In addition to these barriers, the gastrointestinal tract includes immunological defense system, in particular secretory-immunoglobulin A (IgA) [3], which is predominantly produced at intestinal mucosa by the harmonious interaction between ECs and mucosal lymphocytes and blocks microbial infections by inhibiting adherence of mucosal pathogens at the intestinal lumen to host ECs. Secretory IgA (SIgA) can also neutralize toxins produced by gut pathogens by binding to biologically active sites of toxins.

The immunological characteristics of the gastrointestinal tract have focused attention on the development of effective oral vaccines. Oral vaccination offers several advantages over parenteral vaccination, including needle-free delivery, easy and comfortable administration, and the possibility of self-delivery. Most importantly, oral vaccination can induce both mucosal and systemic immunity, leading to the double layers of protective immune responses [4]. In contrast, parenteral immunization primarily yields a systemic immune response. Therefore, effective oral vaccination could establish a first line of immunological defense in the intestinal tract, a major site of pathogen entry, as well as promote immune surveillance perhaps at other mucosal and systemic sites. One of the major strategies of oral vaccine has been induction of pathogen- or toxin-specific SIgA.

The hostile environment of the gastrointestinal tract (low pH, presence of digestive enzymes, and the detergent activity of bile salts) often makes it difficult to induce protective immune responses by oral vaccination with antigen alone. Additionally, effective oral delivery of antigen to the induction site of the mucosal immune system (e.g., gut-associated lymphoid tissues :GALT) is made difficult by the significant dilution and dispersion of antigen that occurs in the lumen since a total interior area of the intestinal wall is thought to be equivalent to over one tennis coat surface. Further, physical barriers, such as mucus and the tight junctions between the ECs prevent the effective delivery of vaccine antigen. To overcome these obstacles, effort has focused on development of effective antigen delivery systems. In this review, we describe the immunological features of gut-associated lymphoid tissue as the most obvious target site of antigen delivery in the development of oral vaccines. We also describe the current strategies being used to develop versatile antigen delivery systems for efficient oral vaccination.

2. Immunological features of GALT

GALTs comprise several different organized lymphoid structures [5]. Among them, Peyer's patches (PPs) are well characterized as sites for the initiation of intestinal IgA responses. Isolated lymphoid tissue (ILF) is another GALT structure, which is also important in the induction of intestinal IgA responses.

2.1. Peyer's patches (PPs)

PPs are considered to be one of the largest organized lymphoid tissues in the gastrointestinal immune system. There are generally 8 to 10 PPs in the small intestine of mice and hundreds in humans [6]. Each PP is composed of several B cell-rich follicles surrounded by a mesh-like structure consisting of T cells known as interfollicular region (IFR) (Fig. 1).

Although PPs share some common immunological and micro-architectural features with peripheral secondary lymphoid organs, they are harboring unique features as the mucosa-associated lymphoid tissue [6]. For example, PPs contain efferent but not afferent lymphatics. To compensate, PPs are covered with a specialized epithelial region, termed follicle-associated epithelium (FAE), containing specialized antigen-sampling microfold or membranous cells (M cells). The M cells are characterized by short microvilli, a thin mucus layer, small cytoplasmic vesicles, and efficient transcytosis activity, allowing the selective and efficient transfer of antigens from the intestinal lumen into PPs (Fig. 2) [7]. Thus, M cells are considered to be a professional antigen sampling and gateway cells for the mucosal immune system.

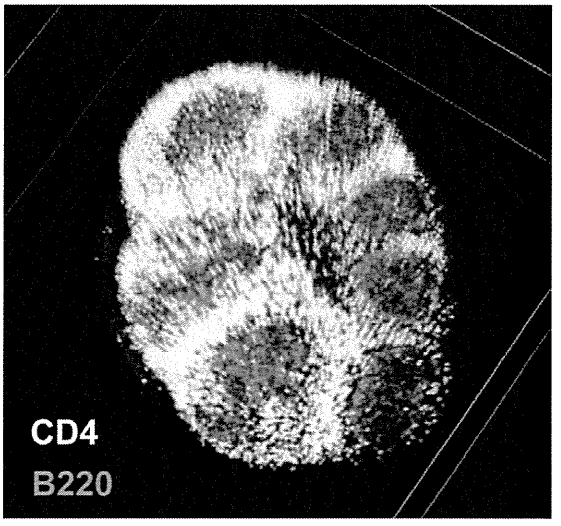

Fig. 1. Microarchitecture of murine Peyer's patches. Purified T cells (green) and B cells (red) were chemically labeled with carboxyfluorescein succinimidyl ester and arboxy SNARF-1, respectively, and adoptively transferred into mice. Fifteen hours after the transfer, cell distribution in the Peyer's patches was observed at the whole tissue level by using macro-confocal microscopy.

Dendritic cells (DCs) are abundant in the subepithelial dome region (SED) under the FAE, which thus can immediately take up orally encountered antigens from M cells and process and present antigenic peptides to mucosal T and B cells for the initiation of antigen-specific immune responses (Fig. 3). DCs are also found in the IFR. They are composed of at least three distinct subsets: $CD11c^+$ DCs in the SED, $CD8\alpha^+$ DCs in the T cell-rich IFRs, and double-negative DCs in both the SED and IFRs [8]. In addition to antigen presentation, DCs in the intestinal tissues express retinal dehydrogenase, an enzyme that converts vitamin A into retinoic acid. Retinoic acid promotes the preferential homing of activated antigen-specific T and B cells into the intestinal lamina propria by inducing the expression of gut imprinting molecules, such as $\alpha4\beta7$ integrin and CCR9 [9,10].

B cells, a major component of PP cells (~75%), are preferentially located in the follicle region (Figs. 1 and 3). Unlike other lymphoid organs, formation of germinal centers (GC) occurs in the PPs even under homeostatic conditions by the continuous stimulation from commensal bacteria, in which leads to the creation of molecular and

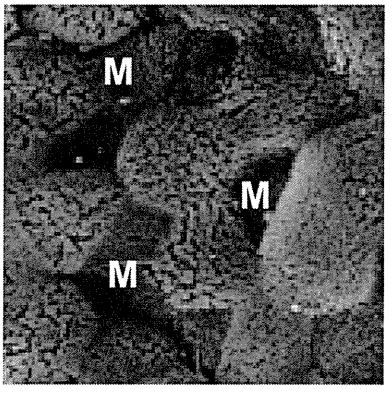

Fig. 2. Scanning electron micrograph of M cells in the Peyer's patches. Scanning electronic microscopy demonstrates that the M cells (indicated as "M") in the Peyer's patches are distinguished from surrounding ECs by their depressed position relative to the ECs, dark brush border, and short microvilli.

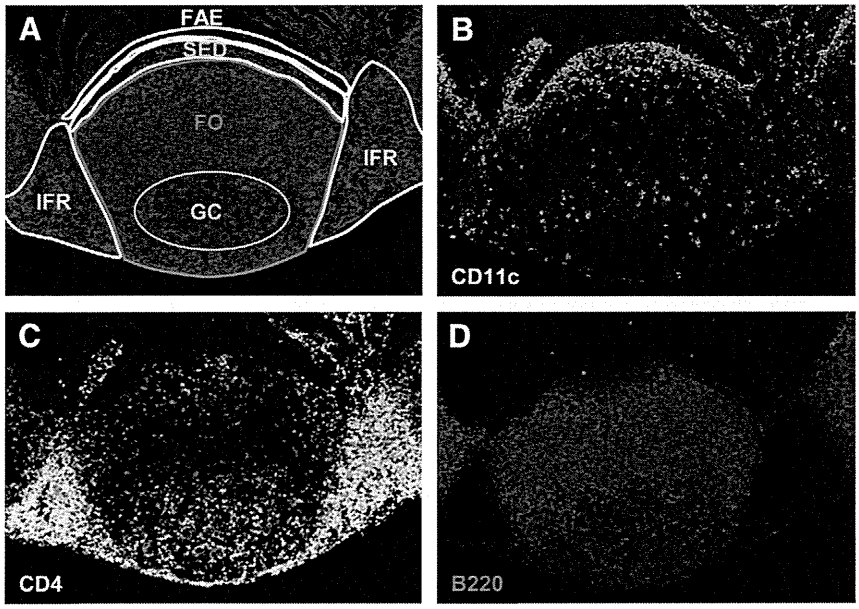

Fig. 3. Distinct cell distribution in the Peyer's patches. Immunohistochemical data on Peyer's patches is shown. (A) Each cell was identified with 4′,6-diamidino-2-phenylindole staining. PP compartments are outlined and labeled as follows: FO, follicle; FAE, follicle-associated epithelium; GC, germinal center; IFR, intrafollicular region; SED subepithelial dome. (B–D) Immunohistochemical staining of PPs for: dendritic cells (anti-CD11c; B), T cells (anti-CD4; C), B cells (anti-B220; D).

cellular environment for class switching of B cells from IgM to IgA (Fig. 3). Thus, PPs contain B cells at several differentiation and maturation stages: IgM^+B220^+ (~70%), $IgM^+IgA^+B220^+$ (~1%), IgA^+B220^+ (~3%), and IgA^+B220^- (~0.5%).

Approximately 20% of PP cells are T cells. Some portions of T cells are found in the IFRs of the PPs, which contain mainly naive T cells (Figs. 1 and 3) [11]. In addition to naïve T cells, other T cells exhibit active phenotype, including IFN-γ-producing Th1, IL-4-producing Th2, and IL-10-producing $Foxp3^+$ regulatory T cells [12]. A recent study demonstrated that at least some portions of $Foxp3^+$ regulatory T cells differentiated into follicular helper T cells which facilitate the B cell class switching to IgA^+ B cells in the GC [13].

Organogenesis of PPs is initiated in the embryonic stage. In mice, clustering of mesenchymal-lineage $VCAM\text{-}1^+ICAM\text{-}1^+$ PP organizer (PPo) cells starts at the site of tissue anlagen at embryonic days 14–16 [14]. PP inducer (PPi) cell are also key cells that initiate PP organogenesis. PPi cells are a component of lymphoid tissue inducer (LTi) cells that express key transcription factors, Id2 and RORγt, as well as a unique pattern of cell surface markers (IL-7 receptor [IL-7R]$^+$, $CD3^-$, $CD4^+$, $CD45^+$, lymphotoxin [LT] α1β2). The interaction between PPi and PPo cells through the IL-7R and LTβ receptors (LTβR) with corresponding cytokines results in production of lymphoid chemokines such as CXCL13 and CCL19/CCL21 from PPo cells. These chemokines recruit lymphocytes and DCs to form the PP micro-lymphoid structure. Several lines of evidence have demonstrated that the loss of any part of the organogenesis pathways results in the disruption or impairment of PP development [14]. Of note, disruption of the PP organogenesis pathway by blockade of IL-7R and/or LTβR signaling during a limited time period leads to the selective loss of PPs without affecting other lymphoid tissue organogenesis [14]. Experiments with PP-deficient mice showed that they failed to develop antigen-specific immune responses against orally administered particle-form antigens but retained their ability to respond to soluble forms of antigens [15,16], suggesting that PPs play an important role in the induction of antigen-specific immune responses against particulate antigen. The finding may provide a clue for the creation of mucosal antigen delivery vehicle which effectively distributes vaccine to appropriate intestinal inductive lymphoid tissues (e.g., GALT or PPs) covered by FAE containing M cells.

