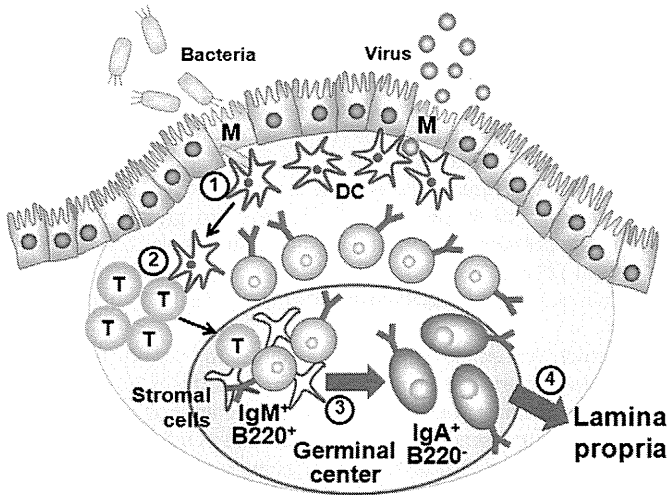

Fig. 4. Sequential processes for initiation of antigen-specific immune responses in Peyer's patches. (1) After transport of antigen by M cells, dendritic cells (DC) take up antigen, and (2) migrate to the T cell region. There, the DCs prime antigen-specific T cells by presenting antigen on MHC molecules and providing co-stimulatory signals. (3) Some of the antigen-primed T cells migrate to the germinal center, where, in coordination with stromal cells and follicular DCs, they induce immunoglobulin class switching and further differentiation of IgM^+ $B220^+$ B cells into IgA^+ $B220^-$ plasmablasts. These germinal center events are dependent on the interaction of CD40 with CD40 ligand, and cytokine activity (in particular TGF-β, IL-4, and IL-21). (4) IgA^+ $B220^-$ plasmablasts modulate their expression of integrins (such as α4β7 integrin) and receptors for chemokines (such as CCR9 and CXCR5) and sphingosine 1-phospahte. These changes promote their emigration from the PPs and trafficking to the intestinal lamina propria where differentiation occurs into plasma cells producing polymeric IgA.

stay in the GC or emigrate from the PPs for the migration to distant effector region (e.g., intestinal lamina propria) [40,41].

After emigration from the PPs, expression of gut-homing molecules (e.g., α4β7 integrin and CCR9/CCR10) on IgA^+ plasmablasts allows them to home to intestinal lamina propria, where IL-5, IL-6, and IL-10 induce terminal differentiation into plasma cells that produce dimeric or polymeric IgA. Polymeric IgA binds polymeric-immunoglobulin receptors expressed on the basal membrane of ECs and is transported to the intestinal lumen as the form of SIgA.

In contrast to events in the PPs, T cell help is not required for the IgA production in the ILFs. As described above, ILFs contain few T cells [17]. A previous study showed that stromal cells could be activated by LTβR-mediated interaction with $ROR\gamma t^+$ LTi and bacterial stimulation through toll-like receptors. This activation resulted in recruitment of DCs and B cells for the subsequent formation of ILFs [18]. Another study demonstrated that simultaneous stimulation of stromal cells with bacteria and retinoic acid induced production of CXCL13, TGF-β, and BAFF and led to preferential generation of IgA^+ B cells [42]. These events occurred in the absence of T cell help [42]. T cell-independent antigens, such as polysaccharides, have been thus considered for use as vaccine antigens [43]. Thus, induction of T cell-independent IgA responses via ILFs could be a novel strategy for the development of oral vaccines.

4.2. GALT-independent IgA production pathway

In addition to conventional B cells (named B-2 cells) which generally located in the organized lymphoid tissues (e.g., PPs), the peritoneal cavity contains large numbers of B-1 cells, another major source of intestinal IgA, especially against T cell-independent antigens [44]. A site for IgA class switching of peritoneal B cells has been elusive, but several lines of evidence indicate the involvement of DCs in the intestinal lamina propria for the creation of class switching molecular and cellular niche. Among the several types of DCs, those that express TNFα and inducible nitric oxide synthase, Tip-DCs, and $TLR5^+$ DCs, induce IgA^+ B cells by producing key molecules, such as APRIL, BAFF, IL-6, and retinoic acid without the involvement of organized lymphoid structure such as PPs [45,46]. However, it was previously reported that lamina propria DCs are capable of initiating systemic IgG responses, whereas antigen transport by M cells into the PPs is required for the initiation of intestinal IgA production pathway [47], which was consistent with another finding that DCs in the PPs are responsible for the intestinal IgA synthesis system [48]. Therefore, although it is generally accepted that lamina propria DCs act as antigen-presenting cells for intestinal antigens and are capable of inducing antibody responses, it is still obscure how lamina propria DCs regulate the induction of intestinal IgA and systemic IgG responses.

As ILF-mediate initiated IgA responses, GALT-independent IgA responses are involved in the immune responses against T cell-independent antigens, such as polysaccharides and phosphoryl choline [49]. Since these T cell-independent antigens have been considered as vaccine antigens such as *Streptococcus pneumonia*[43], the use of GALT-independent IgA induction pathway could be an additional strategy for the development of oral vaccines.

5. Application of drug delivery systems to the development of oral vaccines

Antigen delivery is central and key to the development of effective and successful oral vaccines. Particulate antigens appear to be more effective than soluble ones. This phenomenon is at least partially due to protection of the antigen from the harsh conditions of the gastrointestinal environment of digestive tract, such as low pH, detergent effects of bile salts, and extensive proteolytic enzyme activity. In addition, particulate antigens are preferentially taken up in the GALTs, especially by M cells serving as a gateway of the mucosal immune system, thus enhancing their antigenic activity. Several systems have been developed for targeting vaccine antigen selectively to the M cells in the FAE of GALTs.

5.1. Passive transport system

A variety of biodegradable antigen delivery systems have been developed for oral vaccines. These include incorporation of antigens into polymer-based particles (e.g., poly-lactide-co-glycolide-microparticles) [50], liposomes [51], ISCOM [52], and chitosan particles [53]. Their utility as oral delivery vehicles is enhanced by the fact that they are biodegradable and can be formulated for controlled drug release. The effect of particle size on passive targeting to M cells has been evaluated. M cells preferentially take up particles with diameters less than 10 μm whereas a few micrometer- or nanometer-sized particles are taken up by ECs as well as M cells [54]. For example, small poly-lactide microparticles (e.g., 4 μm) in diameter enhanced only plasma IgG responses without IgA responses in the intestine. In contrast, 8–10 μm poly-lactide microparticles enhanced IgA responses in the intestine [55]. These findings suggest that the former size of particles is effectively transported antigen to the systemic immune system (or peripheral lymph nodes) via ECs for the initiation of IgG responses, while the latter sizes are successfully taken up by M cells for the initiation of mucosal IgA antibody responses via PPs. The combination of optimal sizing of capsule is important consideration for the development of oral vaccine which can induce simultaneously both mucosal and systemic protective immunity.

In addition to particle size, modifications to chemical features have been exploited to enhance antigen delivery. For instance, enterocoated-type particles were employed to protect the encapsulated antigen from the acidic environment of the upper part of intestine and to allow rapid release of antigen in the small intestine [56]. An additional example is the use of chemical mucoadhesive molecules (e.g., carboxy vinyl polymer) to elongate particles containing protein antigens, thereby prolonging antigen persistence in the intestine [57]. Liposomes can also

be made more stable in acid by constructing them with dipalmitoyl phosphatidylserine, dipalmitoyl-phosphatidylcholine, and cholesterol [58,59].

5.2. Use of M cell-specific ligands

In addition to passive one, active delivery of particles to GALT fascinates the induction efficacy of oral vaccines. In this issue, several mucosal antigen delivery systems have been explored that deliver antigen selectively to M cells (Table 1). Lectins have been widely exploited in vaccines to gain or to enhance access of antigen to M cells. The unique reactivity of UEA-1 to M cells allowed the selective and effective delivery of orally administered microparticles or liposomes to murine M cells [60,61]. A similar approach can be taken by using M cell-specific antibodies. NKM16-2-4 recognizing α1,2-fucose-containing carbohydrates. The NKM16-2-4 antibody can be conjugated to vaccine antigen for efficient delivery of antigen to M cells [22]. Thus the targeting to M cells resulted in the induction of antigen-specific IgA antibody responses by the use of low amount of vaccine antigen when compared with the non-targeting form of oral vaccine. Additional studies identified GP2, a receptor for some bacteria expressing Fim(H) [27], as a specific marker of M cells [27] [26]. Because anti-GP2 antibodies have been shown to bind to both murine and human M cells [27], they may be useful for oral antigen delivery in both systems.

