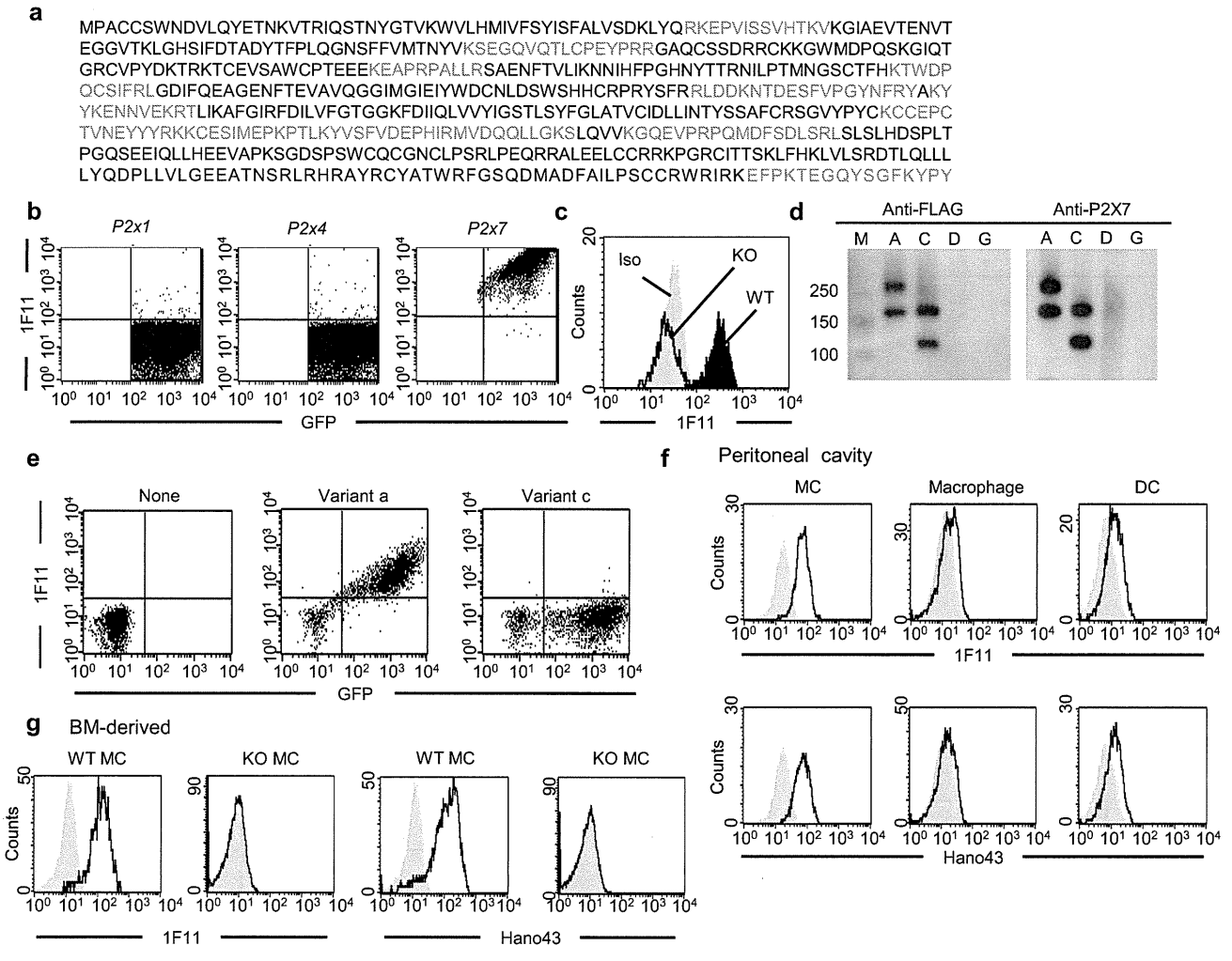
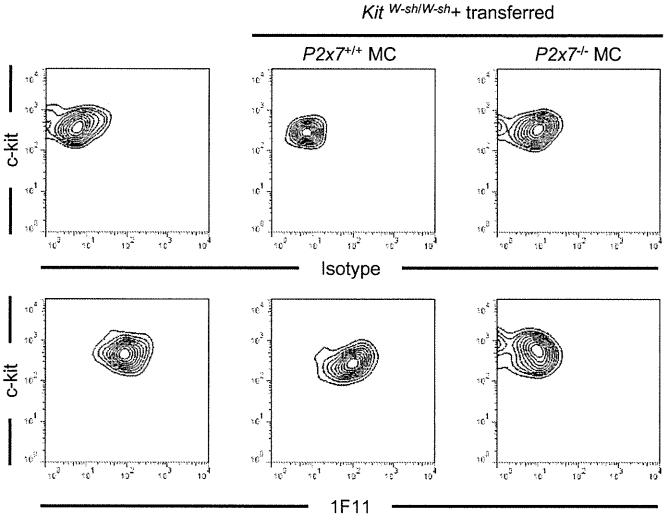
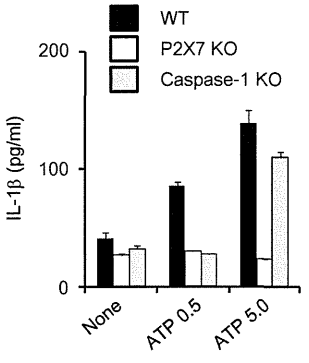


Supplementary Figure S4. Specific recognition of P2X7 purinoceptors by 1F11 mAb.
(a) The amino acid sequence of immunoprecipitants with the 1F11 mAb was determined by using LC-MS/MS. Red indicates the sequence covered by MS analysis. (b) The reactivity of 1F11 mAb to CHO cells expressing murine P2X1, P2X4, and P2X7 was examined. (c) Reactivity of 1F11 mAb to *P2x7*$^{+/+}$ (filled) and *P2x7*$^{-/-}$ (line) BM-derived MCs. Control staining with rat IgG2b is shown in gray. (d) CHO cells expressing flag-tagged variants a, c, and d of the P2X7 purinoceptors were immunoprecipitated with 1F11 mAb and subjected to western blotting with an anti-Flag or anti-polyclonal P2X7 antibody. (e) The reactivity of 1F11 mAb to *P2x7*$^{-/-}$ BM-derived MCs transfected with variants a and c of the P2X7 purinoceptors was examined with flow cytometry. (f) Peritoneal cells and (g) *P2x7*$^{+/+}$ or *P2x7*$^{-/-}$ BM-derived MCs were stained with two different anti-P2X7mAbs, 1F11 mAb and Hano43. Control staining with rat IgG2b is shown in gray.

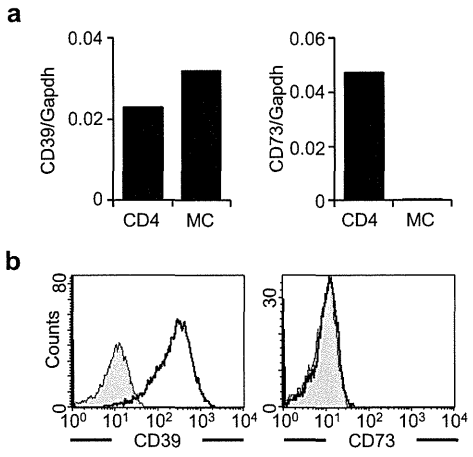


Supplementary Figure S5. P2X7 expression by colonic MCs in reconstituted *Kit*$^{W\text{-}sh/W\text{-}sh}$ mice.

