is the high number of serotonin-containing enteroendocrine cells. After *Campylobacter enteritis* infection, an approximately fivefold increase in enteroendocrine cell number is observed in PI-IBS patients compared with asymptomatic individuals (63), suggesting that bacterial stimulation might regulates the differentiation of enteroendocrine cells and the secretion of the mediators that affect the enteric nervous system (64).

Enterocytes

The surface of the small intestine is covered mainly by absorptive enterocytes. The main role of enterocytes is the construction of a physical and physiological barrier against foreign antigens, especially commensal bacteria. In addition to this, enterocytes are reported to produce alkaline phosphatases that contribute to the detoxification of LPS produced by gut microbiota (65).

Enterocytes also express polymeric immunoglobulin receptor, which binds to the dimeric (or polymeric) form of IgA on the basal membrane of IECs for the formation and subsequent transcytosis of SIgA into the lumen (66). SIgA has been shown to have a critical role as the first line of defense, including the ability to neutralize toxins produced by pathogenic bacteria such as *Vibrio cholerae* and to prevent invasion by pathogenic bacteria such as *S. typhimurium* (67, 68). At the same time, SIgA is involved in and regulates the homeostasis of gut microbiota. High numbers of SFB abnormally colonize in the small intestine of mice lacking activation-induced cytidine deaminase, leading to a lack of IgA isotype switching pathway for the subsequent generation of SIgA production and the development of hyperplastic ILFs (69). Furthermore, a bacteria-specific SIgA can regulate bacterial gene expression: in experiments using *Rag2*-deficient mice inoculated with a hybridoma that produces IgA specific for the capsular polysaccharide (CPS) of *Bacteroides thetaiotaomicron*, this luminal CPS-specific IgA regulated bacterial CPS gene expression and attenuated the expression of host pro-inflammatory genes, such as *NOS2* at the host mucosal surfaces (70).

Epithelial fucoses establish a symbiotic environment

IECs reinforce their physical barrier by expressing a variety of carbohydrate chains on their apical cell surface. In addition, secretory-type IECs, especially goblet cells, release highly glycosylated mucins into the lumen to exclude undesired environmental antigens (14). However, microorganisms, especially commensal bacteria, have evolved to utilize the carbohydrate chains and glycosylated molecules produced by host IECs (71). $\alpha(1,2)$-Fucose is one of the terminal residues of the glycosylated molecules expressed by F-ECs (72). Fucosyltransferase 1 and 2 (Fut1 and Fut2) expressed in the GI epithelium catalyze the addition of $\alpha(1,2)$-fucose to a terminal galactose moiety (73). IECs in the small intestine are selective in their expression of Fut1 and Fut2 genes. Analysis of genetically engineered mouse models of Fut1 and Fut2 showed that M cells on PPs specifically express Fut1, whereas goblet cells and enterocytes express Fut2 (35, 74). Although the specific expression of fucosyltransferase genes has not been identified in each cell type, Paneth cells, enteroendocrine cells, and ileal crypt stem cells are also fucosylated (35). We also note that M cells, goblet cells, enteroendocrine cells, and Paneth cells spontaneously express fucoses, whereas enterocytes do not. However, fucose expression can be induced in enterocytes by environmental stimuli or stresses (35, 75). These reports suggest that (i) all IEC subsets have the potential to express fucoses, whereas the expression of Fut1 and Fut2 are differentially regulated among epithelial cell subsets, and (ii) the mechanism of the gene expression of Fut1 and Fut2 might be different among the cell subsets. Although the expression of Fut2 is regulated by the ERK and JNK pathways and the transcriptional factor AP-1 (76), the detailed mechanisms of the induction of Fut1 and Fut2 are still mostly unknown.