The use of organic molecules or peptides that mimic the functional activity of UEA-1 has also been explored to promote efficient delivery of antigen to M cells (Table 1). In these studies, molecules that bound UEA-1 ligands were identified in mixture-based positional scanning synthetic combinatorial libraries or in phage peptide libraries. The former approach revealed that a digalloyl D-Lysine amide construct and a tetragalloyl D-Lysine amide construct bound effectively to M cells; coating of polystyrene particles with these compounds resulted in the selective and efficient delivery of the particles to M cells [62]. The latter approach yielded peptide sequence (YQCSYTMPHPPV) that selectively bound to the M cell-rich SED region of the PP and enhanced the delivery of polystyrene microparticles to M cells [63]. These accumulative evidences suggest that a combination of intestinal friendly characteristics of chemically modified particle and M cell targeting molecule could be a logical strategy for the development of oral vaccine.

5.3. Applying microbial invasion systems to M cell targeting

Another logical approach has been to use components of microbial invasion systems to deliver synthetic particles to M cells (Table 1). Enhanced antigen uptake was achieved by coating polystyrene nanoparticles with *Yersinia*-derived invasin, a ligand for β1 integrins that is expressed on the apical side of M cells [64]. Similarly, mucosal immune responses were significantly increased by mucosal immunization with an antigen coupled to σ1, a protein derived from reoviruses, which are known to be an invading molecule for the virus to enter the M cells [65]. Long polar fimbria (LPF) mediates the binding of *Salmonella* and adherent-invasive *E. coli* to M cells [24,66], but additional pathways appear to exist, as long polar fimbria-deficient *Salmonella* still invade through M cells [67]. In this issue, FimH, the adhesin portion of long polar fimbria, was found to be involved in the binding of FimH(+) *E. coli* and *Salmonella* to M cells [27]. FimH binds to glycoproteins in a mannose-dependent manner and mediates binding to GP2 expressed on M cells [27,68]. Thus, just as for GP2-specific antibodies, FimH is a candidate targeting bacterial molecule for specific delivery of antigen to M cells.

Recently, we employed genetic analyses to identify indigenous commensal bacteria that specifically localized inside of PPs. *Alcaligenes* species, for example, were observed predominantly inside of PPs, in contrast to their absence on the surface as well as other tissues [69]. It has been suggested that at least some component of *Alcaligenes* was taken up by DCs, which induced IL-6 and BAFF expression for the enhancement of IgA production [69]. These findings suggested an interesting possibility that *Alcaligenes* species can be used as a new form of commensal flora based vaccine antigen-delivery micro-vehicle specifically transport vaccine to PPs.

In related to our new observation for the intra-tissue co-habitation of commensal flora, mucosal IgA antibodies have been suggested to play a critical role for guiding and colonizing *Alcaligenes* in PPs since immunoglobulin-deficient mice showed a significant reduction of *Alcaligenes* in the PPs [69]. It is thus possible that antibody-mediated pathway appears to be involved in the uptake of *Alcaligenes* into the PPs [69]. It was previously revealed that immunogloblins preferentially adhere to M cells [70,71], implicating that *Alcaligenes* was taken up by M cells into the PPs via immunoglobulin-mediated pathway. In addition, it was demonstrated that secretory IgA was recognized by DC-SIGN on DCs [72], implicating that M cells and DCs cooperatively use IgA antibody to efficiently enhance the gut immune responses. In line with this, it was previously reported that coating particles with immonoglobulins would target oral vaccines to M cells and consequently enhanced antigen-specific immune responses [73,74].

Table 1
Tools for M cell targeting.

Ligand	Receptor	Reference
UEA-1 lectin	α1,2 fucose	20, 58, 59
Antibody (LM112)	Sialyl Lewis A	21
Antibody (NKM-16-2-4)	α1,2 fucose-containing carbohydrate	20
Antibody (3G7-H9, 2F11-C3)	Glycoprotein 2	26, 27
Digalloyl D-lysine amide	Unknown	60
Tetragalloyl D-lysine amide	Unknown	60
Peptides (YQCSYTMPHPPV)	Unknown	61
σ1 protein (reovirus)	α2,3 sialic acid	25, 63
Invasin (*Yersinia*)	β1 integrin	23, 62
Long Polar fimbriae (*E. coli, Salmonella*)	Unknown	24, 64
FimH (*E. coli, Salmonella*)	Glycoprotein 2	27
IgA	Immunogloblin receptors	71, 72

6. Conclusion

It is generally accepted that mucosal vaccines are an attractive strategy for protecting against many infectious diseases. Recent advances in biomaterial technologies have allowed the development of versatile antigen delivery systems. In addition, significant progress in our understanding of mucosal immunology and M cell biology has enhanced the possibility of targeting mucosal vaccines to the mucosal antigen-sampling and presenting system including M cells, DCs and ECs. Furthermore, because immunological environment in the intestinal tract is dominantly quiescent by several lines of regulatory/suppressor system to maintain the immunological homeostasis in order to deal with the harsh environment of intestine, we also have to consider the development of mucosal adjuvant/modulator to temporary break the immunological suppression for the initiation of antigen-specific positive responses. Thus, integration of the all knowledge gained in the biomaterial, immunological, and cellular biological fields should facilitate the development of a new generation of mucosal vaccines.

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A Pivotal Role of Vitamin B9 in the Maintenance of Regulatory T Cells *In Vitro* and *In Vivo*

Jun Kunisawa[1,2,3]*, Eri Hashimoto[1], Izumi Ishikawa[1], Hiroshi Kiyono[1,2,3,4]

1 Division of Mucosal Immunology, Department of Microbiology and Immunology, Institute of Medical Science, The University of Tokyo, Tokyo, Japan, **2** Department of Medical Genome Science, Graduate School of Frontier Science, The University of Tokyo, Chiba, Japan, **3** Core Research for Evolutional Science and Technology (CREST), Japan Science and Technology Agency, Tokyo, Japan, **4** Graduate School of Medicine, The University of Tokyo, Tokyo, Japan

Abstract

Dietary factors regulate immunological function, but the underlying mechanisms remain elusive. Here we show that vitamin B9 is a survival factor for regulatory T (Treg) cells expressing high levels of vitamin B9 receptor (folate receptor 4). In vitamin B9-reduced condition *in vitro*, Treg cells could be differentiated from naïve T cells but failed to survive. The impaired survival of Treg cells was associated with decreased expression of anti-apoptotic Bcl2 and independent of IL-2. *In vivo* depletion of dietary vitamin B9 resulted in the reduction of Treg cells in the small intestine, a site for the absorption of dietary vitamin B9. These findings provide a new link between diet and the immune system, which could maintain the immunological homeostasis in the intestine.

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* E-mail: kunisawa@ims.u-tokyo.ac.jp

Introduction

To achieve immunosurveillance and immunological homeostasis at the interface between the interior and exterior of the gastrointestinal tract, the intestinal immune system tightly balances states of immune activation and quiescence [1]. Thus, gastrointestinal tissues contain numerous kinds of T cells, such as Th1, Th2, Th17, forkhead box P3 (Foxp3)$^+$ regulatory T (Treg) cells, IL-10–producing Foxp3$^-$ T regulatory type 1 cells, and T cells expressing γδ T cell receptor, which together create the appropriate immunological environment.

Th17 and Treg cells are observed most frequently in the intestine, and their preferential differentiation is achieved by a unique cytokine environment created by transforming growth factor β (TGF-β), IL-6, and IL-23 [2]. In addition to these host-derived factors, the development and function of the immune system are influenced by crosstalk with environmental factors [3]. For example, stimulation by segmented filamentous bacteria results in the preferential induction of Th17 cells, whereas colonic Treg cells are induced by crosstalk between epithelial cells and Clostridium clusters IV and XIVa [4,5,6].

Nutritional molecules are also considered to be essential environmental factors for the development, maintenance, and regulation of gut immune responses. Thus, deficient or inappropriate nutritional intake increases the risk of infectious, allergic, and inflammatory diseases [7,8]. Among various dietary factors, vitamins are important participants in the regulation of immune responses. For example, vitamin A is converted into retinoic acid (RA) by gut-associated dendritic cells; RA induces the expression of gut-homing molecules (e.g., α4β7 integrin and CCR9) on activated T and B cells [9,10] and promotes the preferential differentiation of Treg cells and the simultaneous inhibition of Th17 cells [11,12,13,14]. Vitamin B6 is required for the metabolic pathway of sphingosine 1-phosphate, a lipid mediator that regulates cell trafficking [15]; disruption of vitamin B6 function results in aberrant T-cell differentiation and cell trafficking in both systemic and intestinal compartments [16,17,18].