2.2. Isolated lymphoid follicles

Mice selectively deficient in PPs retain certain levels of intestinal IgA responses [15,16]; this finding demonstrates the presence of alternative induction pathways for intestinal IgA production that are independent of PPs. In fact, ILFs were identified as an additional inductive tissue for IgA production. ILFs are located throughout the small intestine as clusters of 100–200 lymphocytes [17]. As for PPs, the formation of ILFs is mediated by the crosstalk between LTi cells and organizer cells. Thus, ILF formation was impaired in RORγt-deficient mice, which lack both PPs and ILFs. When RORγt-deficient mice were reconstituted with $ROR\gamma t^+$ LTi, naturally produced intestinal IgA responses were recovered with the newly formed ILFs [18].

ILFs are composed of a single follicle that contains predominantly B cells and some DCs and are covered with a FAE, which contains M cells [17]. In contrast to PPs, ILFs lack T cell-rich IFRs. In agreement with this finding, a recent report indicated that ILFs are a site for T cell-independent IgA production. Indeed, in contrast to PPs, which lack the IgA^+ cells in T cell-deficient mice, many IgA^+ B cells were still noted in the ILFs of TCR-deficient mice [18]. For the delivery of vaccine antigen to the gut mucosal immune system, an interesting strategy might be the selective delivery of T cell-dependent and -independent antigens to PPs and ILFs, respectively.

3. Antigen-sampling system in the gut

3.1. M cells in the GALT are specialized for antigen sampling

As mentioned above, FAE in the PPs contains M cells that act as a portal for uptake of antigen from the intestinal lumen and transfer into the PPs [19]. Approximately 10% (mouse) and 5% (human) of cells in FAE are M cells [19]. In both mouse and humans, M cells have been shown to harboring some biological and immunological uniqueness that distinguishes them from surrounding ECs. For example, M cells are characterized by short microvilli, a thin glycocalyx, and reduced activity of intracellular lysosomes [19]. In addition, M cells exhibit an intra-pocket structure at basal sites, where lymphocytes and/or antigen-presenting cells including DCs locate. These features allow the M cells to easily take particle-form antigens including microorganisms from the

lumen and transport them into the PPs without digestion and processing [19]. M cells also show a unique glycosylation pattern. Thus, ulex europaeus (UEA-1) lectin binds α(1,2) fucose residues that are specifically expressed on mouse M cells and Goblet cells [20]. Similarly, sialyl Lewis A antigen recognized by specific antibody (LM112) is a potential candidate for an M cell marker in humans [21]. We recently developed a murine M cell-specific antibody (NKM 16-2-4) [22]. Intriguingly, the antibody also recognized α(1,2) fucose like UEA-1, but did not bind to Goblet cells that are recognized by UEA-1[20], indicating that additional unique glycosylation pattern exists in M cells. Thus, one interesting and novel approach would be continuous search and characterization of glycoprotein modification patterns of FAE cells for the development of glycosylation targeted vaccine delivery system.

In addition to physiological and morphological features, several receptors important for invasion of pathogens and/or uptake of luminal antigens have been identified on M cells. For example, β1 integrin, identified as a receptor for invasin-mediated infection by *Yersinia*, is expressed on M cells [23]. *Salmonella typhimurium* encodes the specific adhesion molecule, long polar fimbria, which targets M cells [24]. Reovirus derived protein σ1 binds to M cells [25]. Recently, glycoprotein 2 (gp2) was found to be expressed specifically on both human and murine M cells; it recognizes FimH, a component of type I pili on bacterial outer membranes, and thus gp2 acts as a receptor for FimH-expressing bacteria such as *Escherichia coli* and *S. Typhimurium* [26,27].

Several key pathways important in the development of M cells were also recently identified [28]. At the cellular level, studies in B cell-deficient mice suggest that B cells play an important role in the M cell development in PPs. B cell-deficient mice had a decreased number of M cells in PPs and adoptive transfer of B cells reversed this phenotype [29]. At the molecular level, the TNF superfamily plays a critical role in the development of M cells. A recent study demonstrated that CD137 (also known as 4-1BB and induced by lymphocyte activation [ILA]) is required for the functionality of M cells. CD137 deficiency thus resulted in a defect in particle transcytosis by M cells [30]. The fact that the ligand of CD137, 4-1BBL, is expressed on B cells and myeloid lineage cells may explain why M cell development is impaired in B cell-deficient mice. In addition to CD137, another TNF receptor superfamily member, receptor activator of nuclear factor κ-B ligand (RANKL), is reported to be involved in M cell differentiation. The number of M cells in FAE of PPs is reduced in mice lacking RANKL or treated with RANKL-specific neutralizing antibody [31]. These findings will likely yield novel strategies to enhance the M cell development and function, resulting in more efficient antigen delivery in the GALT. Thus, M cell development and function regulating molecules may become new generation of mucosal adjuvants for supporting and enhancing antigen-specific immune responses to orally administered vaccine.

3.2. Epithelial cells and villous M cells

Intestinal ECs not only act as a physiological barrier, but also take part in the immunological function of the intestine by the formation of secretary form of immunoglobulin leading to the secretion of IgA and IgM into the intestinal lumen [1]. Reciprocally, IgG, which is involved in the antigen transport system, is transported from the intestinal lumen via the neonatal Fc receptor (FcRn) expressed on the apical surface of ECs [32]. In addition, ECs release exosomes containing antigen bound to MHC class II. The released MHC-bound antigen is thought to induce tolerance, not activation, of antigen-specific T cell responses [33]. This system might be important aspect of the gut immune system for the creation of immunologically quiescence condition at the harsh environment of intestine.

Among ECs in the villous epithelium, we identified M cells sharing similar characteristic with the M cells originally found in the FAE of PPs (or PP M cells) and termed them villous M cells [34]. Villous M cells are thus morphologically similar to M cells in the PPs and are recognized by UEA-1 lectin and M cell-specific NKM16-2-4 antibody, a marker of murine M cells. The specificity for UAE-1 and NKM 16-2-4 antibody suggests that villous M cells most likely harbor identical α(1,2) fucose based glycosylation molecules. Like M cells, villous M cells were capable of taking up *Salmonella*, *Yersinia*, and *Escherichia coli* expressing invasin. In addition, they are found in villous epithelium in PP-deficient mice, which allow them to still induce antigen-specific IgA responses [15,16]. Thus, villous M cells are an alternative antigen-sampling site and can be consider as the additional targeting site for oral vaccine delivery.

We recently reported that M cell-like α(1,2) fucose based glycosylation can be induced on intestinal ECs by environmental stimuli such as colonization with commensal biota, treatment with cholera toxin, or treatment with dextran sodium sulfate and termed these cells as fucosylated ECs (F-ECs) [35]. Although a functional role of F-ECs in the induction of immune responses against intestinal antigens needs further investigation, these findings suggest additional possible strategies to induce F-ECs for the enrichment of antigen-sampling system at the intestinal epithelium to vaccine administered via oral route.

3.3. Intraepithelial DCs

It is also known that the gut immune system is full of antigen-presenting cells including different subsets of DCs [8]. Some DCs are observed in the epithelium of the terminal ileum, where they extend their dendrites into the lumen and thus capable of taking-up intestinal microorganisms. Among the several subsets of DCs, epithelial DCs uniquely express CX3CR1. They penetrate the epithelial layer without disrupting the epithelial barrier connected with highly sophisticated tight junction molecules such as occludin, claudin 1 and zonula occludens 1, and capture luminal bacteria [36,37]. Because of their unique histological positioning at intestinal epithelium, these DCs can be called as "intraepithelial DCs". Unlike other DCs, $CX3CR1^+$ intraepithelial DCs are a non-migratory and gut-resident population, suggesting that the $CX3CR1^+$ population might play a critical role in the initiation or modulation of local immune responses in the intestinal epithelium or lamina propria regions [38]. Thus, these $CX3CR1^+$ DCs resided in the intestinal epithelium could also be useful targeted cell population for oral vaccine delivery.

4. Induction and regulation of IgA-mediated immune responses in the gut

4.1. GALT-mediated induction of IgA responses

A highly integrated sequence of processes of cellular and molecular interaction occurs in the PPs that lead to the initiation of antigen-specific immune responses (Fig. 4). Antigen transport from intestinal lumen by M cells at the FAE of PPs is an initial step for the induction of antigen-specific immune responses after oral immunization. Antigen is then taken up by DCs that are localized in the pocket of M cells or underneath M cells. Resultant up-regulation of CCR7 chemokine receptor expression on the DCs, allows them to move to the T cell region via locally produced corresponding chemokines (CCL19 and CCL21) in the PP or mesenteric lymph nodes and then present the processed peptide antigen for the generation of antigen-specific T cells [39].

Antigen-primed T cells support IgA class switching and somatic hyper mutation of B cells in the GC through antigen-specific interactions, CD40/CD40 ligand interaction, and cytokine expression (e.g., TGF-β, IL-4, and IL-21) [5]. Simultaneously, retinoic acid derived from PP DCs induces the expression on primed T and B cells of the gut-imprinting molecules α4β7 integrin and CCR9 [9,10]. B cells also alter their expression of receptors for other chemokines (e.g., CXCR5 and CCR10) and sphingosine 1-phosphate, thus determining whether they

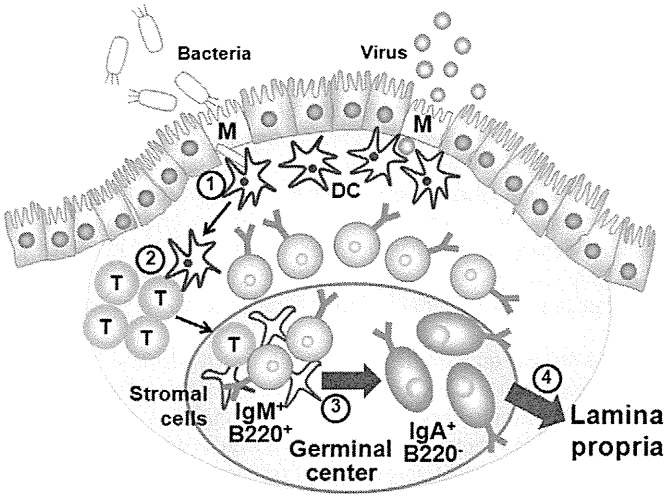

Fig. 4. Sequential processes for initiation of antigen-specific immune responses in Peyer's patches. (1) After transport of antigen by M cells, dendritic cells (DC) take up antigen, and (2) migrate to the T cell region. There, the DCs prime antigen-specific T cells by presenting antigen on MHC molecules 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.