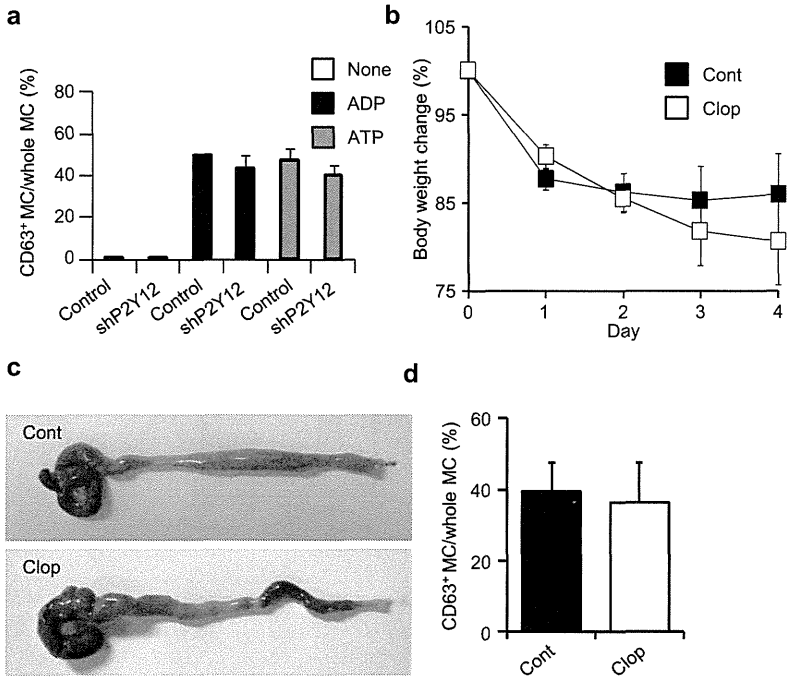
After reconstitution of *Kit*$^{W\text{-}sh/W\text{-}sh}$ mice with *P2x7*$^{+/+}$ or *P2x7*$^{-/-}$ bone marrow derived mast cells, P2X7 expression by colonic c-kit^{+} FcεRIα^{+} MCs was examined by using flow cytometry.



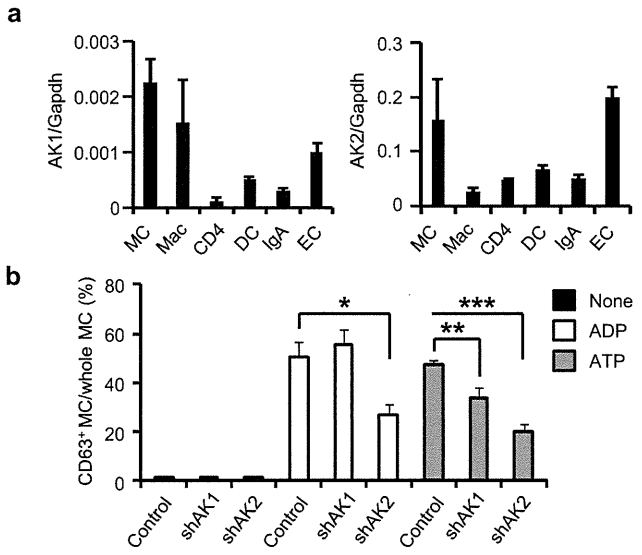
Supplementary Figure S6. Caspase-1-independent IL-1β production in MCs.
BM-derived MCs from wild-type, *P2x7*$^{-/-}$, and *caspase-1*$^{-/-}$ mice were stimulated with LPS followed by various concentrations of ATP. Production of IL-1β was measured by ELISA (n = 3). Data are shown as means ± s.e.m.



Supplementary Figure S7. Expression of CD39 and CD73 in colonic MCs.
(a) mRNA expression of CD39 and CD73 in colonic CD4 and MCs was determined by using quantitative RT-PCR. (b) CD39 and CD73 expression on colonic MCs was determined with flow cytometry. Control staining is shown in gray.

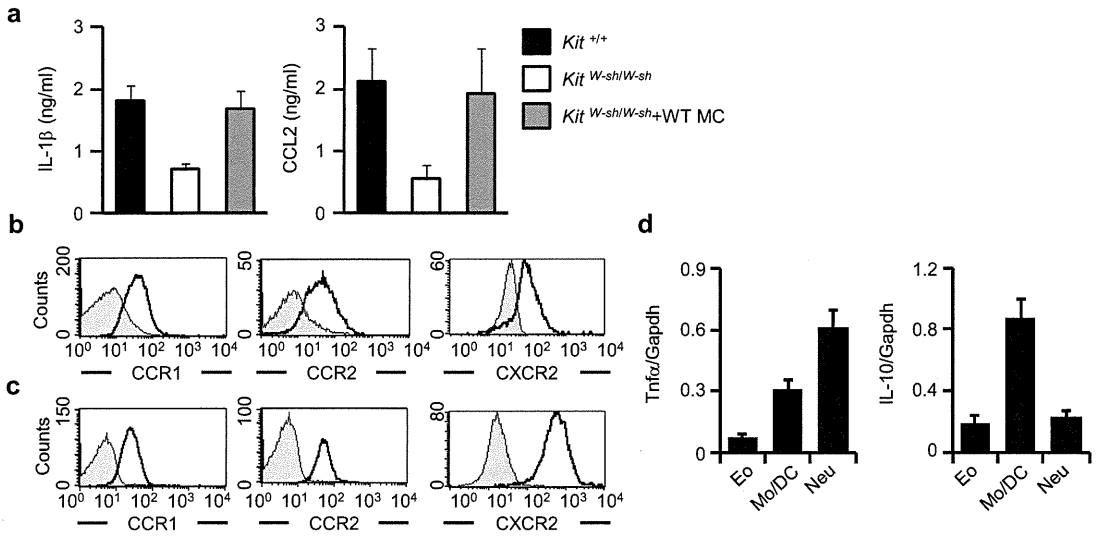


Supplementary Figure S8. P2Y12 inhibition does not affect colitis development.
(a) BM-derived MCs were transduced with shRNA expression lentivirus vector for P2Y12 or were mock-transduced and then stimulated with ADP or ATP for flow cytometric analysis of CD63 expression (n = 4). Data are shown as means ± s.e.m. (b–d) Mice received the P2Y12 inhibitor clopidogrel (Clop) or control vehicle (Cont) during colitis induction. (b) Body weight changes were measured. Data are shown as mean percentages of baseline weight ± s.e.m. (n = 3 for control; n = 5 for Clop). (c, d) Representative pictures of whole colons (c) and percentages of $CD63^+$ MCs in all MCs (d) 4 days after TNBS administration. Data are shown as means ± s.e.m.



Supplementary Figure S9. Involvement of adenylate kinase in P2X7-dependent MC activation.

(a) Expression of adenylate kinase 1 (AK1, left) and 2 (AK1, right) in colonic MCs, macrophages (Mac), $CD4^+$ T cells (CD4), DCs, IgA^+ plasma cells (IgA), and epithelial cells (EC) was measured by using quantitative RT-PCR (n = 4). Data are shown as means ± s.e.m. (b) BM-derived MCs were transduced with shRNA expression lentivirus vector for AK1 or AK2 or were mock-transduced and then stimulated with ADP or ATP for flow cytometric analysis of CD63 expression (n = 4). * $P = 0.0409$. ** $P = 0.0072$, *** $P < 0.0001$ (two-tailed Student's *t*-test). Data are shown as means ± s.e.m.



Supplementary Figure S10. Inflammatory cytokine and chemokine production in MC-deficient and MC-reconstituted mice.

(a) MC-deficient or MC-reconstituted mice were intrarectally administered TNBS and their colon tissues were incubated for 1 day to measure the production of IL-1β (left; n = 10) and CCL2 (right; n = 7) in the culture supernatant. Data are shown as means ± s.e.m. (b, c) CCR1, CCR2, and CXCR2 expression in $CD11b^{+}$ $Gr\text{-}1^{high}$ neutrophils in the colons (b) and blood (c) of TNBS-treated mice was examined by flow cytometry. Data are representative of three experiments. (d) TNFα (left) and IL-10 (right) expression in purified colonic eosinophils (SSC^{high} $CD11b^{+}$ $Gr\text{-}1^{neg}$), monocytes and dendritic cells (Mo/DC; SSC^{low} $CD11b^{+}$ $Gr\text{-}1^{+}$), and neutrophils (SSC^{low} $CD11b^{+}$ $Gr\text{-}1^{high+}$) was determined by using quantitative RT-PCR. Data are shown as means ± s.e.m. (n = 4).