Terminal fucoses are effectively utilized by specific commensal bacteria (71). *B. thetaiotaomicron*, a predominant bacterial species in the ileum and colon of humans and mice, induce Fut2 expression in epithelial cells for the production of fucoses (77). These bacteria utilize epithelial fucoses as nutrients, and the level of available fucoses regulate the expression of multiple bacterial genes (45) (*Fig.* 2*A*). In addition, *Bacteroides fragilis* synthesizes fucosylated capsular polysaccharides, components of the bacterial outer membrane, from these intestinal fucoses, which confers competitive advantages to these bacteria for their preferential colonization in the intestine (44) (*Fig.* 2*A*). Indeed, the close association of commensal bacteria and epithelial-attached and -secreted molecules containing fucoses occurs under normal conditions for the creation of a suitable environment for cohabitation (*Fig.* 2*B*).

In addition to commensal bacteria, several pathogenic bacteria can also colonize a host GI tract by interacting with fucosylated molecules. *Helicobacter pylori* express blood group antigen-binding adhesin that binds to $\alpha(1,2)$-fucose containing glycocalyx expressed on the gastric epithelium and also binds to mucins (46). Supporting these data, the adhesion of *H. pylori* is impaired in Fut2$^{-/-}$ mice (78). *Campylobacter jejuni* is an intestinal pathogenic bacteria that causes severe diarrhea. *C. jejuni* binds to $\alpha(1,2)$-fucosylated H type 2 antigens and

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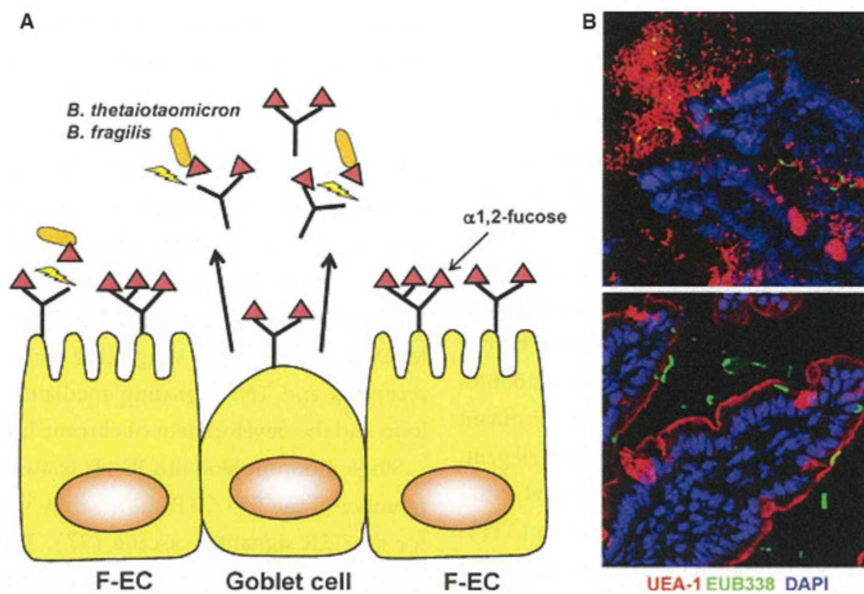

Fig. 2. Interaction between epithelial fucoses and commensal bacteria. (A) Fucosylated columnar epithelial cells (F-ECs) express fucoses on their apical cell surface. Goblet cells produce fucosylated mucins. Commensal bacteria, especially *Bacteroides* (two species are shown), cleave terminal fucoses and utilize them as a nutrient for gene expression and as a component of the bacterial membrane. (B) Histochemical images of ileal tissues. Fucosylated molecules are stained with lectin *Ulex europaeus* agglutinin-1 (UEA-1), and bacteria were detected by using the bacterial specific probe EUB338. Upper panel shows the association of commensal bacteria and fucosylated mucuses. Lower panel shows the mutual relationship between commensal bacteria and F-ECs. DAPI, 4′, 6-diamidino-2-phenylindole.

colonizes the intestine. Interestingly, this colonization is inhibited by the administration of human milk, which contains fucosyloligosaccharides (47). Studies show that intestinal fucosylated molecules may also provide a niche for the infection of pathogenic bacteria, but at same time, an artificially produced fucosylated carbohydrate moiety might be a novel therapeutic agent for the control of infection caused by pathogenic microorganisms.