Vitamin B9 (also known as folate and folic acid) is a water-soluble vitamin derived from both diet and commensal bacteria [19]. Vitamin B9 is essential for the synthesis, replication, and repair of nucleotides for DNA and RNA and is thus required for cell proliferation and survival [20]. Methotrexate (MTX) acts as a vitamin B9 antagonist and blocks vitamin B9–mediated nucleotide synthesis, making MTX useful as an anti-tumor [21] and anti-rheumatoid arthritis agent [22]. Vitamin B9 deficiency also reduces the proliferative responses of lymphocytes and natural killer cell activity [23,24]. Additionally, the vitamin B9 receptor folate receptor 4 (FR4) is both a marker of Treg cells and is immunologically functional [25]; however, how it functions in the intestinal immune system is largely unknown. In this study, we examined the role of vitamin B9 in the regulation of Treg cell *in vitro* and *in vivo*.

Materials and Methods

Mice and experimental treatment

Female Balb/c mice (7–9 wk of age) were purchased from Japan Clea (Tokyo, Japan). Vitamin B9–deficient and control

diets composed of chemically defined materials (Oriental Yeast, Tokyo, Japan) were used within 3 months. All animals were maintained in the experimental animal facility at the University of Tokyo, and the experiments were approved by the Animal Care and Use Committee of the University of Tokyo and conducted in accordance with their guidelines (Approval #20–28).

Lymphocyte isolation

Lymphocytes were isolated from the lamina propria (LP), as previously described [18,26]. Briefly, lymphocytes were isolated from dissected PPs by enzymatic dissociation using collagenase (Wako, Osaka, Japan). To isolate lymphocytes from the LP of jejunum/duodenum, PPs were removed and the remaining intestinal tissue was cut into 2-cm pieces and stirred in RPMI 1640 medium containing 1 mM EDTA and 2% fetal calf serum (FCS). The tissue pieces were then stirred in 0.5 (for small intestine) or 1.0 (for large intestine) mg/mL collagenase, and the dissociated cells were subjected to centrifugation through a discontinuous Percoll gradient. Lymphocytes were isolated at the interface between the 40% and 75% Percoll layers.

Flow cytometry and cell sorting

Flow cytometry and cell sorting were performed as previously described [18,26]. Cells were pre-incubated with anti-CD16/32 antibodies and then stained with fluorescent antibodies specific for CD4, ICOS, and GITR (BD Biosciences, San Jose, CA) and FR4 (Biolegend). A Via-probe solution (BD Biosciences) was used to discriminate between dead and living cells. Intracellular staining of Foxp3 (eBioscience, San Diego, CA), phosphorylated STAT5, Ki67 and Bcl2 (BD Biosciences) was performed in accordance with the manufacturers' instructions. Flow cytometry and cell sorting were carried out using the FACSCantoII and FACSAria systems (BD Biosciences), respectively.

Vitamin B9 measurement

To measure vitamin B9 concentrations, intestinal washes were collected by washing 12 cm of jejunum/duodenum or whole colon with 1 mL of PBS. The vitamin B9 concentration in intestinal washes and serum was measured with a RIDASCREEN enzyme immunoassay kit (R-Biopharm AG, Darmstadt, Germany) in accordance with the manufacturer's instructions. To measure the amounts of intracellular vitamin B9, 5×10^6 purified cells were washed twice with PBS, and a cell lysate was obtained by homogenizing cells in PBS containing 0.01% NP-40. After cell debris was removed by centrifugation, vitamin B9 amounts in the supernatant were measured with a RIDASCREEN enzyme immunoassay kit.

In vitro culture

For the induction of Treg cells from naïve T cells, $CD62L^{hi}CD4^+$ naïve T cells (10^5 cells/well) were cultured for 4 days with 5 μg/mL of immobilized anti-CD3 antibody and 1 μg/mL of an anti-CD28 antibody (BD Biosciences) plus 2 ng/mL of human TGF-β (PeproTech, Rocky Hill, NJ) in vitamin B9–null or normal RPMI 1640 medium containing 10% FCS. To examine the maintenance of differentiated Treg cells, purified $CD25^+CD4^+$ T cells (10^5 cells/well) were cultured for 4 days with 5 μg/mL of immobilized anti-CD3 antibody with or without 1000 units/mL of IL-2 (Peprotech) in vitamin B9–null or normal RPMI 1640 medium containing 10% FCS in the presence or absence of 100 nM MTX.

Statistics

Results were compared with the Student's *t*-test by using GraphPad Prism (GraphPad Software, San Diego, CA). Statistical significance was established at $P<0.05$.

Results

Vitamin B9 is required for the survival of $Foxp3^+$ Treg cells

$Foxp3^+$ Treg cells express high levels of FR4, which is essential for their maintenance [25]. We therefore examined whether vitamin B9 is required for the differentiation of Treg cells from naïve T cells, the survival of differentiated Treg cells, or both. To address this, we initially performed an *in vitro* T-cell differentiation assay. Purified naïve $CD4^+$ T cells were stimulated with anti-CD3 and anti-CD28 antibodies plus TGF-β in complete or vitamin B9–reduced medium. Although a small amount of vitamin B9 is supplied from fetal calf serum (FCS) even in vitamin B9–null medium (0.2 ppb, compared with 25 ppb in normal medium), the total cell number was decreased in the condition with reduced vitamin B9 compared to the control; however, $Foxp3^+$ Treg cells were generated at a normal frequency (Fig. 1A).

To investigate the effects of vitamin B9 on differentiated Treg cells, we cultured $CD25^+$ Treg cells with anti-CD3 antibodies. The total cell number was significantly lower in the vitamin B9–reduced condition than in the control condition (Fig. 1B). The reduction in cell number occurred predominantly among the $Foxp3^+CD4^+$ Treg cells (Fig. 1B). The reduction of $FR4^{hi}Foxp3^+$ T cells was dependent on the dose of vitamin B9 (Fig. 1C).

We then measured the expression of Ki67 and anti-apoptotic Bcl-2 to investigate whether decreased number of $Foxp3^+CD4^+$ Treg cells in vitamin B9–reduced medium was due to the defects of cell proliferation, survival, or both. We found that both Ki67 and Bcl2 were decreased in $Foxp3^+CD4^+$ Treg cells cultured in vitamin B9 vitamin B9–reduced medium, but magnitude of Bcl2 reduction was higher than Ki67 reduction (Fig. 2A and B). These findings suggest that vitamin B9 is preferentially but not exclusively required for the survival of Treg cells *in vitro*.

Vitamin B9 carrier-mediated pathway is not specifically involved in the survival of Treg cells

Because vitamin B9 is highly hydrophilic, mammalian cells must actively mediate the entry of vitamin B9 into cells by carrier- or receptor-mediated pathways [27]. Carriers include the proton-coupled folate transporter and the reduced folate carrier [27]. To examine whether a carrier-mediated pathway is involved in maintaining Treg cells, we employed MTX, an antagonist of vitamin B9 that is transported mainly via the reduced folate carrier and rarely via folate receptors [28,29]. MTX treatment reduced the numbers of both Treg and non-Treg cells (Fig. 3), suggesting that the carrier-mediated pathway does not specifically maintain Treg cells.

Vitamin B9 is an IL-2–independent survival factor for Treg cells

Treg cells could vigorously proliferate in some circumstances (e.g., antigen-specific activation through their highly sensitive TCR signaling [30] and IL-2-mediated activation [31]), which led to a hypothesis that Treg cells simply require large amounts of vitamin B9 as a source of nucleotides, and thus Treg cells might express FR4 as an additional means of acquiring vitamin B9. If so, $FR4^{hi}$ Treg cells should contain a larger amount of vitamin B9 in the intracellular compartments; however, the amount of intracel-