Supplementary Table S1. Primer sequences

Gene	Forward 5' – 3'	Reverse 5' – 3'
P2Y1	CTGTGTGGACCCCATTCTTT	TCGGGACAGTCTCCTTCTGA
P2Y2	GGCCTGTGCATATGTGAGTG	TCCAGGTCTGCTGCCATT
P2Y4	CGGCGACTGTATCGACCT	GAGAGAACGGAGCCGAGAA
P2Y6	GCAGTCTTTGCTGCCACA	GTGGGCTCAGGTCGTAGC
P2Y12	CCCGGAGACACTCATATCCTT	TTGTAGTCTCTGACGCACAGG
P2Y13	ATGTGTGAGATGGGGAAAGG	CTGACTACTGTGGTGGTCTTCG
Pannexin1	AGACCAAGGGAGAGGACCA	GCTGCTCAGGTCCAAATCTT
Connexin32	ACCCATTTCGGACCAACC	AATCCATCTTGTCCTCTGGATG
Connexin43	TCCTTTGACTTCAGCCTCCA	CCATGTCTGGGCACCTCT
CCL2	CATCCACGTGTTGGCTCA	GATCATCTTGCTGGTGAATGAGT
CCL7	TTCTGTGCCTGCTGCTCATA	TTGACATAGCAGCATGTGGAT
CXCL2	AAAATCATCCAAAAGATACTGAACAA	CTTTGGTTCTTCCGTTGAGG
CD39	GCAAGCAGAGACAGCAAAAAC	GCAAAATCTCTTCACCTTAGAATCC
CD73	ATGAACATCCTGGGCTACGA	GTCCTTCCACACCGTTATCAA
Adenylate kinase1	TCCTGATCGACGGCTACC	AGTGTGGGCTGTCCAATCTT
Adenylate kinase2	TCCAAGACTCGCTGCTGAT	GGTAGGACCGGCCACTCT
TNFα	TGCCTATGTCTCAGCCTCTTC	GAGGCCATTTGGGAACTTCT
IL10	CAGAGCCACATGCTCCTAGA	TGTCCAGCTGGTCCTTTGTT
GAPDH	TGAACGGGAAGCTCACTGG	TCCACCACCCTGTTGCTGTA

ELSEVIER

Immune regulation and monitoring at the epithelial surface of the intestine

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The intestinal enterocytes and other epithelial cells create physical barriers, including tight junctions and mucus layers. These cells also actively transport antibodies across the epithelium and simultaneously produce antimicrobial peptides and enzymes. These functions maintain intestinal homeostasis by allowing the selective absorption of nutrients and simultaneously preventing pathogenic infections. Recent evidence has revealed that both host-derived factors (e.g., cytokines) and gut environmental factors (e.g., commensal bacteria, dietary materials, and their metabolites) regulate the physical and immunological functions of the epithelium. Understanding the interactions between host cells and these environmental factors should help us to develop new strategies to prevent and treat immune diseases of the intestine.

The surface of the gastrointestinal tract is covered by a single layer of epithelium that separates the outside world from interstitial tissues. The intestinal epithelium is mainly composed of absorptive enterocytes (ECs) but also includes enteroendocrine, goblet, and Paneth cells [1]. Cross-communication among these cells enables the selective absorption of nutrients while simultaneously preventing the penetration of antigens and pathogens. The defense against pathogenic materials is at least partly achieved by the physical barriers of the epithelium, which include tight junctions and mucus layers. A large number of pathogens disrupt these barriers to access deeper tissues for dissemination [2,3]. The barriers also contribute to the establishment and maintenance of mucosal homeostasis. Indeed, a leaky intestinal barrier is one of the characteristics of chronic intestinal inflammatory diseases, such as inflammatory bowel disease and celiac disease [4,5].

Intestinal tissues also show intense immunological activity, and ECs contribute to the intestinal immune system by transporting and processing antibodies and associated antigens, by producing immunologically functional molecules, and by interacting with immunocompetent cells in the intestine [6]. Accumulating evidence has revealed that both host-derived factors (e.g. cytokines) and gut environmental factors (e.g. commensal bacteria, dietary materials, and their metabolites) engage in molecular crosstalk with the intestinal epithelium and affect intestinal barrier function and immune responses [7,8]. In this review, we focus on the immunological functions of ECs in the intestine and their regulation by commensal bacteria and dietary materials.

Physical barriers at the intestinal epithelium

Tight junctions

ECs provide a physical barrier to prevent the paracellular transport of luminal antigens and pathogens. Tight junctions are multifunctional complexes that are crucial for the maintenance of barrier integrity because they form a seal between adjacent ECs [9]. The tight junction regulates the absorption of nutrients, ions, and water while preventing the entry of pathogens into the host.

Tight junctions are composed of numerous interacting cellular proteins, including claudin, occludin, and zonula occludens (ZO) proteins (Fig. 1). Claudin and occludin are transmembrane proteins that seal the paracellular space between adjacent ECs. Among

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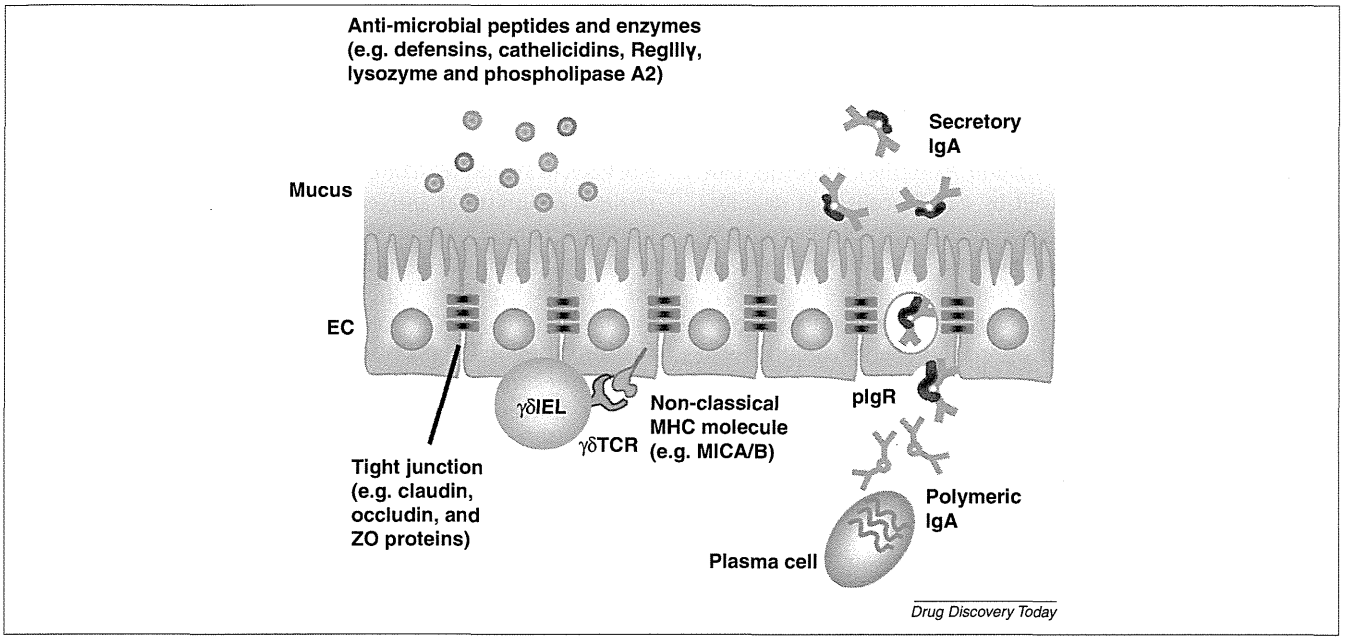