Crosslinking between fucosylated glycocalyx produced by host IECs and disease susceptibility has been suggested for humans. About 20% of Europeans have the 428G → A (Trp143 → stop) non-sense-mutated allele of *FUT2* (79). This mutation leads to a non-secretor phenotype that is not able to produce H antigens in body fluids such as saliva. Direct evidence for disease association shows that the *FUT2* polymorphism is associated with susceptibility to viral infection. A previous study investigated the Norwalk virus infection and compared the susceptibility of secretor and non-secretor individuals to the virus. The results show that non-secretor individuals are resistant to Norwalk-virus infection (48). *FUT2* polymorphism also affects the plasma vitamin B_{12} level (80). Because vitamin B_{12} is generally absorbed by IECs in the ileum (81), intestinal secretory fucoses and fucosylated carbohydrate moieties on IECs might regulate vitamin B_{12} absorption. In addition, this *FUT2* polymorphism is associated with the onset of human IBD. Recent reports from GWAS have highlighted the involvement of *FUT2* in the development of Crohn's disease but not ulcerative colitis (9, 82). In these studies, the non-secretor 428G → A non-sense-mutated allele was identified as a risk factor, and *FUT2* was identified as a susceptibility locus for Crohn's disease (9, 82). It is still unclear how impairment of intestinal fucoses increases the risk of onset of Crohn's disease but not ulcerative colitis. To assess this mechanism, it might be important to clarify the mechanisms of the biological events surrounding the induction and regulation of *FUT2* synthesis, the interaction of intestinal fucoses and microbiota, and the contribution of microflora to IEC fucosylation, because commensal bacteria are closely associated with the induction of both physiological inflammation (e.g. maintenance of normal mucosal immunity) and pathological inflammation (e.g. IBD) (83–85). Recently, one report described the microbiota of individuals with the non-secretor mutation (86). Although the total number of fecal bacteria was relatively higher in the feces of these individuals, the number and diversity of the *Bifidobacterium* species were significantly reduced compared with the samples from control individuals (86). The alteration of gut microbiota caused by aberrant intestinal glycosylated molecules might predispose individuals to develop a variety of mucosa-associated diseases.

Maintenance of epithelial barrier against host pathogenesis

Metabolites of microbiota affect the epithelial barrier

As described above, the functions of IECs are affected by commensal bacteria, and bacterial MAMPs such as LPS, lipoteichoic acid, and CpG DNA contribute to this process. In addition to producing these structural components, living bacteria produce metabolites such as short-chain fatty acids and polyamines, which are speculated to affect the host's epithelial barrier systems (87–90). A recent study investigated the metabolites of several species of *Bifidobacterium*, which is the predominant commensal bacteria in the human intestine, by using elegant, multiple 'omics' technologies (91). The bifidobacterial strain *Bifidobacterium longum* has unique adenosine triphosphate (ATP)-binding cassette carbohydrate transporters that are responsible for the production of acetate (91). *B. longum*-produced acetate suppressed the epithelial apoptosis induced by Shiga toxin produced by *E. coli* O157:H7 (91). Another report showed that acetate binds to G protein-coupled receptor 43 on mucosal immune cells and ameliorates DSS-induced colitis (92), implicating the importance of metabolites produced by commensal bacteria in the maintenance of appropriate balance between the host epithelial barrier and intestinal environment.