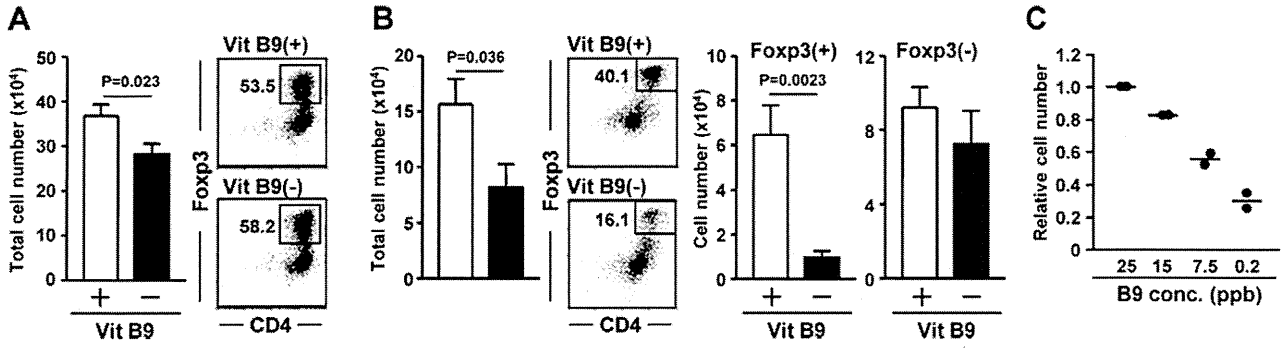

Figure 1. Requirement of vitamin B9 for the maintenance of Treg cells. (A) Purified naïve $CD4^+$ T cells were stimulated with anti-CD3 and anti-CD28 antibodies plus TGF-β in the presence of normal [Vit B9(+)] or reduced [Vit B9(−)] amounts of vitamin B9. After 4 days, total cell numbers were calculated, and the differentiation into $Foxp3^+$ Treg cells was examined by flow cytometry. Data are means ± SEM (n = 4). (B) $CD25^+CD4^+$ T cells were cultured with anti-CD3 antibodies in Cont or B9(−) medium. The frequencies of $Foxp3^+$ and $Foxp3^-CD4^+$ T cells (B) were determined by flow cytometry. Cell numbers were calculated using the total cell number and flow cytometric data. Data are means ± SEM (n = 6). (C) Experiments similar to that shown in (B) were performed with different concentrations of vitamin B9. The relative cell number of $Foxp3^+$ Treg cells is expressed as a ratio to the cell number in control medium. The values and means are indicated with dots and lines, respectively. Similar results were obtained from 2 independent experiments.
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lular vitamin B9 was equivalent between $FR4^{hi}$ Treg and $FR4^{low/-}$ non-Treg cells (Fig. 4A). Thus, FR4 might have an additional specific function for the survival of Treg cells.

IL-2 stimulation enhance the survival of Treg cells [31,32,33]. The FR4-mediated vitamin B9 signal might undergo crosstalk with IL-2-mediated signaling to maintain the survival of $FR4^{hi}Foxp3^+$ Treg cells. To test this, Treg cells were cultured with an anti-CD3 antibody together with IL-2. Although the absolute cell numbers were low in the reduced vitamin B9 condition, the magnitude of the IL-2-mediated enhancement of Treg cell growth was similar in the control and vitamin B9-reduced conditions (Fig. 4B). Consistent with this finding, comparable expression of phosphorylated STAT5 was noted in the control and vitamin B9-reduced conditions (Fig. 4C).

Dietary vitamin B9 maintains $Foxp3^+$ Treg cells in the small intestine

To examine whether vitamin B9 affects Treg cells *in vivo*, we maintained mice on a vitamin B9–depleted diet for 8 wk. Mice maintained with vitamin B9(−) diet showed less vitamin B9 in the small-intestinal wash than controls (Fig. 5A). In contrast, the amounts of vitamin B9 in the large-intestinal wash and serum were not different in those mice (Fig. 5A), presumably due to vitamin B9 production from commensal bacteria [19].

We then focused on Treg cells in the mice maintained with vitamin B9(−) diet. Consistent with our *in vitro* data, the small intestines of mice maintained with vitamin B9(−) diet had fewer $Foxp3^+$ Treg cells than those of control mice ($p = 0.018$), and there was no statistical difference ($p = 0.3022$) in the number of $Foxp3^-CD4^+$ non-Treg cells (Fig. 5B). The number of Treg and

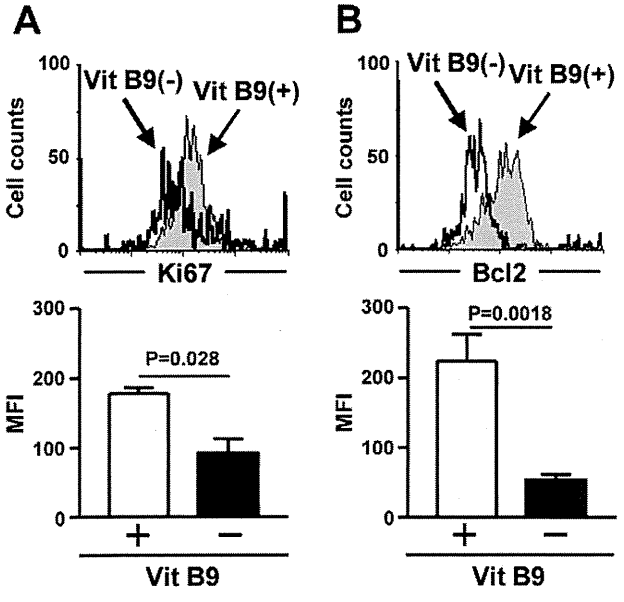

Figure 2. Vitamin B9 is essential for the survival of Treg cells. $CD25^+CD4^+$ T cells were cultured with anti-CD3 antibodies in Vit B9(+) or Vit B9(−) medium. The expression of Ki67 (A) and Bcl2 (B) in $Foxp3^+CD4^+$ T cells were determined by flow cytometry (top panels) and graphs show the means fluorescent intensity (MFI; bottom panels). Data are means ± SD (n = 3). Data are representative of 4 independent experiments.
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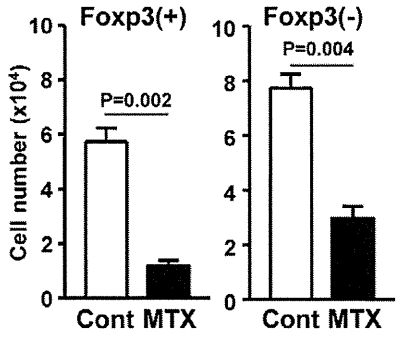

Figure 3. Vitamin B9 carrier-mediated pathway is not specific pathway in the maintenance of T cell survival. $CD25^+$ $CD4^+$ T cells were cultured with an anti-CD3 antibody in complete medium containing 100 nM methotrexate (MTX), and the frequency and absolute cell numbers of $Foxp3^+$ and $Foxp3^-$ $CD4^+$ T cells were determined. Data are means ± SEM (n = 4). Data are representative of two independent experiments.
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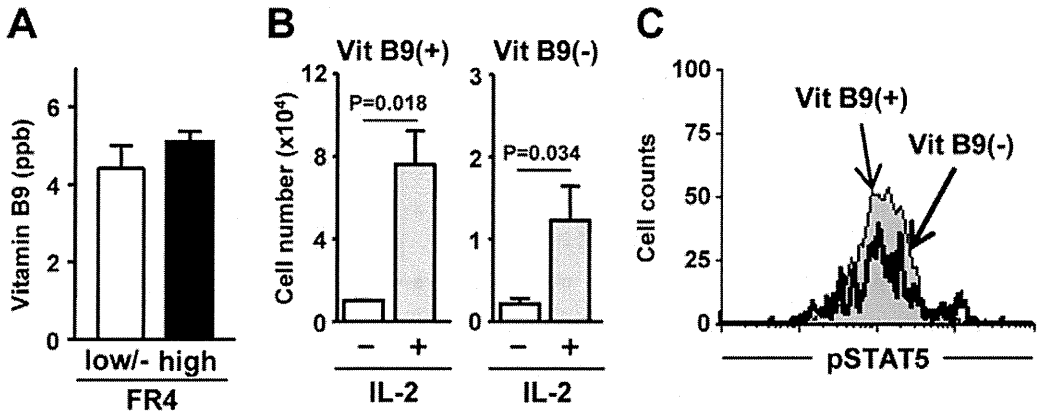