FIGURE 1

Physical and immunological barriers mediated by ECs. ECs (including Paneth cells) produce several molecules that create physical barriers in the intestine. They also produce antimicrobial peptides and enzymes, such as defensins, cathelicidins, RegIIIγ, lysozyme, and phospholipase A2 to kill the bacteria and establish a mucus layer to prevent bacterial attachment to the ECs. Tight junctions among ECs prevent bacterial penetration between the cells. ECs also have immunological functions. They express polymeric immunoglobulin receptor (pIgR), which binds and transports polymeric IgA produced from plasma cells into the intestinal lumen. ECs exposed to stresses (e.g. infection or cancer) express non-classical MHC molecules (e.g. MICA/B). MICA/B acts as a ligand for γδ T cell receptors, which are uniquely expressed on intraepithelial lymphocytes (γδIELs). *Abbreviations*: EC, enterocytes; MHC, major histocompatibility complex; ZO, zonula occludens.

the various types of claudins, claudin-1, -2, -3, -4, -5, -7, -8, -12, -15, -18, -20, and -23 are expressed in the intestinal epithelium [10,11]. ZO proteins are adaptors that connect transmembrane proteins; in particular, ZO-1 interacts with the claudin proteins and with F-actin in the intestinal ECs [12,13].

The physical barriers created by ECs are at least partly regulated by the immunological stimulation provided by commensal bacteria and dietary materials. Indeed, commensal and probiotic bacteria, their metabolites, food extracts, and dietary materials (e.g. fatty acids, polysaccharides, and flavonoids) have been shown to promote intestinal barrier integrity by increasing the expression of tight junction proteins [10].

Mucus

The mucus layer has been recognized as an important component in the intestine (Fig. 1). Mucin 2 (MUC2), a large glycoprotein characterized by variable O-linked glycans, is abundantly expressed by goblet cells located in the intestinal epithelium [14]. Generally, mucus can be divided into two layers. Although both layers have similar protein composition, the outer mucus layer is loose, whereas the inner mucus layer adheres firmly to the surface of the ECs. The firm mucus in the inner layer is an efficient barrier against pathogens [15]. In addition to the physical and biological barrier function of mucus, mucus also ensures the concentration of antimicrobial peptides and IgA antibodies at the surface of ECs. As similar to tight junctions, mucus expression is regulated by commensal bacteria, and the mucus layer of germ-free mice is thicker than that of specific pathogen-free mice [15].

Production of antimicrobial molecules at the epithelium

Antimicrobial peptides

The epithelium also secretes a variety of antimicrobial peptides [e.g. defensins, cathelicidins, and RegIIIγ (Fig. 1)]. The production of these peptides is mainly mediated by ECs and Paneth cells [16]. Paneth cells reside at the base of the crypt regions of the intestine, where they constitutively produce α-defensins. This does not require bacterial stimulation, because Paneth cells produce normal amounts of α-defensin in germ-free mice [17]. By contrast, ECs require microbial stimulation for the production of β-defensins [16]. ECs also produce cathelicidin, the expression of which is regulated by short-chain fatty acids produced when polysaccharides are metabolized by fermenting bacteria [18]. Both defensins and cathelicidin are cationic small peptides that exhibit antimicrobial activity by damaging and permeabilizing the bacterial cell membrane by pore formation [19].

RegIIIγ is a C-type lectin produced by ECs and Paneth cells in the ileum, where it kills Gram-positive bacteria by binding to surface-exposed carbohydrate moieties of peptidoglycans [20]. Commensal bacteria, especially Gram-negative bacteria, induce RegIIIγ expression on ECs, and a recent study demonstrated that MyD88 intrinsically expressed on ECs controls the production of RegIIIγ, which establishes the physical separation between the microbiota and the intestinal epithelial surface [21].

Unlike RegIIIγ, which specifically targets Gram-positive bacteria, bactericidal and/or permeability-increasing protein (BPI) shows antimicrobial activity against Gram-negative bacteria. The high affinity of BPI for lipopolysaccharide (LPS) leads to the

destabilization of the outer membrane of Gram-negative bacteria and also neutralizes LPS-induced inflammation [22].

Antimicrobial enzymes

Antimicrobial activity is also mediated by bacteriolysis enzymes (e.g. secretory phospholipase A2 and lysozyme). Phospholipase A2 is a small enzyme produced by Paneth cells that degrades bacterial phospholipids and subsequently disrupts the integrity of Gram-positive and -negative bacteria [23]. Phospholipase A2 enzyme activity is normal in the intestine of germ-free rats [24], but caloric restriction increases the gene expression of lysozyme and phospholipase A2 [25]. Therefore, it is likely that nutritional conditions rather than commensal bacteria regulate the activity of these antimicrobial enzymes in the intestine. Lysozyme is produced by Paneth cells and ECs. Its bactericidal activity derives from its cleavage of the glycosidic linkage between *N*-acetylglucosamine and *N*-acetyl muramic acid of peptidoglycan. Because Gram-positive bacteria express more peptidoglycan than Gram-negative bacteria, lysozyme acts preferentially on Gram-positive bacteria.

Transport of antibodies through ECs

IgA transport mediated by polymeric immunoglobulin receptors

One function of the epithelial immune barrier is to transport antibodies across the barrier. ECs express polymeric immunoglobulin receptors (pIgR) for the transport of polymeric forms of IgA (pIgA) and IgM (pIgM) in the basal-to-apical direction in association with an extracellular proteolytic fragment of the pIgR (known as the secretory component) [26]; together, the IgA and the secretory component form secretory immunoglobulin A (S-IgA). After S-IgA is secreted into the intestinal lumen, it inhibits adherence of pathogens to host ECs in the intestine and neutralizes pathogenic toxins by binding to their biologically active sites (Fig. 1) [27]. Additionally, IgA is able to exclude antigens and pathogens from the intestinal secretions while it is transported through ECs, and it also prevents viral replication inside ECs [28,29].

In addition to the function of S-IgA in the immunosurveillance, several lines of evidence demonstrate that S-IgA has a key role in preventing the penetration and/or growth of commensal bacteria [30]. These functions of S-IgA achieve the immune responses against commensal bacteria restricted in the intestinal but not systemic immune compartments in normal mice, while IgA-deficient mice exhibited systemic IgG responses against commensal bacteria [31–33]. A recent study also demonstrated that, in the absence of IgA, commensal bacteria-derived stimulation induced the increased expression of interferon-regulated genes in the ECs for the compensatory immunosurveillance with simultaneous reduction of lipid metabolism-related Gata4-regulated genes, which resulted in the lipid malabsorption and decreased lipid deposition [34]. Thus, S-IgA mediates the regulation between ECs and commensal bacteria, which is important not only for the maintenance of immunological homeostasis but also for metabolism [34].

Neonatal Fc receptor for IgG transport

Another receptor for immunoglobulin is the neonatal Fc receptor for IgG (FcRn). Although early studies in rodents indicated that FcRn was responsible for the passive acquisition of IgG neonatally, subsequent studies indicated that FcRn is also expressed by adult human epithelium and antigen-presenting cells in the intestine and thus is not strictly limited to neonatal life [35]. Unlike pIgR mentioned above, human FcRn binds IgG and the transport pathway is bidirectional, both apical to basal and basal to apical [36]. The bidirectional transport of IgG enables retrieval of intestinal antigens in a complex with IgG into the intestinal lamina propria, where the antigen and/or IgG complexes are subsequently taken up by antigen-presenting cells to prime T cell responses [37].

Intraepithelial T lymphocytes

The epithelium also includes lymphocytes that are commonly termed intraepithelial lymphocytes (IELs) [38]. IELs reside between the basolateral surfaces of ECs, and one IEL occurs for every 4–10 ECs in the small intestine and for every 30–50 ECs in the large intestine.