Beneficial and deleterious functions of NFκB in the maintenance of the epithelial barrier

Elevated numbers of apoptotic epithelial cells have been observed in the colon of Crohn's disease patients (93). This high frequency of epithelial apoptosis is reduced after treatment with anti-TNF antibodies, reflecting its contribution to the pathology of the Crohn's disease (93). This finding suggests an association between the aberrant epithelial apoptosis and the development of intestinal inflammation that could be the consequence of unusual penetration of commensal bacteria through the breakage of the epithelial barrier via accelerate apoptotic IECs and subsequent hyperactivation of mucosal immune cells to produce acute and chronic pathological inflammatory conditions. To avoid breaches of the epithelial barrier, IECs have an endogenous anti-apoptotic signaling system. Nuclear factor-κB (NFκB) signaling molecules negatively regulate epithelial apoptosis. For example, mice with IECs lacking IKKγ (NF-κB essential modulator, NEMO) spontaneously develop colitis (94). NEMO-deficient epithelial cells are prone to undergo apoptosis, suggesting that canonical NFκB signaling prevents the transition of apoptosis. This hypothesis is supported by another important study that showed that epithelial cell-specific deletion of TAK1, a signaling molecule upstream of the IKK complex, also leads to spontaneous development of intestinal inflammation (95). This is known as pro-survival signaling produced by NFκB (96) (Fig. 3). Genetic disruption of NEMO in IECs leads to penetration of commensal bacteria into the intestinal tissues (94). This ectopic, intense bacterial signaling leads to hyperproduction of inflammatory cytokines such as TNF in mucosal immune cells, promoting further destruction of the epithelial barrier system. Development of colitis in mice lacking NEMO is rescued by impairment of MyD88 and TNFRI, suggesting that bacterial recognition receptors and TNF signaling mediates this positive feedback loop and the development of chronic inflammation (94).

Single immunoglobulin IL-1R-related receptor (SIGIRR) is a member of the IL-1/TLR superfamily that acts as a decoy target for the TLR signaling cascade (97). This regulatory molecule prevents excess NFκB activation caused by the destruction of epithelial barrier and infiltration of commensal bacteria (Fig. 3). Indeed, *Sigirr*$^{-/-}$ mice develop severe colonic inflammation after inflammation-inducing molecule dextran sodium sulfate (DSS) treatment (98, 99). The inhibitory activity of SIGIRR works in the IECs because transgenic expression of SIGIRR rescues the DSS-sensitive phenotype of mice lacking SIGIRR (99). This complementary negative signaling system works in the normal condition, because constitutive NFκB and JNK activation and abnormal proliferation of IECs are observed in mice lacking SIGIRRs (99). This abnormal activation of NFκB is rescued by antibiotic treatment leading to the removal of the causative intestinal flora (99). *A20* is another negative regulator of NFκB and is reported to be a susceptibility gene for Crohn's disease (100). IEC-specific deletion of *A20* leads to high sensitization to DSS-induced colitis (101). Collectively, these data reveal the dual functions of NFκB: (i) intrinsic NFκB signaling prevents apoptosis of IECs, and (ii) excessive NFκB activation of IECs promotes detrimental intestinal inflammation (96). Importantly, this dual role of NFκB in IECs is managed by commensal bacteria. How the intensity and quality of the epithelial cell-intrinsic NFκB is regulated awaits further clarification. In addition, ablation of TNF signaling does not fully restore the phenotypes of mice lacking epithelial-specific TAK1 (95), implicating the existence of other complementary molecules that mediate NFκB signaling.

Bacterial recognition receptor, autophagy, and endoplasmic reticulum stress

The primary function of IECs is to act as a physical barrier to prevent abnormal infiltration of foreign antigens. As this epithelial barrier excludes deleterious non-self antigens,