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

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].

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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Microbe-dependent CD11b$^+$ IgA$^+$ plasma cells mediate robust early-phase intestinal IgA responses in mice

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Intestinal plasma cells predominantly produce immunoglobulin (Ig) A, however, their functional diversity remains poorly characterized. Here we show that murine intestinal IgA plasma cells can be newly classified into two populations on the basis of CD11b expression, which cannot be discriminated by currently known criteria such as general plasma cell markers, B cell origin and T cell dependence. CD11b$^+$ IgA$^+$ plasma cells require the lymphoid structure of Peyer's patches, produce more IgA than CD11b$^-$ IgA$^+$ plasma cells, proliferate vigorously, and require microbial stimulation and IL-10 for their development and maintenance. These features allow CD11b$^+$ IgA$^+$ plasma cells to mediate early-phase antigen-specific intestinal IgA responses induced by oral immunization with protein antigen. These findings reveal the functional diversity of IgA$^+$ plasma cells in the murine intestine.

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Immunoglobulin (Ig) A is an antibody found predominantly in the intestinal lumen, where it protects the host against pathogenic infections[1,2]. It also has an important role in the creation and maintenance of immunological homoeostasis by shaping homeostatic communities of commensal bacteria[3–5]. Indeed, some patients with IgA deficiency show marked susceptibility to infections with pathogens such as *Giardia lamblia*, *Campylobacter*, *Clostridium*, *Salmonella* and rotavirus; they also have increased incidences of intestinal immune diseases such as coeliac disease and inflammatory bowel diseases[6].

Peyer's patches (PPs) are the major sites for the initiation of antigen-specific intestinal IgA production, mainly in a T cell-dependent manner[7]. Intestinal IgA also originates from B1 cells. B1 cells differ from B2 cells in terms of origin, surface markers (for examples, B220, IgM, IgD, CD5, CD11b and CD23), growth properties and V_H repertoire[8–10]. B1 cells are predominantly present in the peritoneal cavity (PerC) and traffic into the intestinal compartment for the production of IgA against T cell-independent antigens such as DNA and phosphatidylcholine[11]. T cell independent antigen-specific IgA responses are also initiated in the isolated lymphoid follicles (ILFs), which are small clusters of B2 cells in the intestine[12].

Upon Ig class switching from μ to α, IgA^+ B cells acquire the expression of type 1 sphingosine-1-phosphate receptor, CCR9 and α4β7 integrin, allowing them to migrate out from the PPs or PerC and traffic to the intestinal lamina propria (iLP)[11,13,14]. In the iLP, they further differentiate into IgA-secreting plasma cells (PCs) under the influence of terminal differentiation factors (for example, IL-6)[15]. As these locally produced IgA antibodies are continuously transported and secreted by epithelial cells as a form of secretory IgA into the intestinal lumen, stably high levels of IgA production are required for the maintenance of sufficient amounts of IgA; this production is determined by the generation, survival and function of IgA PCs.

Several lines of evidence have demonstrated that the function and survival of PCs in the systemic compartments (for example, spleen and bone marrow (BM)) are not only determined by intrinsic factors but are regulated by the presence of environmental niches[16]. As with systemic PCs, differentiation of IgA PCs in the iLP is regulated by exogenous factors such as IgA-enhancing cytokines (for example, interleukin (IL)-5, IL-6, IL-10, IL-15, a proliferation-inducing ligand (APRIL) and B cell activating factor (BAFF))[7,15]. In addition, microbial stimulation is required for the full effects of intestinal IgA. Indeed, germ-free (GF) mice have decreased intestinal IgA responses with immature structures of PPs and ILFs[17,18]. Previous studies in mono-associated GF mice have indicated that only a small proportion of the total amount of intestinal IgA is reactive to monoassociated bacteria; microbe-dependent IgA production is therefore mediated by polyclonal stimulation through innate immune receptors such as toll-like receptors, rather than through B cell receptors specific for microbial antigens[19,20]. Accumulating evidence has revealed the molecular and cellular pathways of IgA production mediated by innate immunity, including the involvement of myeloid differentiation primary response gene 88 (MyD88) in the regulation of tumour necrosis factor/inducible nitric oxide synthase-producing DCs in the iLP[21] and follicular DCs in the PPs[22]. However, the effects of microbial stimulation on the regulation of differentiated IgA^+ PCs remain to be investigated. Here, we identified unique microbe-dependent subsets of IgA^+ PCs, which add a new level of complexity to the intestinal IgA system of mice.

Results

Microbe dependency of intestinal IgA^+ cells. To examine the immunological elements of intestinal IgA production associated with commensal bacteria, we initially compared the IgA^+ cells of specific pathogen-free (SPF) and GF mice. Flow cytometric analysis showed that $CD11b^+$ IgA^+ cells accounted for about 30% of IgA^+ cells, and we found a lack of $CD11b^+$ IgA^+ cells in the iLP of GF mice (Fig. 1a). Similarly, the numbers of intestinal $CD11b^+$ IgA^+ cells were reduced in both antibiotic-treated SPF mice and MyD88 KO mice (Fig. 1b–d). Immunohistological analysis indicated that $CD11b^+$ IgA^+ cells were dispersed throughout the iLP of wild-type (WT) mice (Fig. 1d), although their frequency appeared lower than expected from the flow cytometric data, probably because of difference in methodological sensitivity. These findings collectively suggest that $CD11b^+$ IgA^+ cells are unique subset that requires MyD88-dependent microbial stimulation for its development and maintenance.

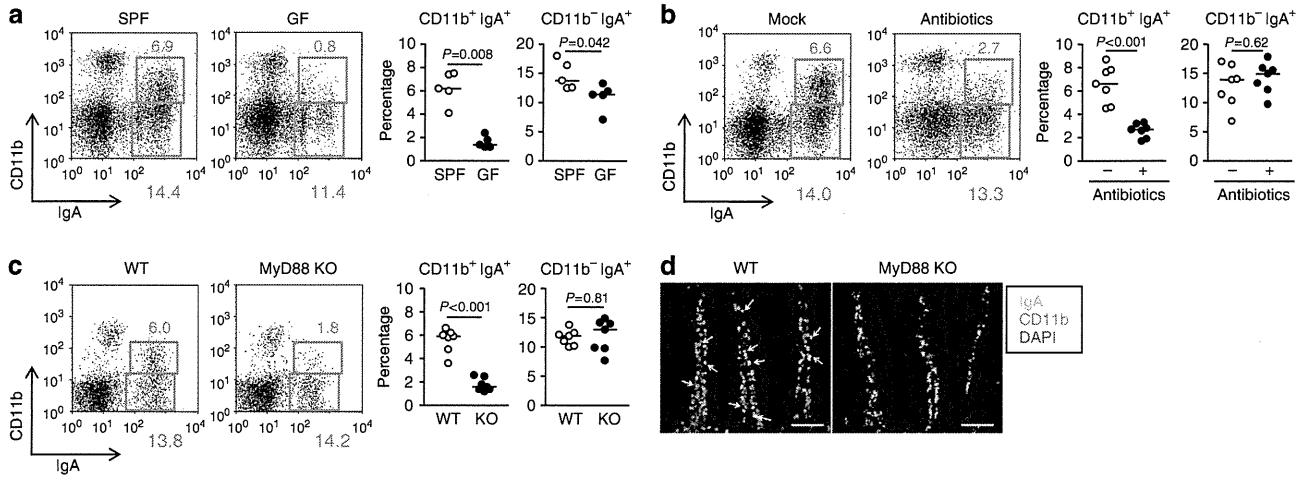

Figure 1 | Intestinal $CD11b^+$ IgA^+ cells require microbial stimulation. (**a**–**c**) Mononuclear cells were isolated from the small intestines of SPF or GF mice (**a**), mock- or antibiotic-treated SPF mice (**b**), or MyD88 WT or knockout (KO) mice (**c**) for analysis of IgA and CD11b expression by flow cytometry. Graphs show data from individual mice, and bars indicate median. Statistical analyses were performed with Mann–Whitney's *U*-test. (**d**) Specimens of small intestinal tissues of WT and MyD88 KO mice were stained for IgA and CD11b, and counterstained with 4′,6-diamidino-2-phenylindole. Data are representative of three independent experiments. Scale bars, 50 μm.

Intestinal CD11b$^+$ IgA$^+$ cells are PCs. We next aimed to characterize the CD11b$^+$ and CD11b$^-$ IgA$^+$ cells in the iLP. In addition to a gating strategy to exclude the possibility that the CD11b$^+$ IgA$^+$ cells detected by flow cytometry were doublets (Supplementary Fig. S1), we further performed a cytospin analysis and confirmed that both CD11b$^+$ and CD11b$^-$ IgA$^+$ cells had homogeneous morphology that was the same as that of PCs (for example, large irregular nuclei with prominent nucleoli), whereas CD11b^{hi} IgA$^-$ cells were composed of different kinds of cells, including eosinophils and macrophages (Fig. 2a). We also confirmed that both CD11b$^+$ and CD11b$^-$ IgA$^+$ cells did not express markers for macrophages (F4/80), DCs (CD11c) or eosinophils (CCR3) (Fig. 2b). Thus, CD11b$^+$ IgA$^+$ cells are neither doublets nor myeloid cells decorated by bound IgA on their surfaces.

CD11b$^+$ and CD11b$^-$ IgA$^+$ cells were identical in cell size and density, as determined by forward scatter (FSC) and side scatter (SSC), respectively, and by their surface expression patterns (CD19^{int}, B220$^-$, CD138$^+$, CD38^{hi} and CD40^{int}) (Fig. 2c). Although PCs in the systemic compartments (for example, the spleen) generally express little or no surface immunoglobulin[23], we previously confirmed that CD38$^+$ CD138$^+$ cells in the iLP express IgA both on the cell surface and in the intracellular compartment (Supplementary Fig. S2)[13]. These findings indicated that both CD11b$^+$ and CD11b$^-$ IgA$^+$ cells could be classically categorized as PCs. This view was further supported by our finding that both populations expressed equal levels of Blimp1, a master transcription factor for PCs (Fig. 2c)[23].