Figure 4. Vitamin B9 is IL-2-independent survival factor for Treg cells. (A) The amounts of intracellular vitamin B9 were measured using purified $CD4^+FR4^{hi}$ Treg or $CD4^+FR4^{low/-}$ non-Treg cells. Data are means ± SEM (n = 4). (B, C) Experiments similar to those shown in Fig. 1B were performed in the presence of anti-CD3 antibody stimulation with or without IL-2 stimulation. Cell number of $Foxp3^+CD4^+$ T cells (B) and the expression of phosphorylated STAT5 (pSTAT5) in $Foxp3^+CD4^+$ T cells (C) were determined. Data in (B) are means ± SEM (n = 6). Similar results were obtained from 3 separate experiments.
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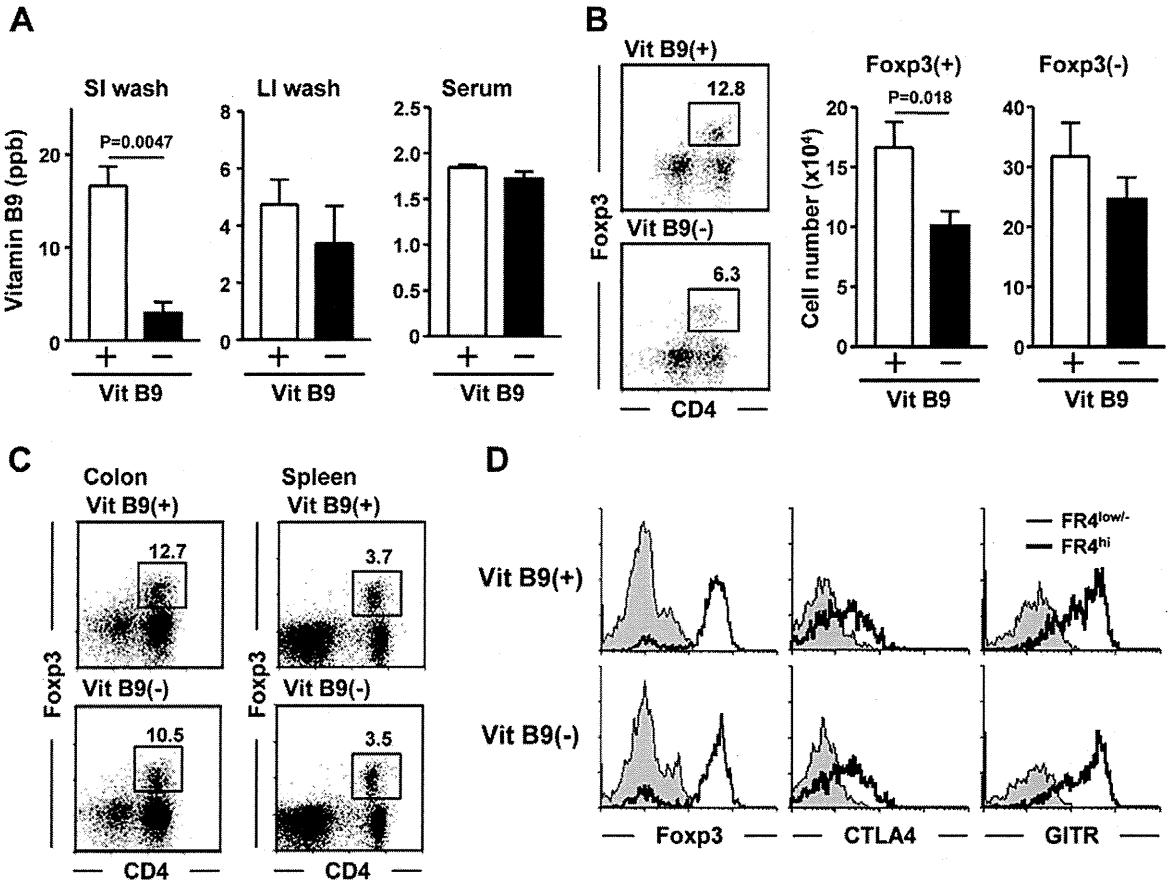

Figure 5. Depletion of dietary vitamin B9 selectively reduces Treg cells in the small intestine. Mice were maintained on a control [Vit B9(+)] or vitamin B9-depleted [Vit B9(−)] diet for 8 wk. (A) Vitamin B9 concentrations were measured in intestinal washes of the small intestine (SI), large intestine (LI), and serum. The data are mean ± SEM (n = 6). (B, C) The frequency and cell numbers of $Foxp3^+$ and $Foxp3^-$ $CD4^+$ T cells in the small intestine (B), colon, and spleen (C) were calculated using the total cell number and flow cytometric data (mean ± SEM, n = 6). (D) Flow cytometric analysis was performed to determine the expression levels of Foxp3, CTLA4, and GITR on the surface of $FR4^{low/-}$ (thin line) and $FR4^{hi}$ (thick line) $CD4^+$ T cells in the LP. Similar results were obtained from 3 separate experiments.
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non-Treg cells was not significantly changed in the colon and spleen of mice maintained with vitamin B9(−) diet (Fig. 5C), which could be explained by the similar concentration of vitamin B9 in the large-intestinal washes and sera of both groups of mice. We also found that Foxp3 and the inhibitory molecules CTLA4 and GITR, which are specifically expressed on Treg cells, were comparable between those mice (Fig. 5D).

Discussion

We have shown that vitamin B9 is crucial for the maintenance of Treg cells. Intriguingly, vitamin B9 was required for the survival of differentiated Treg cells, but was not necessary for the differentiation of naïve T cells into Treg cells. This selective effect of vitamin B9 on Treg cells is opposite to the effect of RA, a vitamin A metabolite, which enhances the differentiation of naïve T cells into Treg cells [11,12,13,14]. RA also induces the expression of gut-homing molecules (e.g., $\alpha4\beta7$ integrin and CCR9) on B and T cells activated by gut dendritic cells [9,10]. Because CCR9 was expressed normally on Treg cells in the LP of mice maintained with vitamin B9(−) diet (data not shown), the deficiency of dietary vitamin B9 did not affect the RA-mediated expression of gut-homing molecules and, predictably, the induction of Treg cells in the small intestine.

Treatment with the vitamin B9 antagonist MTX affected survival of both Treg cells and non-Treg cells, suggesting that the carrier-mediated pathway maintains sufficient amounts of intracellular vitamin B9 for cell survival regardless of the T-cell subset. The indiscriminate effects of MTX could be explained by the ubiquitous expression of the folate carrier [29,34]. As the mechanism of FR4-mediated Treg-cell maintenance, we considered initially that the proliferative activity of Treg cells could require large amounts of vitamin B9 as a source of nucleotides for DNA and RNA. However, the amounts of intracellular vitamin B9 were identical between Treg and non-Treg cells, implying that FR4 specifically recognizes extracellular vitamin B9 for the maintenance of Treg cell survival, consistent with a report that FR4 expressed on Treg cells contributes to their immune function and survival [25]. Additionally, the specific biological functions of vitamin B9 receptors (FR1, FR2, and FR4) have been predicted on the basis of their ~70% amino acid sequence identity, but the expression of each receptor is rigidly restricted, with narrow tissue and cell specificity [35,36]. Because FR1, FR2, and FR4 are glycosyl phosphatidylinositol–anchored proteins [37], adapter molecules may assist FR4 in the maintenance of Treg cell survival. We found that vitamin B9/FR4 was not associated with IL-2–mediated signaling in Treg cells. We will continue to study how FR4-mediated vitamin B9 regulates the survival of Treg cells.

Mammals must obtain vitamin B9 from the diet or from commensal bacteria. The absorption of vitamin B9 from the diet occurs mainly in the small intestine, whereas the uptake of microbial vitamin B9 predominantly occurs in the colon [38]. This explains why depletion of dietary vitamin B9 specifically decreased Treg cells in the small intestine, but not in the colon. It has been proposed that bacterial vitamin B9 absorbed in the colon affects the vitamin B9 status of the host [39,40], which may explain the lack of changes in vitamin B9 in the serum and splenic Treg cells in mice maintained with vitamin B9(−) diet. *Bifidobacterium*, one of the most important genera of commensal bacteria to be used as a probiotic, is well-studied as a vitamin B9 producer [41], and colonic Treg cells are specifically induced by immunological crosstalk with commensal bacteria, especially *Clostridium* clusters IV and XIVa [5]. Although whether *Clostridium* clusters IV and XIVa produce vitamin B9 remains unclear, our current findings suggest that vitamin B9 is an essential survival factor for Treg cells and, in vivo situation, diet vitamin B9 establishes an immunological network in the maintenance of Treg cells specifically in the small intestine.

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Author Contributions

Conceived and designed the experiments: JK. Performed the experiments: JK EH II. Analyzed the data: JK EH II. Wrote the paper: JK HK.

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Review

Immunological Function of Sphingosine 1-Phosphate in the Intestine

Jun Kunisawa [1,2,*] and Hiroshi Kiyono [1,2,3,4,*]

[1] Division of Mucosal Immunology, Department of Microbiology and Immunology, Institute of Medical Science, The University of Tokyo, Tokyo 108-8639, Japan

[2] Department of Medical Genome Science, Graduate School of Frontier Science, The University of Tokyo, Chiba 277-8562, Japan

[3] Graduate School of Medicine, The University of Tokyo, Tokyo 113-0033, Japan

[4] Core Research for Evolutional Science and Technology (CREST), Japan Science and Technology Agency, Tokyo 102-0076, Japan

* Authors to whom correspondence should be addressed;
E-Mails: kunisawa@ims.u-tokyo.ac.jp (J.K.); kiyono@ims.u-tokyo.ac.jp (H.K.);
Tel.: +81-3-5449-5274 (J.K.); +81-3-5449-5270 (H.K.); Fax: +81-3-5449-5411.

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Abstract: It has been shown that dietary materials are involved in immune regulation in the intestine. Lipids mediate immune regulation through a complex metabolic network that produces many kinds of lipid mediators. Sphingosine-1-phosphate (S1P) is a lipid mediator that controls cell trafficking and activation. In this review, we focus on the immunological functions of S1P in the regulation of intestinal immune responses such as immunoglobulin A production and unique T cell trafficking, and its role in the development of intestinal immune diseases such as food allergies and intestinal inflammation, and also discuss the relationship between dietary materials and S1P metabolism.