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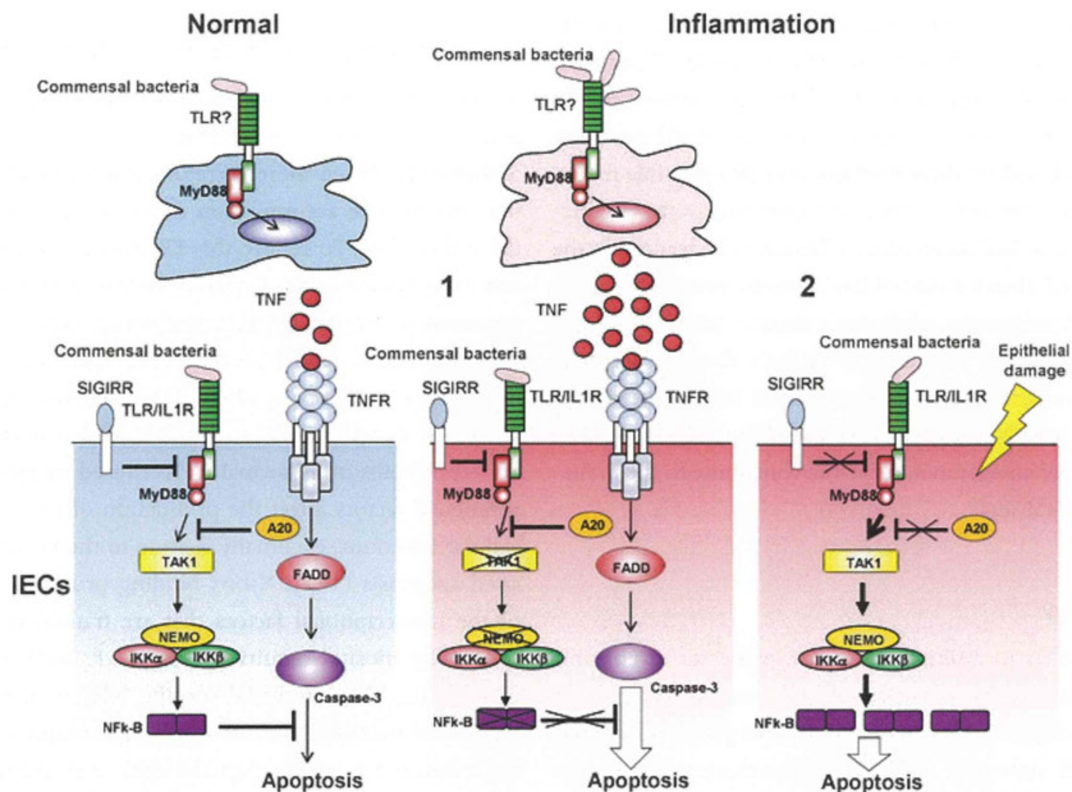

Fig. 3. Epithelial homeostasis is maintained by commensal bacteria and the nuclear factor-κB (NFκB) signaling cascade. In the steady-state condition (normal), commensal bacteria induce the production of physiologically optimal concentration of inflammatory cytokines [e.g. tumor necrosis factor (TNF)] in mucosal immune cells for the creation of homeostatic environment 'physiological inflammation'. Epithelial nuclear factor-κB (NFκB) has an important role in the anti-apoptotic function that is mediated by TAK1 and the inhibitor of NF-κB kinase (IKK) complex including NF-κB essential modulator (NEMO), IKKα, and IKKβ. Epithelial cell-specific inactivation of transforming growth factor-β-activated kinase 1 (TAK1) or NEMO leads to apoptosis that is at least partially mediated by TNFR (inflammation, 1). Single-Ig-interleukin-1 related receptor (SIGIRR) and A20 negatively regulate the NFκB signaling cascade. Disruption of these molecules results in excessive NFκB signaling and contributes to increased susceptibility to dextran sodium sulfate (DSS)-induced colitis (inflammation, 2). Appropriate NFκB signaling regulates the homeostasis of intestinal epithelial cells (IECs). TLR, Toll-like receptor; FADD, Fas-associated death domain protein.

disruption of this barrier system predisposes the host to bowel diseases. It is therefore not surprising that various molecules involved in the bacterial–epithelial interaction and physiology of IECs are reported to be susceptibility genes for IBD such as Crohn's disease and ulcerative colitis (8, 9). Indeed, the roles of the genes responsible for bacterial recognition receptors, autophagy, and ER stress in IECs, especially in Paneth cells, in the development of IBD have been recently highlighted (102).