The phenotypes of IgA$^+$ cells in the iLP differed from those of IgA$^+$ cells in the spleen. Splenic CD11b$^-$ IgA$^+$ cells exclusively had a memory phenotype (that is, B220$^+$, CD138$^-$, CD38^{int} and CD40^{hi}), whereas splenic CD11b$^+$ IgA$^+$ cells contained almost equal amounts of B220$^+$ CD138$^-$ CD38^{int} CD40^{hi} memory cells and B220$^-$ CD138$^+$ CD38^{hi} CD40^{low} PCs (Supplementary Fig. S3). These results indicated that CD11b$^+$ IgA$^+$ cells in the iLP were unique PCs that had an immunologically different status from splenic CD11b$^+$ IgA$^+$ cells.

Intestinal CD11b$^+$ IgA$^+$ PCs require PP lymphoid structure. CD11b$^+$ IgA$^+$ PCs expressed CD18 (Supplementary Fig. S4), which associates with CD11b and acts as a ligand for intercellular adhesion molecule-1 (ICAM-1)[24]. As ICAM-1 is an endothelial adhesion molecule that regulates cell trafficking[24,25], we considered that CD11b$^+$ IgA$^+$ PCs were recent emigrants from IgA-inductive tissues (for example, PPs and PerC) and had migrated into the iLP. To test this possibility, we employed FTY720 to inhibit the trafficking of IgA-committed B cells from PPs and PerC into the iLP. As we previously reported[11,13], FTY720 treatment reduced the numbers of intestinal IgA$^+$ PCs,

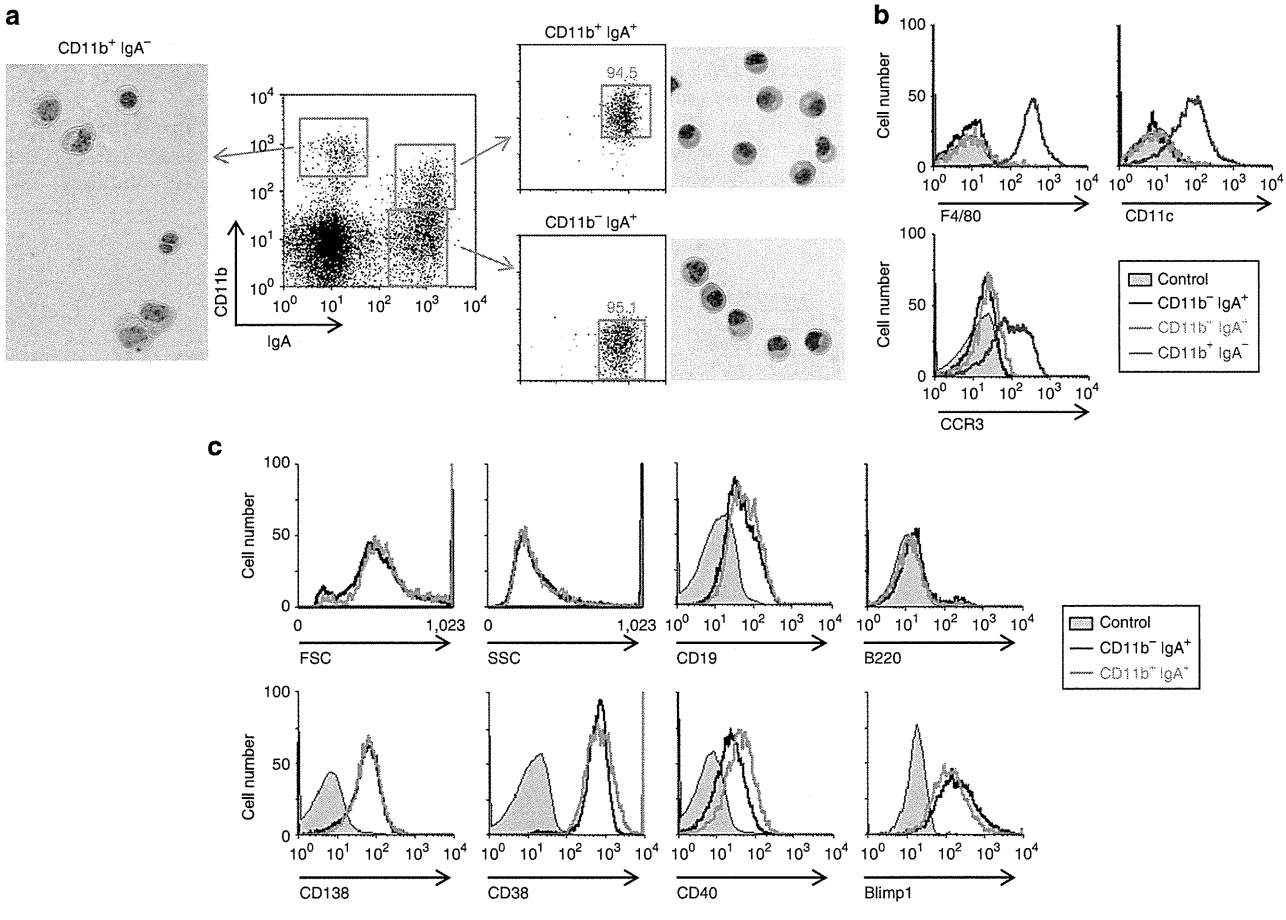

Figure 2 | Both CD11b$^+$ and CD11b$^-$ IgA$^+$ cells in the intestine are categorized as plasma cells. (**a**) Cells were purified by cell sorting from the iLP, and their morphology was examined by haematoxylin and eosin staining after cytospin. Data are representative of three independent experiments. (**b**) Cells were isolated from the iLP for the analysis of F4/80, CD11c and CCR3 expression on CD11b$^-$ IgA$^+$, CD11b$^+$ IgA$^+$ and CD11b$^+$ IgA$^-$ cells. Grey indicates isotype control. Similar results were obtained from three separate experiments. (**c**) Cells were isolated from the iLP for comparisons between CD11b$^+$ and CD11b$^-$ IgA$^+$ cells in terms of cell size (FSC) and density (SSC), and expression of CD19, B220, CD138, CD38, CD40 and Blimp1. Grey indicates isotype control. Similar results were obtained from five separate experiments.

but the effect was not specific to CD11b$^+$ IgA$^+$ PCs (Fig. 3a). These data suggested that CD11b$^+$ IgA$^+$ PCs were not recent emigrants from IgA inductive tissues (for example, PPs and PerC).

The second possibility was that CD11b$^+$ IgA$^+$ PCs originated from B1 cells, because CD11b is a marker of peritoneal B1 cells[26]. To test this possibility, peritoneal CD11b$^+$ B1 cells were purified and adoptively transferred into severe combined immunodeficiency mice. As we reported previously[11], adoptively transferred CD11b$^+$ B1 cells migrated into the intestine, where they differentiated into IgA$^+$ PCs. Although we transferred B cells expressing CD11b, they lost their CD11b expression in the iLP (Supplementary Fig. S5). Although only a few cells were detected in the iLP under these experimental conditions, CD11b expression was likely to be reversible on B cells and was thus not to be a marker of PCs originating from peritoneal CD11b$^+$ B1 cells.

As a third possibility for discriminating between CD11b$^+$ and CD11b$^-$ IgA$^+$ PCs, we examined the T cell dependency of their differentiation and IgA production. For this, we employed TCRβδ mice. Although TCRβ δ mice had decreased levels of intestinal IgA$^+$ cells, the ratio between CD11b$^+$ and CD11b$^-$ IgA$^+$ PCs did not differ between the WT mice and the TCRβ δ mice (Fig. 3b).

We also examined the production of IgA against T cell dependent and T cell independent antigens by CD11b$^+$ and CD11b$^-$ IgA$^+$ PCs. For the analysis of T cell dependent antigen, mice were orally immunized with ovalbumin (OVA) plus cholera toxin (CT). Following three oral immunizations, substantial amounts of OVA-specific IgA antibody-forming cells (AFCs) were detected in the iLP by enzyme-linked immunosorbent spot (ELISPOT) assay; this production was reduced by almost 50% when either the CD11b$^+$ IgA$^+$ or the CD11b$^-$ IgA$^+$ cells were removed before the ELISPOT assay (Fig. 3c). Similar results were obtained when we enumerated IgA AFCs against phosphorylcholine, a typical TI antigen, induced by commensal bacteria (Fig. 3c)[27]. These results collectively suggested that both CD11b$^+$ IgA$^+$ and CD11b$^-$ IgA$^+$ cells almost equally included IgA AFCs producing IgA antibodies specific for T cell dependent and T cell independent antigens.

Next, to examine the involvement of PPs, we established PP-null mice by *in utero* treatment with anti-IL-7Rα antibody[28] and found that PP-null mice had reduced numbers of CD11b$^+$ IgA$^+$ PCs in the iLP (Fig. 3d). In addition, CD11b was not expressed on IgA$^+$ B cells in the PPs (Fig. 3e). We treated mice with anti-IL-7Rα antibody only once *in utero* and confirmed that it did not affect the ILFs[28]. Although it is still possible that CD11b$^+$ IgA$^+$ PCs specifically require IL-7, the most plausible conclusion based on our current findings is that CD11b$^+$ IgA$^+$ B cells require the lymphoid structure of PPs, and CD11b$^-$ IgA$^+$ B cells acquire CD11b expression in the iLP.

As in antibiotic-treated and MyD88 KO mice (Fig. 1), the numbers of CD11b$^-$ IgA$^+$ PCs changed little in PP-null mice (Fig. 3d), suggesting that it is unlikely that CD11b$^+$ IgA$^+$ PCs differentiate back into CD11b$^-$ IgA$^+$ cells in the iLP. This view is further supported by the results of *in vitro* analysis. When purified CD11b$^+$ and CD11b$^-$ IgA$^+$ PCs were separately cultured with different kinds of stimulants (for example, phorbol 12-myristate 13-acetate plus ionomycin, or lipopolysaccharide) little change was noted in CD11b expression (Supplementary Fig. S6). Although the origin of these cells remains to be firmly established, it is plausible that CD11b$^+$ IgA$^+$ PCs act as a separate lineage once they differentiate in the iLP.