Keywords: intestinal immunity; lipid; IgA antibody; intraepithelial T lymphocytes; food allergy

1. Introduction

It is generally accepted that dietary components are involved in immune regulation. The intestinal immune system, especially, seems to be directly affected by the digestion and absorption of dietary materials. Intestinal tissues are primary sites for infection by many pathogenic microorganisms, and commensal bacteria are abundant. Thus, the intestinal immune system has to create harmonious immunological condition, and the disruption of the intestinal immune homeostasis leads to the development of allergic, inflammatory, and infectious diseases [1,2].

Dietary lipids seem to be the dietary materials most involved in the regulation of intestinal immune responses after the conversion into lipid mediators [3]. Among various lipid mediators, sphingosine-1-phosphate (S1P) is a biologically active sphingolipid that regulates cell trafficking and activation [4,5]. S1P is abundantly present in the blood and lymph, which is originated from the cell membranes from sphingomyelin and is produced mainly by platelets, erythrocytes, and endothelial cells [6]. It is degraded by S1P lyase in the lymphoid tissues [7]. This metabolic pathway establishes an S1P gradient between the blood/lymph and lymphoid tissues and mediates cell trafficking.

The S1P gradient is recognized by cells expressing S1P receptors, and these cells migrate toward high concentrations of S1P. Of the five types of S1P receptor, type-1 S1P receptors (S1P1) are preferentially expressed by lymphocytes, and they determine lymphocyte emigration from and retention in the lymphoid tissues [8]. S1P1 is highly expressed in naive lymphocytes, including single-positive thymocytes expressing either CD4 or CD8, and expression is decreased upon lymphocyte activation. S1P1 expression recovers once the activated lymphocytes are fully differentiated and this recovery leads to their emigration from the lymphoid tissues into the blood circulation [4,5]. Studies indicate that the trafficking of macrophages, dendritic cells, and natural killer cells is mediated by S1P2, S1P3, and S1P5, respectively [9–11].

Recent studies have revealed additional functions of S1P in immune regulation that are independent of cell trafficking [4]. For example, differentiation of T cells is regulated by S1P1-mediated signaling [12–14]. It has also been demonstrated that a S1P2-mediated pathway is involved in the activation of mast cells [15] and macrophages [16], and that S1P3 are involved in dendritic cell endocytosis [10]. These findings together suggested that the S1P plays critical role in the activation and differentiation of immunocompetent cells involved in the both innate and acquired phases of immune responses in addition to their function of cell trafficking.

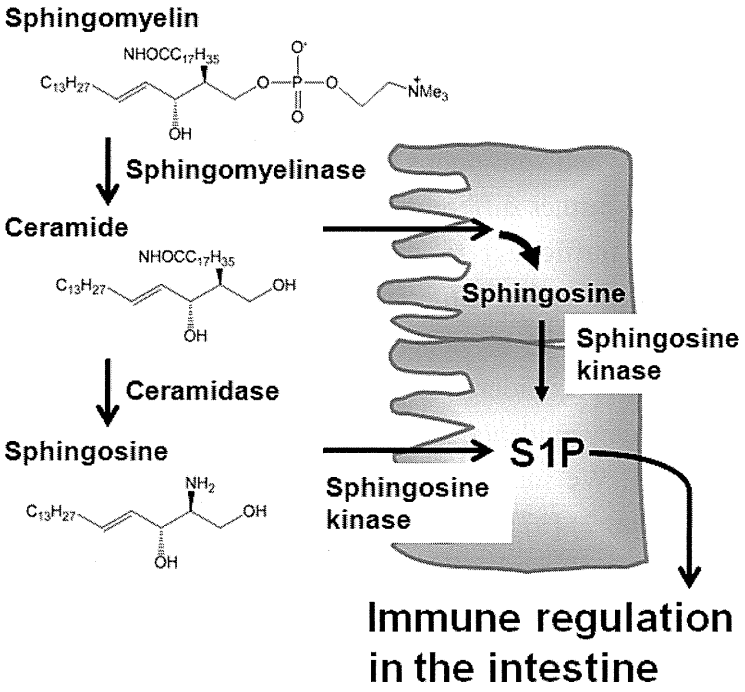
These biological and immunological functions show that S1P is involved in the maintenance of immunosurveillance as well as the development of immune diseases. In this review, we discuss the relationship between dietary materials (e.g., lipids, vitamin, and colorant) and S1P metabolism and describe the immunological functions of S1P, such as regulation of immunoglobulin A (IgA) production and intraepithelial T-lymphocyte trafficking, and its role in the development of intestinal immune diseases such as food allergy and intestinal inflammation.

2. Relationship Between S1P and Dietary Lipids

Several lines of evidence demonstrate that intestinal tissues contain higher levels of sphingolipids, including S1P, than other tissues [17]. There is no evidence of intestinal uptake of sphingolipids from

the blood, and germfree rats have comparable levels of sphingolipids in the intestine to conventional specific pathogen-free (SPF) rats [18]. Therefore, it is plausible that a source of sphingolipids in the intestine could be daily consumed diet. Adult humans ingest around 0.3 to 0.4 g sphingolipids per day, especially sphingomyelin from meat, milk, egg, and fish [19]. Dietary sphingomyelin is not directly absorbed, but is first degraded into ceramide and sphingosine [20,21] by alkaline sphingomyelinase and ceramidase, respectively, which are expressed on the apical membranes of epithelial cells [22,23]. Because epithelial cells express several key enzymes (e.g., sphingosine kinase) in the production of S1P from ceramide and sphingosine [23,24], it is possible that epithelial cells obtain ceramide and sphingosine from dietary sphingomyelin to produce S1P (Figure 1), thereby regulating intestinal immune responses and the associated intestinal immune diseases. Consistent with this, several studies showed that the incidence and severity of intestinal inflammation was changed by the uptake of dietary sphingomyelin [25,26] and the enzymatic activity of sphingomyelinase [27] and sphingosine kinase [28]. In addition, it was reported that dietary cholesterol inhibits the intestinal absorption of sphingolipids [29], implicating that cholesterol-rich Western diets may affect the availability of S1P precursors and consequently interfere with S1P-mediated intestinal immunity.

Figure 1. Dietary sphingolipids in epithelial-cell S1P production. Dietary sphingomyelin is degraded into ceramide and subsequently sphingosine by alkaline sphingomyelinase and ceramidase, respectively, which are expressed on the apical membranes of epithelial cells. In the epithelial cells, absorbed ceramide is metabolized into sphingosine. Together with absorbed sphingosine, sphingosine kinase metabolizes sphingosine into S1P, which then participates in immune regulation in the intestine.



3. Regulation of S1P Metabolism by Dietary Materials

In addition to dietary lipids, other dietary materials are also involved in the regulation of S1P metabolism. For instance, S1P lyase, a key enzyme to degrade S1P and thus keep optimal S1P low

concentration, requires vitamin B6 as a co-factor [7]. Thus, administration of vitamin B6 antagonist impaired S1P lyase activity, which consequently led to the defect of lymphocyte trafficking caused by inappropriate S1P gradient [7]. Similar effect was noted in 2-acetyl-4-tetrahydroxybutylimidazole (THI), a component of caramel food colorant III used in food products. THI inhibits S1P lyase and thus, like treatment with vitamin B6 antagonist, prevents normal lymphocyte trafficking [7]. These findings led to the use of THI for the treatment of immune diseases [30–32].

4. S1P Regulates Innate and Acquired Phases of Intestinal IgA Responses

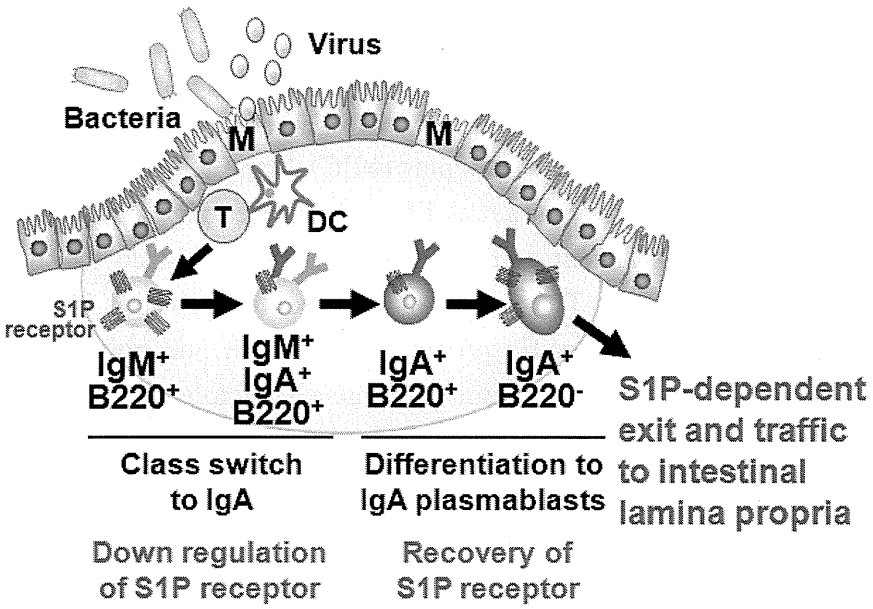
IgA is the most frequently observed antibody isotype in the intestinal compartments and provides the first line of defense against pathogenic microorganisms invading through mucosal tissues. Therefore, the induction of appropriate IgA responses is a logical strategy for the development of oral vaccines [33]. Since IgA antibody is one of the major arms of the mucosal immune system in the digestive tract, which covers a large surface area, the intestinal IgA is originated from several induction sites including Peyer's patches (PPs), isolated lymphoid follicles, and the peritoneal cavity [34].