NOD1 and NOD2

NOD1 and NOD2 are members of the cytosolic receptor family of NLRs, which are sensors of danger-associated molecular patterns (103). A wide variety of cells, including IECs, express NOD1 and NOD2 (103), and muropeptides produced by bacteria have been identified as ligands of these receptors. NOD1 detects the tetrapeptide structure, L-Ala-D-Glu-meso-DAP-D-Ala, which is found in most gram-negative bacteria, whereas NOD2 recognizes muramyl dipeptide, a minimal motif present in the peptidoglycan of almost all bacteria (104). Interestingly, the expression of NOD2 is elevated after colonization of germfree mice by commensal bacteria, suggesting a mutual relationship between NOD2 and commensal bacteria (105). *NOD2* has been identified as a susceptibility gene for Crohn's disease (106–108). Mice lacking *NOD2* display substantially altered microbiota, especially elevations in the numbers of *Firmicutes* and *Bacteroidetes*, which are the two main bacterial phyla in the gut microbiota (105, 109). Interestingly, a similar shift in gut microbiota is observed in Crohn's disease patients homozygous for a *NOD2* mutation (109). Although the mechanism by which *NOD2* mutation contributes to disease susceptibility has not been completely identified, mice lacking NOD2 have abrogated production of

α-defensin in Paneth cells, suggesting a potential role for NOD2 in regulating α-defensin production (110). Consistent with these data, the expression of α-defensin in Paneth cells is abrogated in Crohn's disease patients with the *NOD2* mutation (111). Taken together, these findings suggest a possible mechanism by which the *NOD2* mutation compromises the production of anti-microbial molecules by Paneth cells, leading to the colonization of aberrant microbiota, thereby predisposing the host to the development of Crohn's disease. Mice genetically deficient in *NOD2* do not develop Crohn's disease spontaneously, and thus exogenous stresses such as DSS are needed to induce IBD in *NOD2*-deficient mice (112), suggesting that other genetic or environmental factors contribute to the induction of Crohn's disease.

Autophagy

Autophagy refers to multiple biological processes responsible for maintaining the homeostasis of cells such as the removal of damaged organelles, clearance of microorganisms, and signaling of cell starvation (113). It is therefore an important biological event for the gut epithelial layer, which is constitutively exposed to multiple bacterial stimuli and danger signals from intestinal environment. Indeed, dysfunction of autophagy leads to abnormal effects in intestinal tissues. *ATG16L1*, a mammalian homolog of *Atg16*, is one of the gene clusters involved in autophagy (114, 115). *ATG16L1* is expressed in multiple hematopoietic and non-hematopoietic cells, and recently its function in Paneth cells has been characterized. Although there is no inflammatory sign in mice containing hypomorphic *ATG16L1* ($Atg16l1^{HM}$), Paneth cells in these mice have abnormal microarchitecture (116). $Atg16l1^{HM}$ mice co-infected with murine norovirus are susceptible to DSS-induced colitis compared with control mice co-infected with norovirus (117). Therefore, *ATG16L1* and autophagy prevent the development of IBD by establishing IEC homeostasis. This is supported by the facts that *ATG16L1* is one of the susceptibility genes for Crohn's disease (9) and that epithelial-specific deletion of *ATG5* and *ATG7*, which are both autophagy proteins, also present with the similar abnormal phenotype as $Atg16l1^{HM}$ mice (118). Importantly, the elevated inflammatory states in norovirus-infected and DSS-treated $Atg16l1^{HM}$ mice were rescued by treatment with antibiotics, suggesting the involvement of commensal bacteria. Collectively, these data suggest the importance of environmental factors, especially commensal bacteria with genetic abnormalities that are involved in the alteration of physiological autophagy, in the development of IBD (117).