High proliferation activity of CD11b$^+$ IgA$^+$ PCs. We next performed a gene microarray analysis to assess the uniqueness of CD11b$^+$ IgA$^+$ PCs in the iLP. Gene ontology enrich-

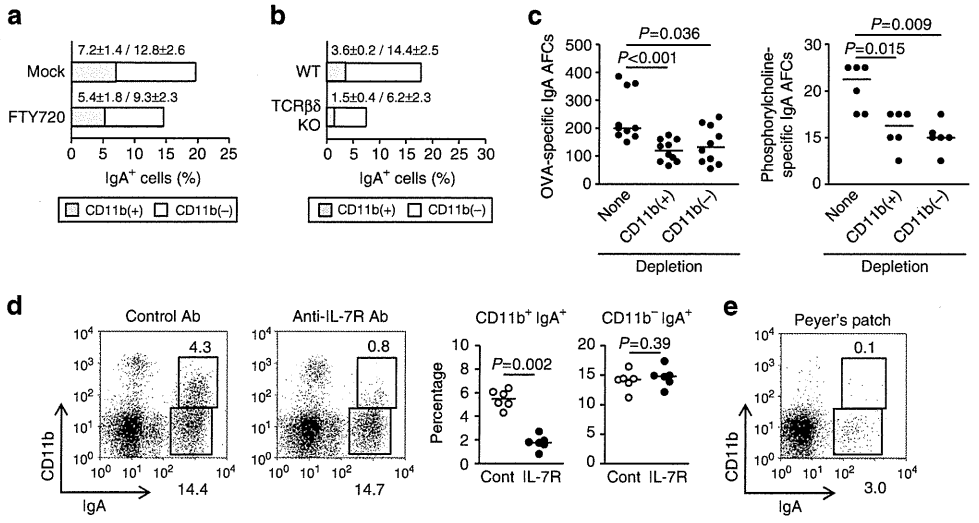

Figure 3 | CD11b$^+$ IgA$^+$ cells require the lymphoid structure of Peyer's patches. (**a**) Mice were treated with FTY720 every day for 5 days. The day after the final treatment, the proportions of CD11b$^+$ and CD11b$^-$ IgA$^+$ cells were measured by flow cytometry. Data are presented as means ± s.d. from four mice. Similar results were obtained from three separate experiments. (**b**) Proportions of CD11b$^+$ and CD11b$^-$ IgA$^+$ cells in the iLP of WT and TCRβδ KO mice were measured by flow cytometry. Data are presented as means ± s.d. from four mice. Similar results were obtained from three separate experiments. (**c**) After three oral immunizations with OVA plus cholera toxin, cells were isolated from the iLP and used in an ELISPOT assay to enumerate OVA-specific IgA AFCs. In some groups of mice, CD11b$^+$ or CD11b$^-$ IgA$^+$ cells were depleted by cell sorting before application of ELISPOT assay. Phosphorylcholine-specific IgA AFCs were measured. Graphs show data from individual mice, and bars indicate median. Statistical analyses were performed with Mann–Whitney's *U*-test. (**d**) Mononuclear cells were isolated from the iLP of Peyer's patch (PP)-normal (control Ab) and -null (anti-IL-7Rα Ab) mice for analysis of IgA and CD11b expression by flow cytometry. Graphs show data from individual mice. Statistical analyses were performed with Mann–Whitney's *U*-test. (**e**) Mononuclear cells were isolated from PPs for analysis of CD11b$^+$ and CD11b$^-$ IgA$^+$ cells by flow cytometry. Similar results were obtained from three separate experiments.

ment score computation analysis showed that the activity of cell-cycle-associated pathways was higher in CD11b$^+$ IgA$^+$ PCs than in CD11b$^-$ IgA$^+$ PCs (Supplementary Table S1). Consistent with this finding, higher expression of cell-cycle-associated genes was noted in CD11b$^+$ IgA$^+$ PCs than in CD11b$^-$ IgA$^+$ PCs; these genes included members of the cell division cycle family (Fig. 4a and Supplementary Table S2). In line with this, these cells expressed higher levels of the proliferation marker Ki67 than did CD11b$^-$ IgA$^+$ PCs (Fig. 4a and Supplementary Table S2). Additionally, CD11b$^+$ IgA$^+$ PCs showed greater uptake of bromodeoxyuridine (BrdU) than did CD11b$^-$ IgA$^+$ PCs (Fig. 4b). CD11b$^+$ IgA$^+$ PCs were preferentially removed by treatment with cyclophosphamide (CPM), which selectively targets proliferating cells (Fig. 4c). These data collectively suggested that CD11b$^+$ IgA$^+$ PCs possessed greater proliferating activity than did CD11b$^-$ IgA$^+$ PCs in the iLP.

Microarray analysis further identified CD150 (also known as signalling lymphocytic activation molecule family member 1, SLAMF1)[29], β1 integrin and CD168 (also known as hyaluronan-mediated motility receptor)[30] as possible candidates uniquely expressed on CD11b$^+$ IgA$^+$ PCs (Supplementary Table S3). Flow cytometric analysis confirmed that CD11b$^+$ IgA$^+$ PCs expressed higher levels of CD150 than did CD11b$^-$ IgA$^+$ PCs, whereas CD11b$^+$ IgA$^+$ and CD11b$^-$ IgA$^+$ PCs identically expressed β1 integrin and no CD168 (Supplementary Fig. S7).

IL-10 is essential for intestinal CD11b$^+$ IgA$^+$ cells. We next aimed to identify key molecules for inducing and maintaining CD11b$^+$ IgA$^+$ PCs in the iLP. As CD11b$^+$ IgA$^+$ PC numbers were reduced in MyD88 mice (Fig. 1c), and MyD88 is expressed in not only hematopoietic cells, including B cells, but also non-hematopoietic cells, including epithelial cells[31], we performed BM chimeric experiments to determine whether MyD88 in non-hematopoietic cells, hematopoietic cells, or both, was required for the generation of CD11b$^+$ IgA$^+$ cells. Similar levels of CD11b$^+$ IgA$^+$ cells were observed in irradiated WT mice receiving WT or MyD88 BM cells and in irradiated MyD88 mice receiving WT BM cells (Supplementary Fig. S8), suggesting that MyD88-dependent molecules commonly expressed in both non-hematopoietic and hematopoietic cells are involved in the microbe-dependent induction of CD11b$^+$ IgA$^+$ PCs.

We then examined the involvement of cytokines known to enhance IgA responses. Among several IgA-enhancing cytokines (for example, IL-5, IL-6, IL-10 and APRIL/BAFF)[7,15], we found that neutralization of IL-10 resulted in preferential reduction in CD11b$^+$ IgA$^+$ PCs, whereas blocking of other cytokines induced a reduction in IgA$^+$ cell numbers regardless of CD11b expression (Fig. 5a). Additionally, CD11b$^+$ IgA$^+$ cell numbers were preferentially reduced in IL-10 KO mice (Fig. 5b). As normal differentiation into IgA$^+$ B cells was observed in the PPs and PerC of IL-10 KO mice (Supplementary Fig. S9), it is plausible that IL-10 targets the maintenance of CD11b$^+$ IgA$^+$ cells in the iLP, but not the induction of IgA$^+$ cells in inductive tissues such as PPs and PerC.

Early-phase robust IgA responses by proliferating IgA$^+$ PCs. To examine the immunological importance of proliferating IgA$^+$ PCs present mainly in CD11b$^+$ IgA$^+$ PCs, mice were orally immunized with OVA plus CT. In this assay, one group received CPM treatment during immunization and the second group received CPM treatment 4 days after the final immunization (Fig. 6a). Because of the high cell-proliferation activity, CPM treatment during oral immunization resulted in efficient killing of peanut agglutinin (PNA^{hi}) B220$^+$ GC B cells and thus a reduction in the numbers of IgA$^+$ IgM$^-$ plasmablasts in the PPs (Supplementary Fig. S10). Thus, treatment with CPM during oral immunization led to an ~90% reduction in the numbers of OVA-specific IgA AFCs (Fig. 6b); this was associated with almost complete disappearance of faecal IgA produced against OVA (Fig. 6c). On the other hand, when mice were treated with CPM 4 days after the final immunization to remove proliferating cells mainly present in CD11b$^+$ IgA$^+$ cells in the iLP, the reduction in numbers of OVA-specific IgA AFCs in the iLP was only about 50% (Fig. 6b). This finding was consistent with our current finding that CD11b$^+$ IgA$^+$ PCs accounted for half the number of OVA-specific IgA AFCs (Fig. 3c). Thus, CPM treatment after the last immunization preferentially depleted CD11b$^+$ IgA$^+$ cells, with little influence on CD11b$^-$

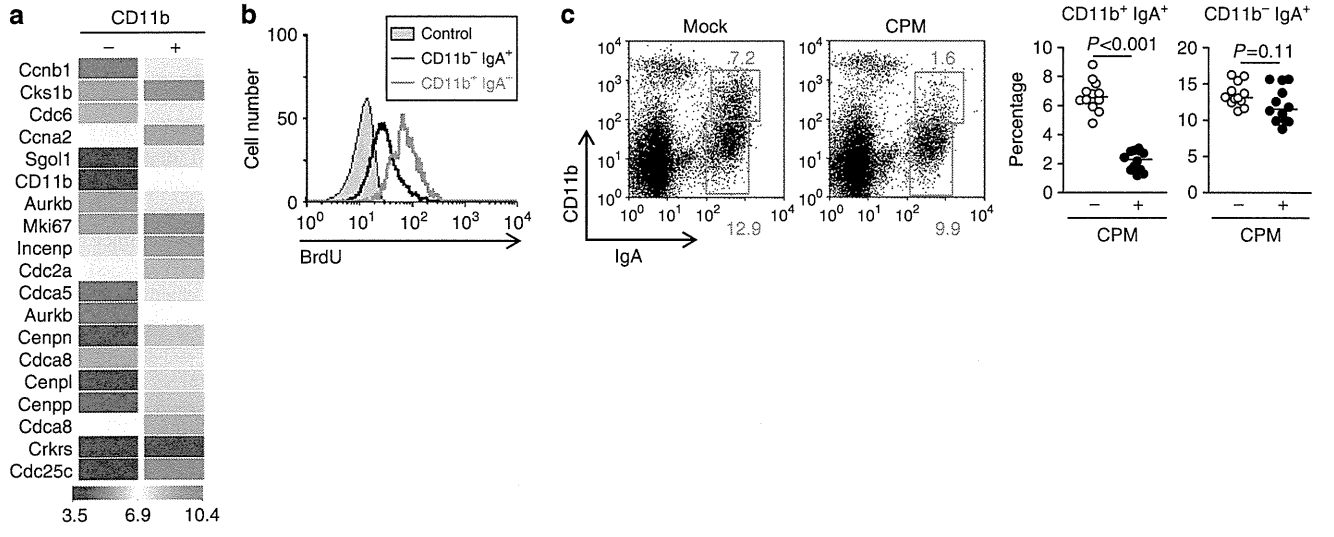

Figure 4 | CD11b$^+$ IgA$^+$ cells are proliferating cells. (**a**) mRNA was purified from small intestinal CD11b$^+$ and CD11b$^-$ IgA$^+$ cells and used for microarray analysis. Data related to the cell cycle and proliferation are shown. Data are representative of two independent experiments. (**b**) Mice were treated with BrdU, and uptake of BrdU by CD11b$^+$ and CD11b$^-$ IgA$^+$ cells was determined by flow cytometry. Data are representative of four independent experiments. (**c**) Cells were isolated from the intestinal lamina propria of mice receiving CPM to analyse CD11b$^+$ IgA$^+$ cells. Similar results were obtained from four separate experiments. Graphs show data from individual mice. Statistical analyses were performed with Mann-Whitney's *U*-test.