A well characterized gut-associated lymphoid tissue (GALT) is PPs. PPs act as induction sites for the initiation of IgA responses against T-cell-dependent antigens [35]. PPs are covered with a specialized epithelium known as follicle associated epithelium (FAE) containing antigen-sampling M cells, which are responsible for the uptake and transport of antigens from the intestinal lumen to antigen-presenting cells such as dendritic cells (DCs) (Figure 2) [36]. Then, DCs capture antigens from the M cells, process and present them to T cells. It has been shown that the formation of PP DC-T cell clusters provide both cellular and molecular environment for the generation of IgA committed B cells in PPs [34]. In this pathway, some of the activated T cells differentiate into follicular helper T cells to help the antibody class switching of B cells in the germinal centers [34]. Because of the unique cytokine environment (e.g., TGF-β, IL-4, and IL-21) and continuous stimulation by commensal bacteria in the intestine, PPs have been shown to equip with efficient molecular and cellular environment for the spontaneous and continuous B cell class switching from IgM to IgA [34,35]. After class switching to IgA, B cells further differentiate into IgA plasmablasts and then migrate out from the PPs for their subsequent trafficking to the intestinal lamina propria, where they terminally differentiate into plasma cells producing dimeric (or polymeric) forms of IgA. This process mainly contributes to the development of T cell-dependent antigen-specific immune responses. Thus, the PP-mediated induction pathway is considered to be a major arm of the acquired IgA response [34].

Our investigation provided new evidence that S1P regulated the B cell trafficking in the PPs for the intestinal IgA production [37]. We initially found that S1P1 expression in B cells changes during differentiation in the PPs (Figure 2) [37]. High levels of S1P1 expression were detected in IgM^+ naive B cells, and expression was down-regulated when B cells started class switching to IgA. The low expressions of S1P1 allowed newly class-switched IgA^+ B cells to retain in the PPs for the sufficient differentiation into the IgA^+ plasmablasts. S1P1 expression was restored on the IgA^+ plasmablasts, resulting in their emigration from the PPs. Mice treated with FTY720, an immunosuppressant inducing S1P1 downregulation [38], show selective accumulation of IgA^+ plasmablasts in the PPs, leading to the disturbance of continuous delivery of IgA committed B cells from the PPs to the lamina propria of intestine. Consequently, the decrease of same population in the intestinal lamina propria was noted,

which associated with the reduction of intestinal antigen-specific IgA responses against orally immunized protein antigen [37].

Figure 2. Sequential changes in S1P1 expression during B-cell differentiation in Peyer's patches. Dendritic cells (DC) take the antigens transported by M cells from intestinal lumen and present them to T cells for their activation. Through the interaction with T cells and DCs, IgM^+ naive B cells show class-switch from IgM to IgA. During this process, S1P1 is expressed at high levels in IgM^+ naive B cells and downregulated on B cells class-switching from IgM to IgA and subsequently recovered on IgA^+ $B220^-$ plasmablasts, resulting in their emigration from the Peyer's patches and traffic into the intestinal lamina propria.



In the IgA production pathway in the gut, peritoneal B cells are an additional source of intestinal IgA [39]. A number of peritoneal B cells belong to a unique B-cell subset, termed as B1 cells, which produces antibodies against T-cell-independent antigens such as lipids and polysaccharides. Because these T-cell-independent antigens are conserved in various microorganisms, B1-cell-derived antibodies undiscriminatingly react to commensal and pathogenic bacteria and prevent their attachment and invasion into the host. This reaction is opposite to antibody responses against protein antigen mediated by PP B cells, which show rigid specificity against microorganisms. Therefore, it has been considered that B1-cell-derived IgA is categorized as to be innate-type antibodies that recognize a wide range of microorganisms in the intestine [39].

Trafficking of peritoneal B1 cells into the intestine requires S1P-mediated signaling [40]. Like B cells in the PPs, peritoneal B1 cells identically expressed S1P1. Thus, trafficking of peritoneal B cells into the intestine and consequent production of intestinal IgA are diminished by treatment with FTY720, mainly because of the inhibition of B1 cell emigration from the parathymic lymph nodes, which drain to the peritoneal cavity [40]. This impaired trafficking in FTY720-treated mice was associated with the decreased IgA responses against phosphorylcholine (a T-cell-independent antigen) induced by oral immunization with heat-killed *Streptococcal pneumoniae* [40].

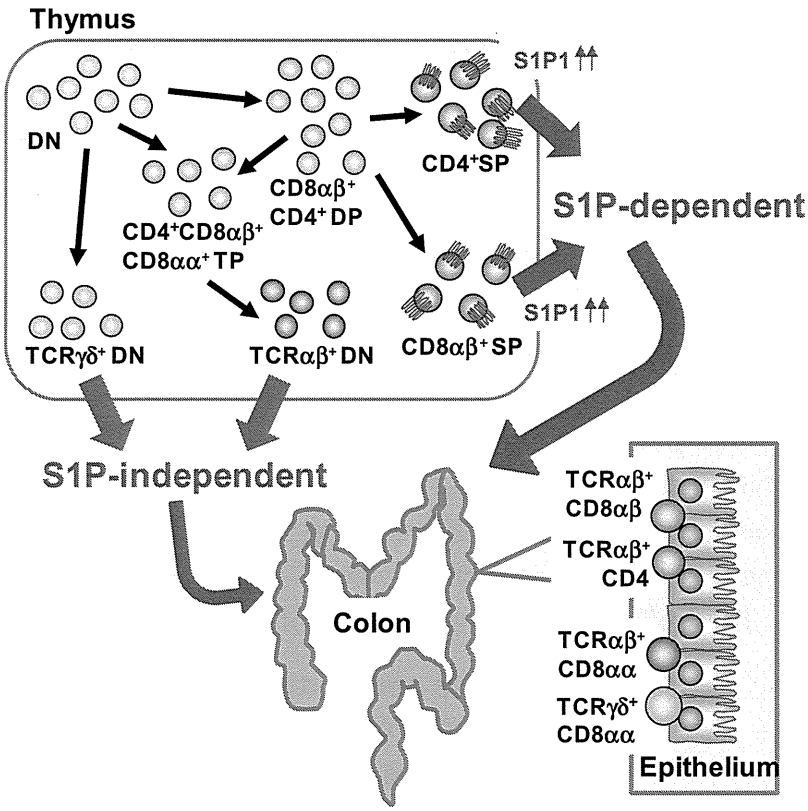
We also found that S1P-mediated regulation of peritoneal B-cell trafficking requires crosstalk with stromal cells in the peritoneal cavity [41]. This interaction mediated by adhesion molecules (e.g., ICAM-1 and VCAM-1) on stromal cells and the expression is regulated by NFκB-inducing kinase (NIK). Therefore, NIK-mutant aly/aly mice show decreased sensitivity to FTY720 in the regulation of peritoneal B-cell trafficking due to the impaired expression of adhesion molecules although peritoneal B1 cells in aly/aly mice expressed comparable levels of S1P1.

5. Distinct S1P Dependency of Trafficking of Intraepithelial T-Lymphocytes in the Gut

Large numbers of lymphocytes are also present in the intestinal epithelium and called as intraepithelial lymphocytes (IELs) [42]. IELs are mostly T cells, but unlike in conventional T cells observed in the systemic compartments (e.g., spleen) which predominantly express the αβ T-cell receptor (αβTCR), in the IEL subset there is an abundance of T cells expressing the γδ T cell receptor (γδTCR) in addition to $\alpha\beta TCR^+$ T cells [42]. αβTCR recognizes peptide antigen presented via major histocompatibility complex (MHC) molecules, whereas γδTCR recognizes non-classical MHC molecules such as MHC class I chain-related proteins (MIC) A and B (MICA/B) in human and Rae-1 in mouse [43]. Unlike MHC molecules that act as ligand by presenting peptide antigen, non-classical MHC molecules act as a ligand by itself and the expression was induced by stress (e.g., infection, tumors, or chemical treatment) [44]. Thus, it is considered that αβTCR is involved in acquired immunity through the activation by specific presentation of antigenic peptides, whereas γδTCR is involved in innate immunity by the ligation of non-classical MHC molecules [42]. A distinctive pattern of CD8 expression has also been noted in IELs. Conventional $\alpha\beta TCR^+$ T cells express CD8 as a heterodimer of α and β (CD8αβ). In contrast, some IELs uniquely express CD8 as a homodimer (CD8αα) [42]. A previous study identified a unique precursor of CD8αα IELs in the thymus [45]. In the thymus, $CD4^-$ $CD8^-$ double-negative thymocytes differentiate into $CD4^+$ $CD8^+$ double-positive thymocytes and then further differentiate into single-positive thymocytes expressing either CD4 or CD8. $CD8\alpha\beta^+$ IELs are derived mainly from $CD8^+$ single-positive thymocytes expressing αβTCR. $CD8\alpha\alpha^+$ IELs, however, originate from double-negative thymocytes expressing either αβTCR or γδTCR that have themselves differentiated from unique $CD4^+$ $CD8\alpha\alpha^+$ $CD8\alpha\beta^+$ triple-positive thymocytes (Figure 3) [45].