Endoplasmic reticulum stress and X-box-binding protein 1

As discussed above, Paneth cells are secretory type IECs that constantly synthesize and produce secretory molecules such as α-defensins. When these secretory molecules are transported into the ER, the accumulation of misfolded proteins stresses the cells (119). To relieve this ER stress, a series of enzymes and transcription factors cooperatively engage multifunctional processes such as the folding, processing, export, and degradation of proteins. These processes are called unfolded protein responses (UPRs) (119, 120). UPRs maintain the homeostasis of the ER environment; however, excessive retention of misfolded proteins or abnormal UPRs caused by genetic or environmental factors affect the production of proteins and other cellular functions, eventually leading to the initiation of undesired apoptosis (121). X-box-binding protein 1 (XBP1), one of the transcriptional factors that are translated from mRNA spliced by inositol-requiring enzyme 1, induces UPR target genes (122, 123). Epithelial-specific deletion of *XBP1* results in the loss of Paneth cells caused by a high frequency of apoptosis, a reduced number of goblet cells, and hyperproliferation of IECs. Consequently, mice lacking XBP1 spontaneously develop enteritis and are sensitive to *Listeria monocytogenes* infection and DSS-induced colitis (124). More importantly, hypomorphic variants of *XBP1* are risk factors for both ulcerative colitis and Crohn's disease in humans (124). ER stress mediated by XBP1, therefore, has a central role in the maintenance of the homeostasis of IECs and the intestine. *XBP1*-deficient Paneth cells lack the bactericidal activity that is induced by LPS, and *XBP1*-knockdown epithelial cells produce lower pro-inflammatory responses against flagellin (124), suggesting that bacterial stimulation might influence the kinetics of XBP1 and ER stress. Indeed, recent reports showed that the expression of XBP1 is induced by bacteria (e.g. *E. coli*, *Mycobacterium tuberculosis*, *Mycobacterium avium*, and *Klebsiella pneumoniae*) (125–127) and is especially regulated by TLR2 and TLR4 on macrophages (128).

Recently, several lines of evidence have suggested a linkage between NOD1 and NOD2 and ATG16L1. NOD1 and NOD2 directly interact with ATG16L1, are recruited to the plasma membrane of IEC at bacterial entry sites, and subsequently initiate the autophagocytic process (129). In addition, NOD2 induces autophagy and promotes bacterial handling and antigen presentation in DCs (130). Because *NOD2* and *ATG16L1* are expressed in IECs, especially in Paneth cells, both genes are thought to affect the function and maintenance of physiologically normal Paneth cells and their disruption leads to Crohn's disease (9, 110, 111, 116). It could be possible that NOD2 is directly associated with ATG16L1 for the physiological control

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of IECs. Furthermore, ATG16L1 and NOD2 are involved in the development of Crohn's disease, but XBP1 is associated with both ulcerative colitis and Crohn's disease (124). Further studies of the specific localization and gene expression involved in autophagy and ER stress might provide important information about the divergence of these two IBD.