Most IELs are T cells. As similar to T cells observed at other sites (e.g. spleen and intestinal lamina propria), some portions of IELs express αβ T cell receptors and act as cytotoxic T lymphocytes by recognizing antigenic peptides presented by classical major histocompatibility complex (MHC) molecules on pathogenic ECs (e.g. microbe-infected cells) and killing them by producing cytotoxic molecules (e.g. perforin and granzymes) [38]. Other IELs express the γδ T cell receptor (and are therefore known as γδIELs) and show minimal pathogen-specific activity [38,39]. The innate immune function of γδIELs enables the rapid removal of infected ECs. To recognize the infected ECs, non-classical MHC molecules, such as MHC class I chain-related protein A/B (MICA/B) in human, act as ligands for γδIELs. MICA/B is generally not expressed on ECs, but is induced by stresses such as heat shock and microbial infections. The activated γδIELs then synthesize an array of cytokines, including interleukin (IL)-2, IL-3, IL-6, interferon (IFN)-γ, tumor necrosis factor (TNF)-α, and transforming growth factor (TGF)-β, and cytotoxic molecules, such as perforin, granzyme, and Fas ligand to kill the microbe-infected ECs [38].

Epithelium senses signals from commensal bacterial in the regulation of T cell differentiation in the intestine

The immune system requires interactions with commensal bacteria for its development. Toll-like receptors (TLRs) act as sensors of commensal bacteria although they were initially discovered as pathogen recognition receptors. ECs express several kinds of TLRs and the ligands from commensal bacteria promote immunological functions of Ecs, such as IgA transport, tight junctions, and expression of antimicrobial peptides [40]. Of note, ECs have unique expression profiles and spatially restricted distribution (apical vs. basolateral) of TLRs together with unique underlying signaling pathways, which enables the prevention of deleterious inflammatory responses in the intestine [40].

Because commensal bacteria express shared molecules which act as a ligand of TLRs, it was previously thought that unspecified commensal bacteria indiscriminately induced the development of the immune system; however, accumulating evidence has demonstrated that individual species of commensal bacteria have specific roles in the determination of immunological balance by regulating T cell differentiation in the intestine [8]. ECs have an important role in this pathway.

Segmented filamentous bacteria induce the differentiation of Th17 cells

Several groups have shown that segmented filamentous bacteria (SFB) induce components of the active immune system, including IgA-producing cells, γδIELs, and IL-17-producing T (Th17) cells [41–43]. SFB colonization on ECs results in the production of serum amyloid A, which acts on intestinal dendritic cells (DCs) to enhance the production of IL-6 and IL-23 [43]. Because these two cytokines are Th17 cell-inducing cytokines, the immunological environment mediated by SFB, ECs, and DCs results in the preferential induction of Th17 cells in the intestine.

Preferential induction of Treg cells in the colon by Clostridium *clusters IV and XIVa*

Another form of crosstalk between ECs and commensal bacteria in the regulation of T cell differentiation is mediated by *Clostridium* clusters IV and XIVa (also known as the *Clostridium leptum* and *coccoides* groups) [44]. By contrast to the effects of SFB, colonization by *Clostridium* clusters IV and XIVa induces regulatory T (Treg) cells in the colon to achieve quiescent immunity. *Clostridium* clusters IV and XIVa form a thin colonizing layer on the epithelium, where they enhance the release of the active form of TGF-β by increasing the expression of matrix metalloproteinases that convert latent TGF-β into the active form. Because TGF-β is an essential cytokine for the differentiation of Treg cells from naive T cells, colonization with these *Clostridium* species converts non-Treg cells into Treg cells locally in the colon with little effect on thymus-derived Treg cells.

Dietary metabolites regulate intestinal immunity through the epithelium

Nutritional materials also influence intestinal immunity, and commensal bacteria are involved in metabolizing indigestible dietary materials into biologically active metabolites. Dietary materials (e.g. polysaccharides, vitamins, and lipids) and their metabolites contribute to the regulation of intestinal immunity (Fig. 2).

Polysaccharides

Dietary polysaccharides and endogenous mucus in the intestine are digested and metabolized into short-chain fatty acids, such as acetate, butyrate, and propanoate, by bacterial fermentation. These short-chain fatty acids are an energy source for ECs and affect immune cell functions. For example, acetate and butyrate maintain epithelial barrier function by stimulating the release of mucin and by facilitating the maintenance of epithelial integrity [45,46]. Acetate and butyrate also regulate the proliferation of ECs and their production of cytokines [47,48]. In addition, acetate modulates the immunological function of neutrophils that express G-protein-coupled receptor 43 [GPR43, also known as free fatty acid receptor 2 (FFAR2)], a receptor for the short-chain fatty acids. Neutrophils lacking GPR43 show decreased levels of phagocytic activity and lower production of reactive oxygen species, but also are more responsive to chemoattractants such as C5a and inflammatory chemokines [49]. Consistent with these findings, intestinal inflammation is exacerbated in GPR43-deficient mice.

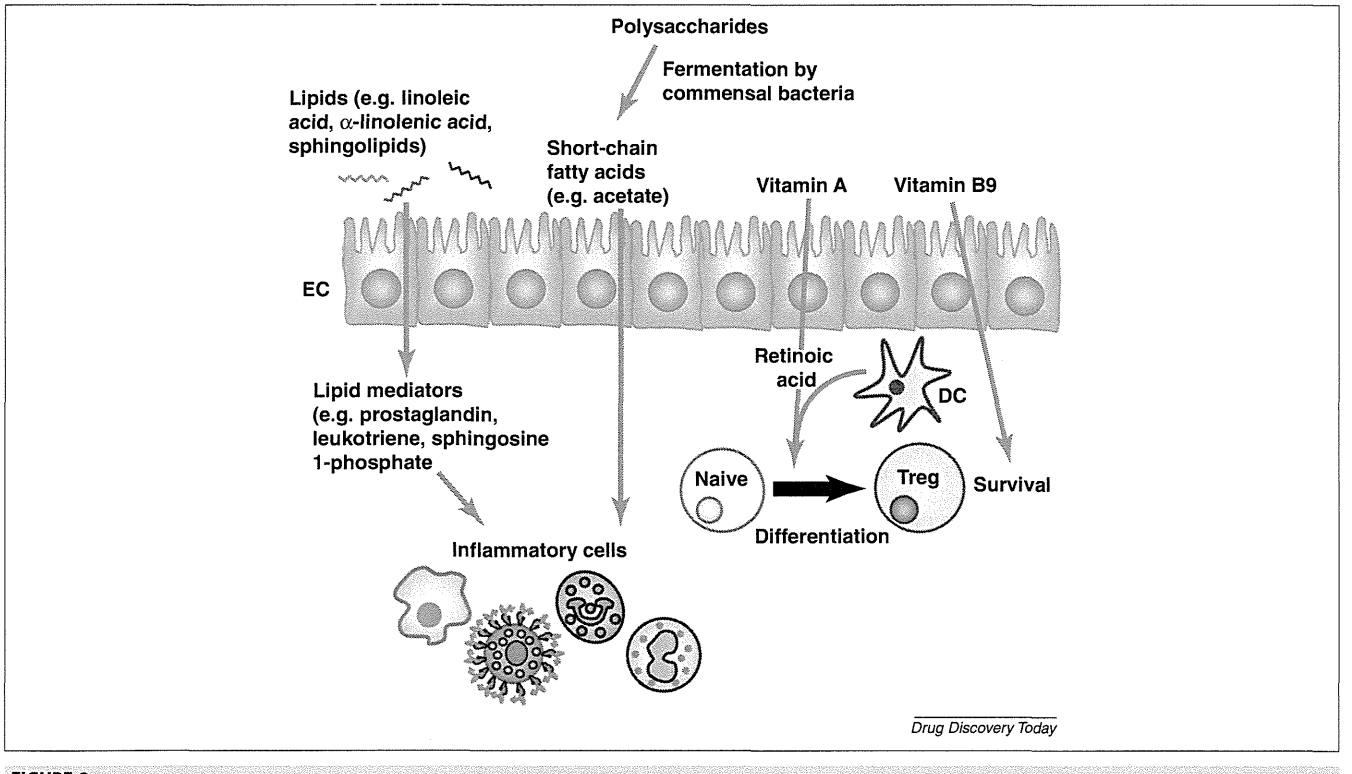

FIGURE 2

Dietary materials in the regulation of EC functions. Dietary lipids are metabolized into lipid mediators, and short-chain fatty acids are generated by fermentation of polysaccharides by commensal bacteria. These products positively or negatively regulate the functions of inflammatory cells. ECs also absorb vitamin A, and both ECs and dendritic cells (DCs) metabolize vitamin A into retinoic acid, which preferentially induces regulatory T (Treg) cells from naive T cells. The differentiated Treg cells require vitamin B9 for their survival. *Abbreviation:* EC, enterocytes.