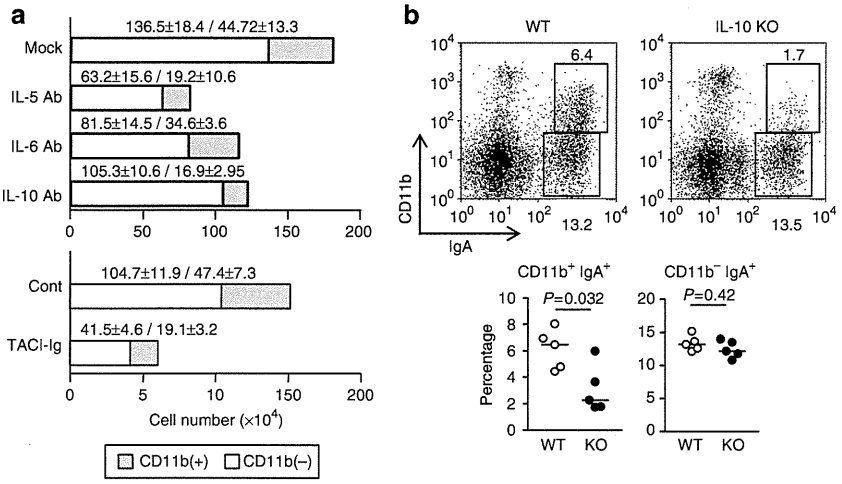

Figure 5 | Role of IL-10 in the maintenance of CD11b$^+$ IgA$^+$ cells in the iLP. (**a**) Mice were treated with antibodies to block IL-5, IL-6, IL-10 or antagonistic TACI-immunoglobulin (TACI-Ig) fusion protein. Mononuclear cells were isolated from the iLP and used for analysis of CD11b$^+$ and CD11b$^-$ IgA$^+$ cells by flow cytometry. Data are presented as means ± s.d. ($n=4$). (**b**) Mononuclear cells were isolated from the iLP of WT or IL-10 KO mice for analysis of IgA and CD11b expression by flow cytometry. Graphs show data from individual mice. Statistical analyses were performed with Mann–Whitney's *U*-test.

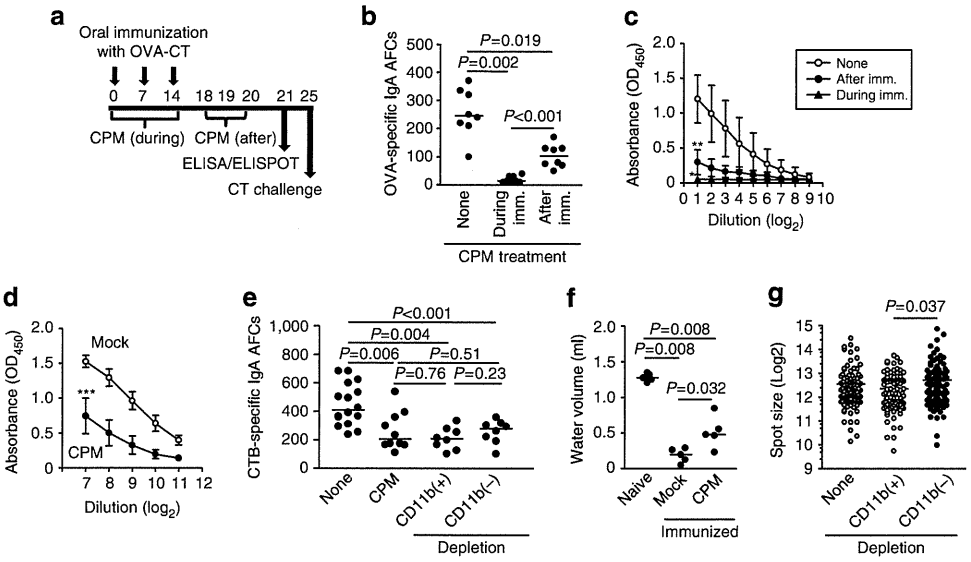

Figure 6 | Proliferating IgA$^+$ cells mediate early-phase IgA responses to oral antigen. (**a**) Experimental schedule for oral immunization and CPM treatment. Mice were orally immunized with OVA plus CT on days 0, 7 and 14. One group received CPM during oral immunization (days 0, 7 and 14) and another received CPM after the last immunization (days 18, 19 and 20). (**b**,**c**) One week after the final immunization (day 21), mononuclear cells were isolated from the iLP to quantify OVA-specific IgA-forming cells by ELISPOT (**b**). Simultaneously, faeces (**c**,**d**) were collected and were used for the detection of the (**c**) OVA-or (**d**) B subunit of CT (CTB)-specific IgA by enzyme-linked immunosorbent assay. Data are from individual mice and bars indicate median (**b**) and represent means ± s.d. ($n=10$) from two separate experiments (**c**,**d**). $^*P<0.001$, $^{**}P<0.01$, $^{***}P<0.05$ (two tailed unpaired *t*-test). (**e**) Mononuclear cells were isolated from the iLP of mock- or CPM-treated mice 1 week after the final immunization to quantify CTB-specific IgA-forming cells by ELISPOT. In some groups of mock-treated mice, CD11b$^+$ or CD11b$^-$ IgA$^+$ cells were depleted by cell sorting before application of ELISPOT assay. Graphs show data from individual mice, and bars indicate median. (**f**) On day 21, mice were orally challenged with 100 μg CT. After 15 h, the volume of intestinal fluid was measured. Graphs show data from individual mice, and bars indicate median. Similar results were obtained from two separate experiments. (**g**) Spot sizes of CTB-specific IgA AFCs were measured by Zeiss KS ELISPOT software. Graphs show data from individual mice, and bars indicate median. Statistical analyses were performed with Mann–Whitney's *U*-test (**e**–**g**).

IgA$^+$ cells. Of note, these mice showed ~90% reduction in OVA-specific IgA content in the faeces compared with mice not treated with CPM (Fig. 6c). We also confirmed that CPM treatment 4 days after final immunization induced a reduction in the production of IgA specific to the B subunit of CT (that is, CTB), which was associated with the halving of the abundance of CTB-specific IgA AFCs in the intestine (Fig. 6d,e). Like OVA-specific IgA responses (Figs. 3c and 6b), similar levels of reduction of CTB-specific IgA AFCs were noted when CD11b$^+$ IgA$^+$ cells were depleted before ELISPOT assay (Fig. 6e). These mice showed reduced resistance to oral challenge with CT and developed watery diarrhoea (Fig. 6f and Supplementary Fig. S11).

These findings led us to hypothesize that CD11b$^+$ IgA$^+$ PCs are capable of producing more IgA than are CD11b$^-$ IgA$^+$ PCs. To test this hypothesis, we measured the size of each spot in CTB-specific IgA AFCs in an ELISPOT assay. The cells in the CD11b$^+$ IgA$^+$ cell-enriched fraction (depletion of CD11b$^-$ IgA$^+$ cells) were bigger than those in the CD11b$^-$ IgA$^+$ cell-enriched fraction (depletion of CD11b$^+$ IgA$^+$ cells) (Fig. 6g). Furthermore, an adoptive transfer experiment demonstrated higher intestinal IgA production in severe combined immunodeficiency mice receiving CD11b$^+$ IgA$^+$ PCs than in those receiving CD11b$^-$ IgA$^+$ PCs (Supplementary Fig. S12), presumably because of both high IgA production and proliferating activity of CD11b$^+$ IgA$^+$ PCs. Although some possibilities (for example, proliferation and CD11b expression of IgA$^+$ cells might be changed during immunization) cannot be excluded, it is plausible that the actual production of IgA secreted into the intestinal lumen was derived mainly from CD11b$^+$ IgA$^+$ PCs in the early phase of the IgA response against orally immunized antigen.

Discussion

PCs could secrete antibodies to provide antigen-specific humoral immune responses in both systemic and mucosal tissues. Here, we demonstrated that intestinal IgA$^+$ PCs in mice could be categorized into two populations on the basis of CD11b expression. CD11b is an integrin αM that non-covalently associates with CD18 to form αMβ2 integrin (Mac-1) and binds to ICAM-1 (ref. 24). We therefore expected that CD11b$^+$ IgA$^+$ PCs were newly migrating cells whose migration was mediated by endothelial cells expressing ICAM-1, but in fact they were not. We also found no uptake of opsonized bacteria in either CD11b$^+$ or CD11b$^-$ IgA$^+$ cells (Supplementary Table S4 and Supplementary Fig. S13a), although CD11b is a receptor for complement (iC3b)[24]. In addition, unlike in human CD11b$^+$ B cells, which stimulate T cells strongly[32], major histocompatibility complex (MHC) class II (I-A^d) and costimulatory molecules (for example, CD80) were identically expressed on both CD11b$^+$ and CD11b$^-$ IgA$^+$ cells (Supplementary Table S4 and Supplementary Fig. S13b).

A similar subset of CD11b$^+$ IgA$^+$ cells was observed in the systemic murine compartments (for example, spleen), but the immunological characteristics of these cells differed from those of the cells in the intestine. Indeed, intestinal CD11b$^+$ IgA$^+$ cells consisted exclusively of PCs, but not memory B cells, whereas splenic CD11b$^+$ IgA$^+$ cells included both PCs and memory B cells. We further found that CD11b could not be used as a marker of B1 cells in the intestine. Our current findings show for the first time that CD11b could be a specific marker for discriminating IgA$^+$ PCs that require microbial stimulation and IL-10, and presumably contribute to the early phase of the intestinal IgA response in mice.

