 times) to FTY720 than WT mice (Figure 1). We found that NIK-mediated signaling in stromal cells was involved in the emigration, but not immigration, step of S1P-mediated trafficking of peritoneal B cells (Figure 4). Although our results showed that the specific involvement of NIK-mediated signaling in stromal cells in the emigration of peritoneal B cells is a critical and major factor determining less sensitivity (~5 times) of *aly* mice to FTY720, it is simply possible that elevated numbers of peritoneal B cells in *aly* mice (~10 times) may at least partly provide additional explanation for the lower sensitivity of *aly* mice to FTY720.

Our current study also revealed that *aly* peritoneal B cells are functionally normal and therefore show normal S1P₁ expression and reactivity to S1P (Figure 2). In addition, *aly* peritoneal B cells show normal expression of CXCR5, a receptor for CXCL13.²³ In contrast, functional defects of *aly* stromal cells led to impaired S1P-mediated peritoneal B-cell trafficking in *aly* mice, although stromal cells did not express any types of S1P receptors (Figure 5 and data not shown). Regarding this issue, we found 2 possible major defects in stromal cell expression of adhesion molecules (ICAM-1 and VCAM-1) and chemokine, CXCL13. First, in agreement with previous findings that the expression of VCAM-1 and ICAM-1 was positively regulated by NF κ B/NIK pathway,^{31,32} *aly* stromal cells showed reduced expression of VCAM-1 and ICAM-1, thereby weakening the attachment of peritoneal B cells to stromal cells in their trafficking pathway (Figure 5B). Second, although CXCL13 expression was decreased in the spleens of *aly* mice compared with WT control (Figure 5C), which is in agreement with a previous work,²³ *aly* stromal cells showed increased CXCL13 expression compared with WT stromal cells (Figure 5C). Underlying mechanisms of the opposite effect of NIK mutation on CXCL13 expression between stromal and spleen cells remain enigmatic and represent challenges for future studies. But our current findings indicate that NIK-mediated signalings are involved in both positive and negative regulation of CXCL13 expression and which is used depends on cell types. This idea is supported by a previous report that the regulation of inflammatory cytokine-mediated CXCL13 expression was different among cell types (eg, bone marrow stromal cells and osteoblasts).³³ Taken together with these facts that S1P and CXCL13 mutually regulate marginal B-cell trafficking and the S1P function is dominant to CXCL13,³⁴ it seems that, under normal conditions, the disruption of S1P₁-mediated signaling by FTY720 treatment may allow peritoneal B cells to react to the CXCL13 gradient between the peritoneal cavity and other sites (eg, spleen), leading to B-cell emigration from the peritoneal cavity through the interaction with stromal adhesion molecules (eg, ICAM-1 and VCAM-1). Our results indicated that the functional defects of stromal cells in this pathway caused decreased sensitivity of *aly* mice to FTY720 and thus

replacement of *aly* stromal cells with WT rescued normal S1P-mediated emigration of peritoneal B cells.

The molecular mechanisms of peritoneal B-cell trafficking for intestinal IgA production remain enigmatic.^{1,2} In this regard, the enhanced expression of β_2 integrin induced by peritoneal environment plays a role in establishing the commitment of peritoneal B cells to home back to the peritoneal cavity as well as migrate to the intestine.⁸ In addition, gut-associated dendritic cells (eg, Peyer patches and mesenteric lymph nodes) can allow B cells to migrate into the intestine by inducing the expression of $\alpha_4\beta_7$ integrin and CCR9 through retinoic acid.²⁵ However, the molecules involved in the peritoneum-dependent gut tropism of peritoneal B cells remain unknown. In a previous study,²⁴ NIK-dependent stromal cell activation was required for the direct migration of bone marrow-derived B cells to the intestinal lamina propria but not for the migration of B cells primed in the Peyer patches. Our current results similarly suggest that the peritoneum-mediated trafficking of B cells into the intestine involves NIK-dependent stromal cells. The present study therefore shows that both NIK-dependent signaling in stromal cells and S1P were required for B-cell trafficking from the peritoneal cavity to the intestine for intestinal IgA production, especially in the step of B-cell emigration from the peritoneal cavity (Figure 4). Therefore, molecular interaction among S1P, NIK in stromal cells, and unknown gut-imprinting molecules likely uniquely coordinates the trafficking of B cells from the peritoneum into the intestine for subsequent intestinal IgA production.

Considering all previous and current findings together, we suggest that the destiny of peritoneal B cells is controlled by a pleonastic regulatory network comprising S1P, chemokines, and integrins. In this pathway, NIK-mediated signaling in stromal cells regulates the S1P-mediated emigration of B cells from the peritoneum to the intestine for subsequent production of intestinal IgA.

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Authorship

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

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