Vitamins

Vitamins are supplied by both the diet and commensal bacteria. Several lines of evidence have shown that vitamins are involved in regulating immune responses through the epithelium. For example, retinoic acid, a metabolite of vitamin A, is involved in the preferential induction of regulatory T cells and the inhibition of Th17 cells [50]. Both ECs and DCs in the intestine are the major cell types that express retinaldehyde dehydrogenase, a key enzyme for the conversion of vitamin A into retinoic acid, suggesting that the unique gut environment mediated by ECs, DCs, and vitamin A preferentially induces Treg cells for maintaining quiescent immunity in the intestine. Because it was reported that Treg cells enhanced the differentiation of IgA^1 B cells in the intestine [51,52] and retinoic acid induced the expression of gut-homing molecules (e.g. CCR9 and $\alpha4\beta7$ integrin) on IgA-committed B cells as well as T cells [53,54], it is likely that retinoic acid directly and indirectly enhances intestinal IgA responses.

Vitamin B9 is another important vitamin in the maintenance of Treg cells. Vitamin B9 receptor (folate receptor 4) is exclusively expressed on Treg cells and can therefore be used as a cell surface marker of Treg cells [55]. We recently showed that vitamin B9 is an essential survival factor for Treg cells [56]. Indeed, Treg cells differentiate from naive T cells but fail to survive in vitamin B9-reduced conditions. Because vitamin B9 is supplied from both the diet and commensal bacteria, and dietary vitamin B9 is predominantly absorbed by ECs in the jejunum and duodenum, depletion of dietary vitamin B9 results in the reduction of Treg cells in the small intestine.

Lipids

Dietary lipids also involved in the regulation of intestinal immune responses. The ratio of omega-3 polyunsaturated fatty acids (ω-3 PUFA) to ω-6 PUFA in the diet may determine the presence and/or levels of inflammatory conditions. Dietary linoleic acid is the parent fatty acid of ω-6 PUFA which is metabolized into proinflammatory lipid mediators, whereas ω-3 PUFA, which is derived from dietary linolenic acid, is metabolized into anti-inflammatory mediators [57]. A possible molecular mechanism is that ω-3 PUFA exert anti-inflammatory effects through binding to GPR120, which is mostly expressed by macrophages, thereby inhibiting the production of inflammatory cytokines [58].

Another lipid metabolite with important immunological function is sphingosine 1-phosphate (S1P), which regulates cell trafficking, activation, and survival. Intestinal tissues contain higher levels of sphingolipids, including S1P, than other tissues and diet could be a major source of sphingolipids in the intestine, especially sphingomyelin from meat, milk, eggs, and fish [59]. Because ECs express alkaline sphingomyelinase and ceramidase to degrade dietary sphingomyelin into ceramide and sphingosine, respectively, and also express several key enzymes in the production of S1P from ceramide and sphingosine (e.g. sphingosine kinase), it is possible that ECs produce ceramide, sphingosine, and S1P for the regulation of intestinal immune responses.

Concluding remarks

ECs in the intestine have both physical and immunological barrier functions, which are achieved by immunological communication with both immunocompetent cells and gut environmental factors (e.g. commensal bacteria, dietary materials, and their metabolites). Elucidation of the complex networks established by commensal bacteria, dietary molecules, and the host immune system will provide new insights in gut environment-based mucosal immunology and should lead to new strategies to prevent and treat infectious and immune diseases in the intestine.

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REVIEW ARTICLE
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Alcaligenes is commensal bacteria habituating in the gut-associated lymphoid tissue for the regulation of intestinal IgA responses

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Secretory-immunoglobulin A (S-IgA) plays an important role in immunological defense in the intestine. It has been known for a long time that microbial stimulation is required for the development and maintenance of intestinal IgA production. Recent advances in genomic technology have made it possible to detect uncultivable commensal bacteria in the intestine and identify key bacteria in the regulation of innate and acquired mucosal immune responses. In this review, we focus on the immunological function of Peyer's patches (PPs), a major gut-associated lymphoid tissue, in the induction of intestinal IgA responses and the unique immunological interaction of PPs with commensal bacteria, especially *Alcaligenes*, a unique indigenous bacteria habituating inside PPs.

Keywords: Peyer's patch, IgA, commensal bacteria

INTRODUCTION

Secretory-immunoglobulin A (S-IgA) is predominantly observed in the intestine where it participates in immune defense (Mestecky et al., 2005; Brandtzaeg, 2010). S-IgA inhibits adherence of pathogens to host epithelial cells in the intestinal lumen and neutralizes pathogenic toxins by binding to the toxins' biologically active sites. Based on the immunological importance of S-IgA in immunosurveillance in the intestine, the development of oral vaccines has focused on the induction of antigen-specific S-IgA responses (Kunisawa et al., 2007). In addition to the immunosurveillance in the intestine, S-IgA antibody contributes to the establishment of beneficial gut commensal microbiota and thus dysfunction of S-IgA formation resulted in the alteration of normal bacterial flora (e.g., the reduction of Lactobacillus and increase of segmented filamentous bacteria, SFB; Suzuki et al., 2004).

Peyer's patches (PPs) are major gut-associated lymphoid tissue (GALT) where intestinal IgA responses are initiated and regulated by unique immunological crosstalk via cytokines [e.g., interleukin-4 (IL-4), IL-6, IL-21, and transforming growth factor-β (TGF-β)] and cell–cell interactions (e.g., via CD40/CD40 ligand interactions) among dendritic, T, and B cells (Kunisawa et al., 2008; Fagarasan et al., 2010). Thus, oral delivery of antigens to PPs is considered an important strategy for the effective induction of antigen-specific intestinal IgA responses (Kunisawa et al., 2011).

In addition to host-derived factors, microbial stimulation is also required for the maximum production of S-IgA in the intestine (Cebra et al., 2005). Indeed, germ-free (GF) mice have decreased intestinal IgA responses with immature structure of GALT when compared with mice housed under SPF or conventional conditions (Weinstein and Cebra, 1991). Although it was reported that some commensal bacteria [e.g., SFB and altered Schaedler flora (ASF), a combined eight culturable bacteria] and bacterial products (e.g., peptidoglycan, CpG oligonucleotide, and LPS) stimulated the intestinal IgA production (Michalek et al., 1983; Talham et al., 1999; Butler et al., 2005), it is obscure which bacteria is involved in this process indigenously. Because predominant commensal bacteria in the intestine is uncultivable, it was difficult to determine by culture-based method which bacteria regulated specific immune responses. However, recent advances in the genomic analysis allowed us to identify the uncultivable bacteria, which revealed key bacteria in the regulation of specific immune responses (Ivanov et al., 2009; Atarashi et al., 2011) as well as the development of immune diseases (Chow et al., 2010; Hill and Artis, 2010). Using genomic and immunological methods, we recently found that the microbial community inside PPs is different from those on the epithelium of PPs or in the intestinal lumen (Obata et al., 2010).