We have identified unique CD11b$^+$ IgA$^+$ PCs in mice; the next question is whether or not the same population of IgA$^+$ PCs exists in humans. Our preliminary experiments have shown that no human intestinal IgA$^+$ cells express CD11b, but that some IgA$^+$ cells express Ki67, a marker of proliferating cells (unpublished data). One possible explanation for this difference between human and mice is difference in the composition of commensal bacteria. In this regard, we examined the involvement of segmented filamentous bacteria (SFB), which are a known major IgA stimulus in mice, but has not yet been confirmed as part of the human microbiota[19]. As expected, SFB stimulated IgA production following colonization of SFB-deficient C57BL/6 mice from the Jackson laboratory (JAX mice) with bacterial suspensions from SFB-monoassociated mice (JAX + SFB mice)[33]; however, we found that CD11b is expressed on IgA$^+$ cells independently of SFB colonization (Supplementary Fig. S14). It is possible that other commensal bacteria such as *Lactobacillus* (abundant in mice) and *Bifidobacterium* (abundant in human) are responsible for the species-specific expression of CD11b on IgA$^+$ cells. It is important to recognize the differences between the mouse and human immune systems, but it is obvious that proliferating IgA$^+$ cells are present in the iLP of both mouse and human. The immunological function of human proliferating IgA$^+$ cells in the intestine will therefore be the subject of our next study.

In the initial step of the antibody response to T cell dependent antigens, B cells are activated by antigens and form GCs in the lymph nodes[7]. As depleting antigen-specific GC B cells by CPM treatment during oral immunization resulted in complete loss of the IgA response to orally immunized antigen, it is likely that both CD11b$^+$ and CD11b$^-$ IgA$^+$ PCs against T cell dependent antigen are derived from GC B cells. We also found that depletion of proliferating CD11b$^+$ IgA$^+$ PCs by CPM treatment after final immunization led to a decrease in the early-phase IgA response, although it is possible that proliferation activity and/or CD11b expression on IgA$^+$ cells might be wobble during immunization. Our *in vivo* findings indicated that the reduction in CD11b$^+$ IgA$^+$ PC numbers in MyD88 KO, IL-10 KO and PP-null mice did not affect the numbers of CD11b$^-$ IgA$^+$ PCs (Figs 1c, 3d and 5b). These findings, together with our *in vitro* data (Supplementary Fig. S6), indicate that it is likely that CD11b$^+$ IgA$^+$ PCs act as a separate lineage once they differentiate in the iLP.

Proliferating CD11b$^+$ IgA$^+$ PCs required microbial stimulation in the intestine. As proliferation is one of the characteristics of plasmablasts, it was possible that CD11b$^+$ IgA$^+$ cells have been recently committed to the PC fate. Notably, intestinal IgA$^+$ cells expressed MHC class II molecules; this expression is one of the unique characteristics of plasmablasts. Therefore, it is likely that intestinal IgA$^+$ PCs partly retain their plasmablast features. However, our findings indicated that CD11b$^+$ and CD11b$^-$ IgA$^+$ cells expressed identical levels of Blimp-1 and MHC class II. In addition, similar reduction was noted in CD11b$^+$ and CD11b$^-$ IgA$^+$ cells when cell trafficking from IgA inductive tissues (for example, PPs and the PerC) into the iLP was inhibited by treatment with FTY720. Thus, our findings suggest that CD11b$^+$ IgA$^+$ cells uniquely exhibit high proliferating and IgA-producing activity, although their other immunological features as PCs are similar to those of CD11b$^-$ IgA$^+$ PCs.

Proliferating CD138$^+$ PCs have been detected in the spleens of NZB/W mice with signs of systemic lupus erythematosus, but not in naive mice[34]. In contrast, the number of non-proliferating CD138$^+$ PCs is unchanged in the intestines of GF mice, as it is in the spleens of NZB/W mice[34]. These findings suggest that MyD88-dependent homeostatic stimulation of commensal bacteria determines the fate of proliferating CD11b$^+$ IgA$^+$ CD138$^+$ PCs in the intestine. Several lines of evidence have revealed the cellular and molecular mechanisms of microbe-dependent initiation of IgA responses. B cells express several toll-like receptors, and B cell-intrinsic MyD88-mediated signalling has been implicated in enhanced antibody production in some studies[35,36]. However, our current findings indicated that MyD88-mediated signalling in hematopoietic cells, including B cells, was not essential for intestinal CD11b$^+$ IgA$^+$ PC production. Additionally, we found IL-10 as a key molecule inducing CD11b$^+$ IgA$^+$ PC production. Previous studies have demonstrated that IL-10 promotes the proliferation of activated B cells and subsequent IgA production *in vitro*[37,38], which are consistent with our current findings of high-level proliferation of, and IgA production by, CD11b$^+$ IgA$^+$ PCs. Thus, our current findings proved that IL-10 functions in IgA production *in vivo* and that CD11b$^+$ IgA$^+$ PCs are the main targets in this

pathway. Despite these findings, our preliminary study demonstrated that treatment of CD11b$^+$ or CD11b$^-$ IgA$^+$ PCs with IL-10 alone did not induce their reciprocal differentiation into each other, and IL-10 KO mice with colitis possessed CD11b$^+$ IgA$^+$ PCs (J.K., unpublished data). Thus, IL-10 is redundant in some cases and additional factors are required for the maintenance of CD11b$^+$ IgA$^+$ PCs. Our current findings identified CD150 as a surface molecule that is highly expressed on CD11b$^+$ IgA$^+$ PCs. CD150 is a 70-kDa glycoprotein expressed on some B and T cells, thymocytes and macrophages[29]. Homophilic interaction of CD150 induces proliferation of, and antibody synthesis by, B cells[39], and notably IL-10 synergistically enhances CD150-mediated B cell proliferation[39]. Thus, it is likely that, at least partly, IL-10 and CD150 determine the unique features (for example, proliferation and high IgA production) of CD11b$^+$ IgA$^+$ PCs in the iLP. In addition, accumulating evidence has revealed an important immunological function of stromal cells as survival niches for PCs in the BM[40] and intestine[41,42]. It is possible that complex immunological communications among commensal flora, epithelial and stromal cells, and the cells involved in innate and acquired immunity determine the differentiation and maintenance of IgA PCs in the intestine.

Taken together, our results provide new insights into the nature of IgA$^+$ PCs in the murine intestine, and especially into the regulation of the early-phase IgA responses to intestinal antigens and requirement of microbe-dependent stimulation, IL-10, and the PP lymphoid structure. These findings add a new level of complexity to the intestinal IgA system of mice.

Methods

Mice. SPF and GF Balb/c mice were obtained from Japan CLEA (Tokyo, Japan). MyD88 KO mice, IL-10 mice (Balb/c background) and TCRβδ mice (C57/BL6 background) were maintained under SPF conditions at the Experimental Animal Facility, The Institute of Medical Science, The University of Tokyo, and WT littermates were used as controls. To deplete gut commensal bacteria, mice received broad-spectrum antibiotics, namely ampicillin (1 g l^{-1}; Sigma-Aldrich, St Louis, MO), vancomycin (500 mg l^{-1}; Shionogi, Osaka, Japan), neomycin sulphate (1 g l^{-1}; Sigma-Aldrich) and metronidazole (1 g l^{-1}; Sigma-Aldrich), in their drinking water for 4 weeks[43]. To establish BM chimeric mice, we injected γ-irradiated (960 rad, Gammacell 40, Atomic Energy of Canada Limited, Ontario, Canada) recipient mice with 5×10^6 BM cells through the tail vein and used them in experiments 8 weeks after injection. Under our experimental conditions, the reconstitution efficacy was about 90–95%. To obtain PP-null mice, pregnant BALB/c mice were injected intravenously and subcutaneously with 1 mg anti-IL-7Rα antibody (A7R34, BioLegend, San Diego, CA) at 14.5 days post coitus, as described previously[28]. We confirmed the disruption of organized PPs and the existence of ILFs in the offspring, as described previously[28]. To neutralize cytokines, mice were treated intraperitoneally with 250 μg of monoclonal antibodies specific for IL-5 (TRFK5), IL-6 receptor (D7715A7) or IL-10 (JES5.16E3) (BioLegend, San Diego, CA); control antibody (Rat IgG2b); or 100 μg of soluble TACI-Fc fusion protein (R&D Systems, Minneapolis, MN) every second day for 2 weeks[44,45]. For assessing the role of SFB, mice purchased from the Jackson laboratory were orally inoculated with bacterial suspensions obtained by homogenizing faecal pellets from SFB-monoassociated mice in water. SFB colonization was confirmed by quantitative PCR[33] and CD11b$^+$ IgA$^+$ cells were analysed in the small intestine 2 weeks post gavage by flow cytometry. All experiments followed the guidelines of the Animal Care and Use Committee, The University of Tokyo and Columbia University.

Oral immunization. Mice were given sodium bicarbonate solution to neutralize stomach acid[11,13]. Thirty minutes later, the mice were orally immunized with 1 mg OVA (Sigma-Aldrich) and 10 μg CT (List Biological Laboratories, Campbell, CA). This procedure was conducted on days 0, 7 and 14. In some groups, mice were intraperitoneally given CPM (35 mg kg^{-1} each time, Sigma-Aldrich). One week after the final immunization, faecal samples and mononuclear cells from the iLP were collected for enumeration of OVA-specific antibody responses by enzyme-linked immunosorbent assay and ELISPOT, respectively[13]. *In vivo* CT challenge was performed by oral challenge of naive or immunized mice with 100 μg of CT as previously described[46].

Cell isolation. To isolate mononuclear cells from PPs, we stirred the tissues in RPMI-1640 medium containing 2% fetal calf serum plus 0.5 mg ml^{-1} collagenase (Wako, Osaka, Japan)[11,13]. To isolate mononuclear cells from the iLP, PPs were carefully removed and the remaining intestines including ILFs were opened longitudinally, washed with RPMI-1640, cut into 2-cm pieces and stirred for 20 min at 37 °C into RPMI-1640 containing 0.5 mM EDTA and 2% fetal calf serum to remove epithelial cells and intraepithelial lymphocytes[11,13]. The tissues were then stirred three times in 0.5 mg ml^{-1} collagenase for 20 min before undergoing discontinuous Percoll gradient centrifugation (40 and 75%). Peritoneal cells were obtained by peritoneal flushing with 8 ml ice-cold phosphate-buffered saline (PBS)[11,13].