S1P has been involved in the regulation of cell trafficking of different subsets of IELs originated from thymus. We found that each type of IEL shows a different dependency on S1P in its trafficking from the thymus to the intestine, especially in the colon (Figure 3) [46]. When mice were treated with FTY720, decreased numbers of $CD8\alpha\beta^+$ IELs were observed. In contrast, the numbers of $CD8\alpha\alpha^+$ IELs were barely affected. These data suggest that, in the colonic epithelium, $CD8\alpha\beta^+$ IELs are S1P dependent and $CD8\alpha\alpha^+$ IELs are S1P independent. Consistent with this finding, $CD8^+$ single-positive thymocytes—the precursors of $CD8\alpha\beta^+$ IELs—express high levels of S1P1 [8], whereas no S1P1 expression has been noted on double-negative thymocytes, the precursors of $CD8\alpha\alpha^+$ IELs [46]. These findings suggest that S1P1 expression was different in different subsets of thymic precursors of IELs and provide versatile immunological pathways in the intestine.

Figure 3. Distinct dependency on S1P in T-cell trafficking into the colonic epithelium. In the thymus, $CD4^-$ $CD8^-$ double-negative (DN) thymocytes differentiate into $CD4^+$ $CD8^+$ double-positive (DP) thymocytes and then into single-positive (SP) thymocytes expressing either CD4 or CD8 and αβTCR. These SP thymocytes express high levels of S1P1 and migrate out from the thymus and into the colon in an S1P-dependent manner. DN thymocytes express TCRαβ or TCRγδ. DN thymocytes expressing TCRαβ are derived from $CD4^+$ $CD8\alpha\alpha^+$ $CD8\alpha\beta^+$ triple-positive (TP) thymocytes differentiated from DN or DP thymocytes. Little or no S1P1 expression is noted in the DN thymocytes expressing TCRαβ or TCRγδ, so traffic to the colonic epithelium proceeds in an S1P-independent manner.

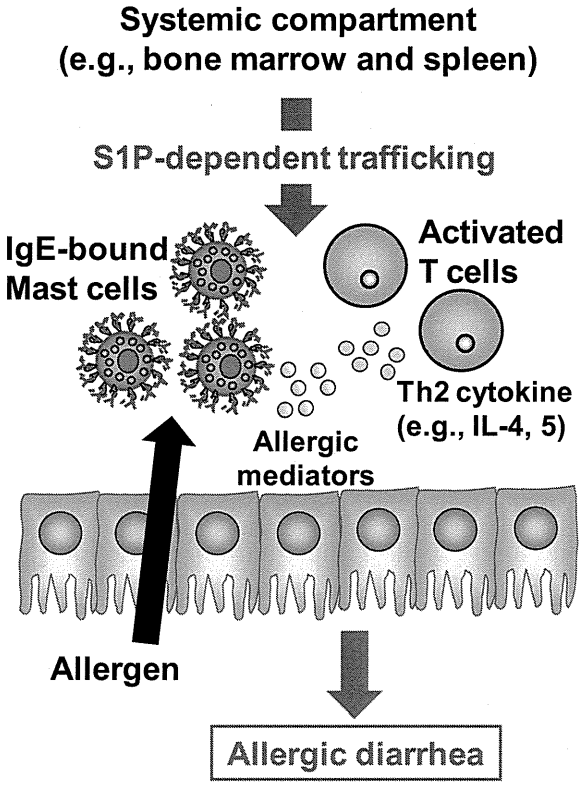


6. S1P-Mediated Regulation in the Development of Intestinal Immune Diseases

Accumulating evidence has revealed the pivotal role of S1P in the development of inflammatory diseases such as autoimmune type 1 diabetes, rheumatoid arthritis, and multiple sclerosis [5]. FTY720 prevents the egress of autoreactive lymphocytes from the lymph nodes into the peripheral circulation and subsequent across the blood–brain barrier into the central nerve system and thus has recently been approved as an oral therapy for multiple sclerosis [47]. In addition to being involved in these immune diseases at the systemic immune compartments, S1P is involved in the development of intestinal immune diseases including food allergies and intestinal inflammation [5]. The number of patients with food allergies has increased not only in children but also in adults; the development of effective preventive and therapeutic strategies for food allergies is therefore required to improve patients' quality of life. Using the ovalbumin-induced murine food-allergy model developed by our group [48], we examined the molecular and cellular mechanisms underlying the development of food allergies and found that, in allergic mice, activated T cells migrate into the colon, where they produced high

amounts of Th2 cytokines such as IL-4 and IL-5 [48]. We demonstrated that the trafficking of pathogenic T cells from the systemic compartments into the colon was mediated by S1P (Figure 4) [49]. Indeed, activated T cells in the colon of allergic mice expressed S1P1 and their infiltration into the colon and subsequent production of Th2 cytokines (e.g., IL-4 and IL-5) were inhibited by the treatment with FTY720 [49]. In addition, the infiltration of mast cells, effector cells in the development of food allergy, into the colon was also prevented in the FYT720-treated mice [49]. As a mechanism of FTY720-mediated inhibition of mast cell infiltration, it was likely that FTY720 directly and indirectly prevented the mast cell infiltration into the colon. Direct effect of FTY720 was predicted by results that mast cells expressed S1P1 and their *in vitro* migration was inhibited by FTY720 [49]. Indirect effect is mediated by activated T cells producing Th2 cytokines which enhanced the proliferation and recruitment of mast cells [50]. Thus, inhibition of activated T cell trafficking into the colon by FTY720 resulted in the reduced recruitment and/or proliferation of mast cells. Taken together, involvement of S1P in the trafficking of both pathogenic T cells and mast cells is a potential target for prevention and treatment of food allergies.

Figure 4. S1P mediates intestinal allergy by regulating pathogenic T and mast cell infiltration into the colon. In murine food allergy model, systemically sensitized T cells migrate into the colon upon the oral challenge with same allergen. This trafficking is mediated by S1P and thus treatment with FTY720 resulted in the inhibition of activated T cell trafficking into the colon. In the colon, these activated T cells produced high amounts of Th2 cytokines such as IL-4 and IL-5 for promotion of mast cell recruitment and proliferation. In addition, mast cell itself expresses S1P1. Therefore, FTY720 treatment directly and indirectly (Th2 cytokine from activated T cells) decreases the numbers of mast cells in the colon. These effects lead to the inhibition of allergic diarrhea.



Similarly, several lines of evidence have demonstrated that the FTY720 treatment prevents the development of intestinal inflammation [51–53]. For example, in a spontaneous colitis model in interleukin-10-deficient mice, administration of FTY720 suppressed the infiltration of pathogenic T cells producing interferon-γ [51]. Infiltration of the colon by pathogenic T cells was also inhibited by treatment with FTY720 in both a dextran sulfate sodium (DSS)-induced colitis model and a T-cell transfer model in mice [52,53]. Although S1P regulates the activation of several inflammatory cells via modulation of the signaling of certain innate receptors such as toll-like receptors, TNF receptor, and protease-activated receptor 1, and S1P itself is produced by activated inflammatory cells [4], collectively these findings suggest that S1P–S1P1 axis participates mainly in the development of intestinal immune diseases at the stage of pathogenic cell trafficking into the colon.

7. Conclusion

It is clear from past and current studies that S1P plays an important role in the regulation of the immune system of the gut in both healthy and disease states. In general, S1P is derived from sphingomyelin and is produced mainly by platelets, erythrocytes, and endothelial cells in the body. However, in the intestine, it is likely that epithelial cells contribute most to the production of S1P. Most importantly, S1P produced by epithelial cells seems to originate from dietary sphingolipids, especially sphingomyelin. Thus, elucidation of the complex networks established by dietary lipids will create a new era in nutrition-based mucosal immunology and should provide a new strategy against intestinal immune diseases.

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Conflict of Interest

The authors declare no conflict of interest.

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