In this review, we discuss initially the immunological features of PPs in the induction and regulation of intestinal IgA responses. In the later part, we focus on the unique cross-communication between PPs and habitat commensal bacteria, *Alcaligenes*, a unique indigenous bacteria habituating inside PPs and regulating dendritic cells (DCs) for the efficient production of intestinal IgA.

IMMUNOLOGICAL FEATURES OF PEYER'S PATCHES

In the intestine, GALT comprise several different, organized lymphoid structures (Spencer et al., 2009; Fagarasan et al., 2010). Among them, PPs are the largest and most well-characterized sites

for the initiation of intestinal IgA responses, especially responses to T cell-dependent antigens (Kunisawa et al., 2008; Fagarasan et al., 2010). There are generally 8–10 PPs in the mouse small intestine and hundreds in the human small intestine. Each PP is composed of several B cell-rich follicles surrounded by a mesh-like structure consisting of T cells known as the interfollicular region (**Figure 1**).

Inside PPs, antigen-sampling M cells located in the follicle-associated epithelium transport luminal antigens to DCs situated in the subepithelium region (Neutra et al., 2001), which then form clusters with T-, B-, and stromal cells in the germinal centers and promote μ-to-α-class-switch recombination of B cells with the help of cytokines such as IL-4, IL-21, and TGF-β (Fagarasan et al., 2010). Upon immunoglobulin class-switching from μ to α, IgA-committed B cells (IgA^+ B cells) begin to express type 1 sphingosine-1-phosphate receptor, CCR9, and α4β7 integrin, allowing them to depart from the PPs and subsequently traffic to the intestinal lamina propria (Mora et al., 2006; Gohda et al., 2008). In the intestinal lamina propria, they further differentiate into IgA-secreting plasma cells under the influence of terminal differentiation factors (e.g., IL-6; Cerutti et al., 2011). DCs play a key role in these processes. For instance, nitric oxide, TGF-β, APRIL, and BAFF produced by TNF-α/iNOS-producing DCs (Tip-DCs) promotes IgA production (Tezuka et al., 2007). Also, DCs in the PPs metabolite vitamin A and produce retinoic acid, which induces the expression of gut-homing receptors (CCR9, and α4β7 integrin) on activated B and T cells (Iwata et al., 2004; Mora et al., 2006). Retinoic acid also induces the preferential differentiation into regulatory T (Treg) cells (Hall et al., 2011), and some of Treg cells differentiated into follicular helper T cells to promote IgA production in the PPs (Tsuji et al., 2009).

The identification of the molecular pathway of PP organogenesis allowed the establishment of PP-deficient mice through the loss of any part of this pathway (Nishikawa et al., 2003). Notably, disruption of the PP organogenesis pathway by blockade of tissue genesis cytokine receptor signaling [IL-7R and/or lymphotoxin-β receptor (LTβR)] during a limited fetus time period results in the selective loss of PPs without affecting other lymphoid tissue organogenesis (Yoshida et al., 1999). Experiments with PP-deficient mice showed that the dependency on PPs in the induction of antigen-specific IgA responses depends on the form of the antigen. For instance, the PP-deficient mice failed to develop antigen-specific IgA responses against orally administered antigens in particle form, but retained their ability to respond to soluble forms of antigens (Yamamoto et al., 2000; Kunisawa et al., 2002). It was also 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 induction of intestinal IgA production (Martinoli et al., 2007). This is consistent with another finding that DCs in the PPs are responsible for intestinal IgA production (Fleeton et al., 2004). Therefore, PPs are considered to be one of the major sites for the initiation of intestinal antigen-specific IgA responses.

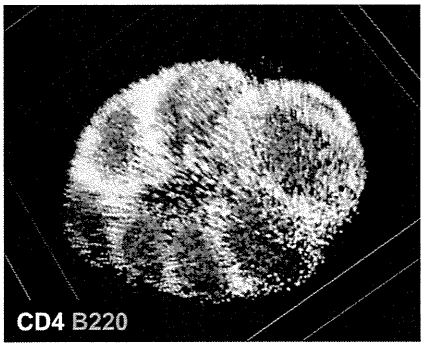

FIGURE 1 | Microarchitecture of murine Peyer's patches. Purified T cells (green) and B cells (red) were chemically labeled with carboxyfluorescein succinimidyl ester and carboxy-SNARF-1, respectively, and adoptively transferred into mice. Fifteen hours after the transfer, cell distribution in the Peyer's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

EFFECT OF MICROBIAL STIMULATION ON THE PRODUCTION OF INTESTINAL IgA

It is well known that microbial stimulation is required for the full production of S-IgA in the intestine. Indeed, GF mice have decreased intestinal IgA responses when compared with mice housed under SPF or conventional conditions (Cebra et al., 2005). Studies using mono-associated GF mice with SFB have demonstrated that only a minor proportion of the total intestinal IgA is reactive to mono-associated bacteria (Talham et al., 1999). In addition, bacterial products produced by commonly expressed on commensal bacteria (e.g., peptidoglycan, CpG oligonucleotide, and LPS) stimulated the intestinal IgA production (Michalek et al., 1983; Butler et al., 2005). In contrast, a recent study using reversible colonization of GF mice with genetically engineered *E. coli* showed that intestinal IgA induced in those mice bound to parent strain but not other bacteria (Hapfelmeier et al., 2010). Therefore, it remains unclear whether intestinal IgA responses induced by commensal bacteria is mediated by polyclonal stimulation and/or by B cell receptors specific for microbial antigens.

As one mechanism of impaired IgA production of GF mice, it was reported that GF mice have structurally immature GALT (e.g., PPs and ILFs) when compared with SPF mice (Weinstein and Cebra, 1991; Hamada et al., 2002). In the PPs, several key pathways for the IgA production require microbial stimulation. For example, Tip-DCs enhance the IgA production by producing nitric oxide, TGF-β, APRIL, and BAFF, which requires microbial stimulation through innate receptors (Tezuka et al., 2007). Indeed, the number of Tip-DCs was much reduced in the intestine of GF and MyD88-deficient mice (Tezuka et al., 2007). Another cell involved the microbe-dependent IgA production is non-hematopoietic follicular DCs (FDCs). It was reported that microbial stimulation of FDCs resulted in expressing chemokine CXCL13, BAFF, and TGF-β for the germinal center formation and B cell class-switching from IgM to IgA (Suzuki et al., 2010).

ALCALIGENES IS A UNIQUE INDIGENOUS BACTERIA INSIDE PPs

Recent advances in genomic technology make it possible to detect commensal bacteria in the intestine, allowing identification of key bacteria involved in the regulation of specific immune responses. For example, SFB was identified as commensal bacteria inducing

Th17 cells (Ivanov et al., 2009), whereas colonic regulatory T cells were induced by *Clostridium* clusters IV and XIV (Atarashi et al., 2011). These commensal bacteria localize at the surface of intestinal epithelium, but we supposed that the immunological crosstalk between host and commensal bacteria might establish in the regulation of intestinal IgA responses in the GALT. In this issue, we analyzed the composition of the microbial community inside PPs and identified *Alcaligenes* as a major commensal bacteria uniquely locating inside PPs (Obata et al., 2010).