Flow cytometry and cell sorting. Mononuclear cells were preincubated with 10 μg ml^{-1} anti-CD16/32 antibody (BD Biosciences, San Diego, CA). They were then reacted with the following antibodies: Pacific blue-rat anti-mouse CD45R (B220) (RA3-6B2, 0.8 μg ml^{-1}), phycoerythrin (PE)-rat anti-mouse CD11b (M1/70, 0.1 μg ml^{-1}), PE-Cy7-hamster anti-mouse CD11c (HL3, 0.4 μg ml^{-1}), PE-rat anti-mouse CD18 (C71/16, 0.8 μg ml^{-1}), PE-rat anti-mouse CD19 (1D3, 0.8 μg ml^{-1}), PE-rat anti-mouse CD38 (90, 0.13 μg ml^{-1}), FITC-rat anti-mouse IgA (C10-3, 2 μg ml^{-1}), PE-Cy7-rat anti-mouse IgM (R6-60.2, 1 μg ml^{-1}), PE-anti-mouse I-A^d (AMS-32.1, 0.4 μg ml^{-1}), APC-Cy7-rat anti-mouse CD11b (M1/70, 1 μg ml^{-1}), APC-Cy7-anti-mouse β1-integrin (HMβ1-1, 4 μg ml^{-1}), APC-anti-mouse CD40 (3/23, 2 μg ml^{-1}), Pacific blue-anti-mouse CD11b (M1/70, 1 μg ml^{-1}), PE-Cy7-anti-mouse F4/80 (BM8, 0.4 μg ml^{-1}) and biotin mouse anti-CD138 (281-2, 10 μg ml^{-1}) (all antibodies from BD Biosciences) followed by incubation with streptavidin-APC (1 μg ml^{-1}, BD Biosciences), PE-anti-mouse CD150 (TC15-12F12.2, 0.1 μg ml^{-1}), Alexa Fluor 647-anti-mouse CD80 (16-10A1, 1 μg ml^{-1}) (BioLegend, San Diego, CA), anti-mouse CD267 (TACI) (8F10-3, 4 μg ml^{-1}) (eBioscience, San Diego, CA), PE-mouse CCR3 (83101, 0.5 μg ml^{-1}) (R&D Systems) or biotinylated anti-peanut agglutinin lectin (1 μg ml^{-1}, Vector Laboratories, Burlingame, CA), followed by staining with streptavidin PE (1 μg ml^{-1}, BD Biosciences). For staining for Blimp-1, cells were fixed and permeabilized with a Cytofix/Cytoperm kit (BD Biosciences) and stained with PE-conjugated anti-Blimp1 goat polyclonal IgG (0.4 μg ml^{-1}, Santa Cruz Biotechnology, Santa Cruz, CA). FSC-H and FSC-A discrimination was used to exclude doublet cells, and ViaProbe cell-viability solution (BD Biosciences) was used to discriminate between dead and living cells. To detect proliferating cells, mice received 1 mg BrdU intraperitoneally 24 h before analysis; the BrdU signal was detected with the manufacturer's protocol (BD Biosciences). Concentration-matched isotype antibodies were used as negative controls. Flow-cytometric analysis and cell sorting were performed with FACSCanto II and FACSAria (BD Biosciences), respectively. We confirmed that cell purity was about 95% (Fig. 2a).

Immunohistological analysis. Intestines were fixed in 4% paraformaldehyde for 15 h at 4 °C, washed with PBS and treated sequentially in 10 and 20% sucrose for 12 h at 4 °C[13]. The tissues were embedded in OCT compound (Sakura Finetechnical Co., Tokyo, Japan). Cryostat sections (7 μm) were pre-blocked with anti-CD16 and CD32 antibody for 15 min at room temperature and stained for 15 h at 4 °C with FITC-rat anti-mouse IgA (C10-3, 2 μg ml^{-1}) and biotin anti-mouse CD11b antibody (M1/70, 1 μg ml^{-1}). This was followed by incubation with horseradish peroxidase (HRP)-conjugated streptavidin (Pierce, Rockford, IL) for 30 min at 4 °C and amplification of the fluorescent signal with Cy3-tyramide (TSA-Direct kit; PerkinElmer, Waltham, MA)[13]. We confirmed that no signal was detected when the specimens were stained with the concentration-matched isotype antibodies. They were then counterstained with 4′,6-diamidino-2-phenylindole (Sigma-Aldrich). Deconvoluted fluorescence images of specimens were obtained by fluorescence microscopy (BZ9000, Keyence, Osaka, Japan).

Detection of antibody responses by enzyme-linked immunosorbent assay and ELISPOT. To measure OVA- or CTB-specific IgA levels in faecal extracts, faeces were homogenized in PBS by vigorous vortexing[11,13]. After centrifugation of the extracts (9,000*g* for 15 min) the supernatants were used as faecal extracts. Plates were coated with 1 mg ml^{-1} OVA or 2 μg ml^{-1} CTB in PBS; this was followed by blocking for 1 h at room temperature with 200 μl PBS containing 1% (w/v) bovine serum albumin. After extensive washing of the plates with PBS containing 0.05% Tween 20, serial sample dilutions were added for incubation overnight at 4 °C. Samples were then incubated for 1 h at room temperature with optimally diluted HRP-conjugated goat anti-mouse IgA (SouthernBiotech, Birmingham, AL). After sample washing, the colour reaction was developed at room temperature with 3,3′,5,5′-tetramethylbenzidine (Moss, Pasadena, MD) and terminated by adding 0.5 M HCl. The colour reaction was measured as the optical density (wavelength 450 nm).

ELISPOT assay was used to enumerate IgA-producing AFCs in the iLP[11,13]. Briefly, various concentrations of mononuclear cells were cultured at 37 °C for 4 h in 96-well nitrocellulose membrane plates (Millititer HA; Millipore, Bedford, MA) coated with 1 mg ml^{-1} OVA and 5 μg ml^{-1} bovine serum albumin-conjugated phosphorylcholine (Biosearch Technologies, Novato, CA). After vigorous washing of the plates with PBS and PBS containing 0.05% Tween 20, HRP-conjugated goat anti-mouse IgA was added; the plates were then incubated overnight at 4 °C. Spots of AFCs were developed with 2-amino-9-ethylcarbazole

(Polysciences, Warrington, PA). The size of each spot was measured with Zeiss KS ELISPOT software (Oberkochen, Germany).

***In vitro* culture**. $CD11b^+$ IgA^+ or $CD11b^-$ IgA^+ PCs (10^4 cells per well) were purified from the iLP and cultured with 100 ng ml^{-1} phorbol 12-myristate 13-acetate plus 300 ng ml^{-1} ionomycin, or 10 μg ml^{-1} lipopolysaccharide (all from Sigma-Aldrich), for 24 h.

For the bacteria uptake assay, fluorescent *Staphylococcus aureus* was opsonized in accordance with the manufacturer's protocol (Molecular Probes). Mononuclear cells isolated from the iLP (2×10^5 cells) were incubated with 1×10^5 opsonized bacteria for 90 min. After being washed, the cells were stained with antibodies for PE-IgA (mA-6E1, 0.5 μg ml^{-1}, eBioscience) and Pacific Blue CD11b, and the bacterial uptake by each population was examined by flow cytometry.

Microarray analysis. Microarray analysis was performed as we previously reported[47]. Briefly, $CD11b^+$ IgA^+ and $CD11b^-$ IgA^+ cells were isolated from the iLP, and total RNA was extracted from them with an RNeasy kit (Qiagen, Dusseldorf, Germany). cRNA was hybridized with DNA probes on a GeneChip Mouse Genome 430 2.0 array (Affymetrix), washed and fluorescence-labelled in accordance with the standard amplification protocol developed by Affymetrix. The fluorescence intensity of each probe was taken to represent the raw expression level and was quantified with GeneChip Operating software (Affymetrix). Data obtained from two independent experiments were analysed with GeneSpring 7.3.1 software (Silicon Genetics). All microarray data have been deposited in the National Center for Biotechnology Information Gene Expression Omnibus database (www.ncbi.nlm.nih.gov/geo/) under the accession no. GSE37225.

Statistics. Results were compared by a non-parametric Mann–Whitney's *U*-test and unpaired *t*-test (two tailed) (GraphPad Software, San Diego, CA).

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Author contributions

J.K. planned the research and experiments, analysed data, wrote the paper and directed the research; M.G., E.H., I.I., M.H., Y.S., Y.G., C.P., I.I.I., R.S., L.A., T.W., S.S., Y.K. and S.S. conducted the immunological experiments; K.T. and S.A. provided key materials; and H.K. wrote the paper.

Additional information

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Review

Immunological Function of Sphingosine 1-Phosphate in the Intestine

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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

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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.

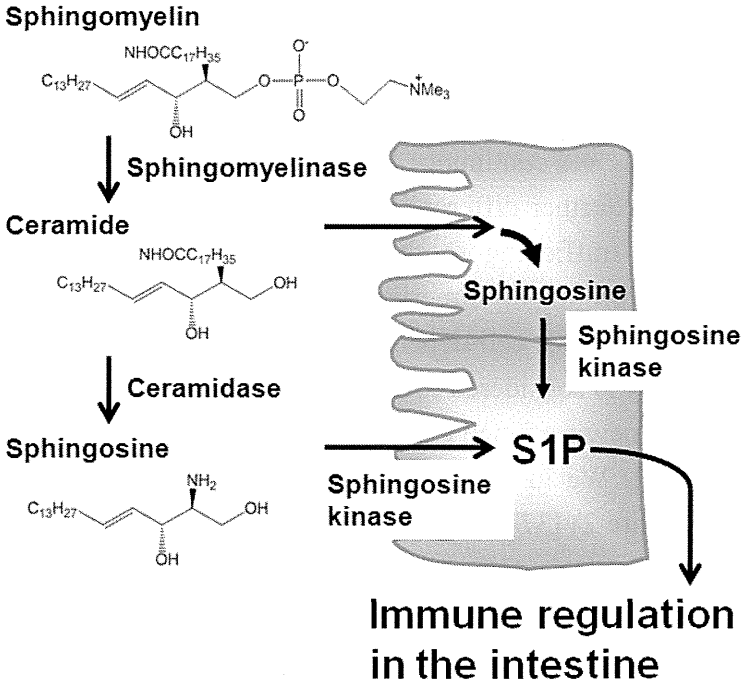
These biological and immunological functions show that S1P is involved in the maintenance of immunosurveillance as well as the development of immune diseases. In this review, we discuss the relationship between dietary materials (e.g., lipids, vitamin, and colorant) and S1P metabolism and describe the 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