By using the 16S rRNA clone library method, SFB are the predominant commensal bacteria co-habitat on FAE of PPs as like small intestinal epithelium. Although the FAE consisted with antigen-sampling M cells, SFB was not found inside of PPs. Instead, *Alcaligenes* are predominant bacteria inside PPs. The result obtained by the 16S rRNA analysis was further confirmed by fluorescence *in situ* hybridization (FISH) method and thus *Alcaligenes* are present exclusively inside PPs, not on the FAE of PPs, and intestinal villous epithelium and intestinal lamina propria (**Figure 2**). Of note, the preferential presence of *Alcaligenes* was observed not only in mouse but also in monkey and human (Obata et al., 2010). One of interesting but unresolved questions is the species specificity of *Alcaligenes*. We are now investigating whether *Alcaligenes* isolated from human or monkey colonize in the PPs to promote IgA production when they are orally fed to GF mice. Inside PPs, a proportion of the *Alcaligenes* seemed to be alive in mice. The presence and growth of *Alcaligenes* were detected in the PPs of GF mice after adoptive transfer of PP homogenates containing *Alcaligenes* from SPF mice. These findings suggest that *Alcaligenes* are indigenous bacteria ubiquitously living inside the PPs of various mammalian species.

ANTIBODY-MEDIATED RECIPROCAL INTERACTION BETWEEN *ALCALIGENES* AND THE HOST IMMUNE SYSTEM

As mentioned above, M cells located on the FAE of PPs transport luminal bacteria into DCs locating at the subepithelial region of FAE (Neutra et al., 2001). 16S rRNA clone library methods consistently revealed that DCs in the PPs predominantly contain *Alcaligenes*, whereas these bacteria are rarely detected in DCs isolated from other lymphoid tissues (e.g., spleen and mesenteric lymph nodes; Obata et al., 2010). We examined the immunological effects of *Alcaligenes* on DCs and found that the production of IgA-enhancing cytokines such as IL-6, TGF-β, and BAFF was increased when DCs isolated from the PPs of GF mice were stimulated with *Alcaligenes* (Obata et al., 2010). Several lines of evidence have revealed that immunological functions of DCs are different between intestinal and other lymphoid tissues (reviewed in Rescigno, 2010), we are now investigating whether immune stimulatory functions of *Alcaligenes* is specific for the PP DCs or not.

In agreement with the uptake of *Alcaligenes* and subsequent production of IgA-enhancing cytokines by DCs, *Alcaligenes*-specific IgA-forming cells were frequently observed in PPs, and consequent IgA production was noted in the intestinal lumen of SPF mice, but not GF mice (Obata et al., 2010). Although biological role of *Alcaligenes*-specific IgA antibody remains to be elucidated, the antibody might be involved in the creation of intratissue co-habitation of *Alcaligenes* in PPs. To this end, the number of *Alcaligenes* inside PPs is decreased in B cell-deficient CBA/N xid and IgA-deficient mice compared with wild-type mice (Obata et al., 2010). Therefore, it is interesting to suggest that *Alcaligenes*-specific IgA antibody mediates the uptake and presence of *Alcaligenes* in the PPs. Since M cells express IgA receptors

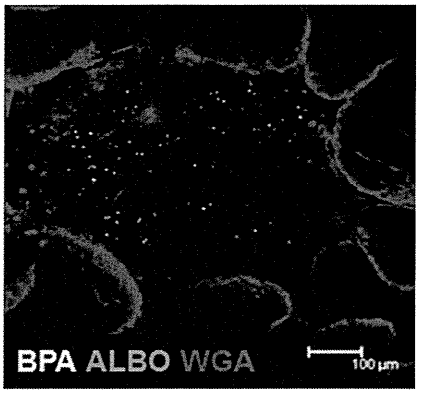

FIGURE 2 | Microarchitecture of murine Peyer's patches. Whole-mount fluorescence *in situ* hybridization was performed to visually analyze the presence of *Alcaligenes* inside PPs. Both BPA and ALBO34a were used as specific probes for *Alcaligenes*. Wheat germ agglutinin (WGA), an *N*-acetylglucosamine-specific lectin, was used to detect epithelial cells. Scale bar indicates 100 μm.

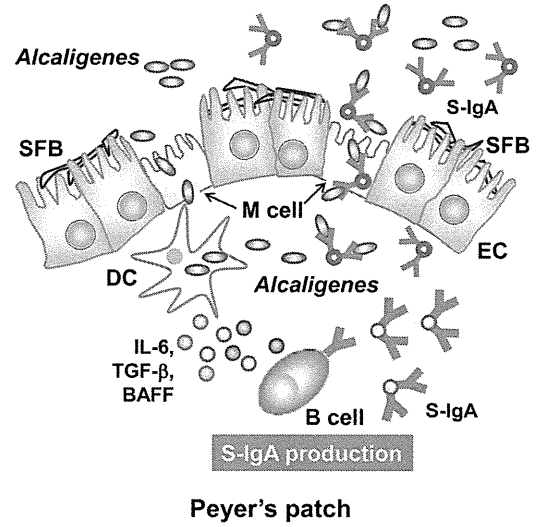

FIGURE 3 | *Alcaligenes* mediates symbiotic communication inside Peyer's patches. On the follicle-associated epithelium of PPs, segmented filamentous bacteria (SFB) is predominantly observed. In contrast, *Alcaligenes* specifically localizes inside Peyer's patches, where some are taken up by dendritic cells (DCs). Stimulation by *Alcaligenes* prompts the DCs to produce IgA-enhancing cytokines [e.g., interleukin-6 (IL-6), transforming growth factor-β (TGF-β)], and B cell activating factor (BAFF), which enhance the intestinal IgA response. The intestinal IgA includes *Alcaligenes*-specific IgA, which might mediate the preferential uptake and presence of *Alcaligenes* in the PPs. The uptake is presumably mediated by M cells.

(Mantis et al., 2002), one possibility is that *Alcaligenes* coated with the *Alcaligenes*-specific antibody are taken up into PPs through M cells. Further, the antigen-specific IgA coating on *Alcaligenes* might be beneficial for the bacteria to create the co-habitation niche since IgA antibody has been shown to non-inflammatory antibody (Mestecky et al., 2005).

CONCLUSION

In this review, we discussed a new concept of symbiotic communication in PPs that is mediated by commensal bacteria-specific IgA antibody. *Alcaligenes*-specific antibodies may mediate the uptake and the presence of *Alcaligenes* in the PPs, and the co-habitation of *Alcaligenes* within the PPs is one of the key factors to promote the intestinal IgA production by enhancing the production of IgA-enhancing cytokines from DCs (**Figure 3**). We still have various questions regarding this co-habitation of *Alcaligenes* in the PPs. For example, it remains unclear whether the presence of *Alcaligenes* inside of PPs is physiologically beneficial or harmful for the host immune system. In this issue, we are now addressing the microbial community in the PPs of mice and human patients suffering from intestinal immune diseases (e.g., intestinal inflammation and allergy). The biological roles of intra-tissue habitation of *Alcaligenes* in the PPs in the appropriate regulation of mucosal immune responses need to be elucidated. The current goal is to elucidate the mechanisms behind the co-habitation of *Alcaligenes* within PPs, and the exact contribution of *Alcaligenes* to educate and guide mucosal immunocompetent cells especially DCs in the PPs for the development, maturation and maintenance of the appropriate host immune system. These studies will provide novel molecular and cellular mechanisms of symbiotic communication with commensal bacteria in the regulation of host immunity.

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Gut-associated lymphoid tissues for the development of oral vaccines ☆

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ABSTRACT

Oral vaccine has been considered to be a prospective vaccine against many pathogens especially invading across gastrointestinal tracts. One key element of oral vaccine is targeting efficient delivery of antigen to gut-associated lymphoid tissue (GALT), the inductive site in the intestine where antigen-specific immune responses are initiated. Various chemical and biological antigen delivery systems have been developed and some are in clinical trials. In this review, we describe the immunological features of GALT and the current status of antigen delivery system candidates for successful oral vaccine.

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1. Introduction

Despite physical and biological barriers, the gastrointestinal tract is a major route of entry for numerous pathogens. Barriers include epithelial cells (EC) joined firmly by tight junction proteins, brush-border microvilli, and a dense layer of mucin [1]. Antimicrobial peptides, such as defensins produced by ECs and Paneth cells, are additional barrier to provide further protection [2].

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