IECs control immunological homeostasis

Another important biological role of IECs is the control of the mucosal immune responses. Intraepithelial lymphocytes and *lamina propria* cells are major cellular components of the mucosal immune system that form molecular and cellular networks with IECs. IECs provide immune modulatory signals to these mucosal immune cells in response to stimulation by commensal bacteria. NOD1 expressed in non-hematopoietic lineage cells, presumably IECs, promotes the formation of ILFs and cryptopatches which suggested to be the precursors of ILFs in response to GMtetraDAP produced by Gram-negative commensal bacteria such as *Bacteroides distasonis* and *E. coli* (131). Indeed, NOD1 regulates the IEC production of β-defensin 3 and CCL20, a chemokine that recruits $CCR6^+$ B cells and lymphoid tissue inducer-like cells to the mucosal compartment (132–134). Mice lacking NOD1 and ILFs have aberrant gut microbiota (131), suggesting that IECs have important roles in the detection and maintenance of homeostasis with gut microbiota and that the dialog between commensal bacteria and IECs has a critical role in the induction and maintenance of secondary lymphoid tissues associated with the mucosal immune system (e.g. MALT). This notion is supported by the observation that the microarchitectures of PPs and the spleen are immature in germfree mice (4). From earlier experiments on the treatment of germfree mice mono-associated with *E. coli* or LPS, it was shown that gut microbial stimulation is necessary for the development of the mucosal immune system (135, 136). Furthermore, a recent study has provided evidence that GMtetraDAP-diffused bacterial structures, rather than live bacteria, are enough to induce ILFs (131); however, it is not well known how this microbial molecule is detected by the cytosolic sensors of IECs.

After the uptake of antigens, antigen-presenting cells, especially DCs, instruct the active immune responses whether to respond or not. Although commensal bacteria constitutively provide biological signals to the intestinal tissues, the activation of mucosal immune cells is maintained at a low, but not unresponsive, level without any signs of pathological reactions. This immunological status is considered to be a part of 'physiological inflammation' (12, 13). Physiological inflammation is a balance between the activating and inhibitory immune responses. The important point is that this immunological homeostasis is initially established by cross-communication between commensal bacteria and IECs.

SFB are indigenous bacteria that especially colonize the rat and murine ileal epithelial layer (137, 138). These bacteria are closely associated with IECs and are known to induce the expression of major histocompatibility complex II molecules and fucosyl asialo GM1 glycolipids in IECs (138). Recently, it has been reported that SFB drive the differentiation of Th17 cells, which are a pro-inflammatory type of immune cell (139, 140). SFB induce serum amiroid A, presumably produced by ileal epithelial cells, which promotes the differentiation of Th17 cells (140) (Fig. 4). Th17 cells are also induced by colonic *lamina propria* $CD70^+CD11c^+$ cells, which are stimulated by ATP produced by commensal bacteria (141). Bacterial flagellin is detected by TLR5 expressed on *lamina propria* $CD11c^{high}$ DCs and induces Th17 cells as well (142). In contrast, clusters IV and XIVa of the *Clostridium* genus are involved in the induction of regulatory T cells (Treg) in the colon (143). These bacteria induce the production of TGF-β by colonic epithelial cells, which promotes the differentiation of $helios^-$ inducible Treg cells (Fig. 4). Recently, polysaccharide A produced by *B. fragilis* has been shown to induce Tregs through the TLR2 on T cells (144, 145). Other reports show that SFB and *Lactobacillus reuteri* also have the potential to induce Treg cells (139, 146) (Fig. 4). These interesting reports indicate that the differentiation of T-cell subsets, at least in the intestine, is controlled by local colonization of specific commensal bacteria and IECs (147). The detailed mechanisms of the cross-regulation of Th17 and Treg cell differentiation by certain commensal bacteria and IECs remain to be explored. In addition to Th17 and Treg cells, mucosal Th2 cells might also be regulated by bacteria–IEC interactions. IECs, especially at the proximal colon, constitutively express thymic stromal lymphopoietin (TSLP) (148), and the expression of TSLP in the gut epithelium is induced by exposure to bacteria (149, 150) (Fig. 4). In *in vitro* experiments, TSLP produced by IECs educates DCs to instruct Th2 cells to produce cytokines such as IL-4, IL-5, IL-10, and IL-13 (149, 150). Indeed, mice lacking TSLP receptor defect the Th2 responses against *Trichuris muris* infection and develop severe intestinal inflammation induced by DSS (148).

Conclusion

Three-way interactions among commensal bacteria, IECs, and mucosal immune cells provide the platform for the establish-

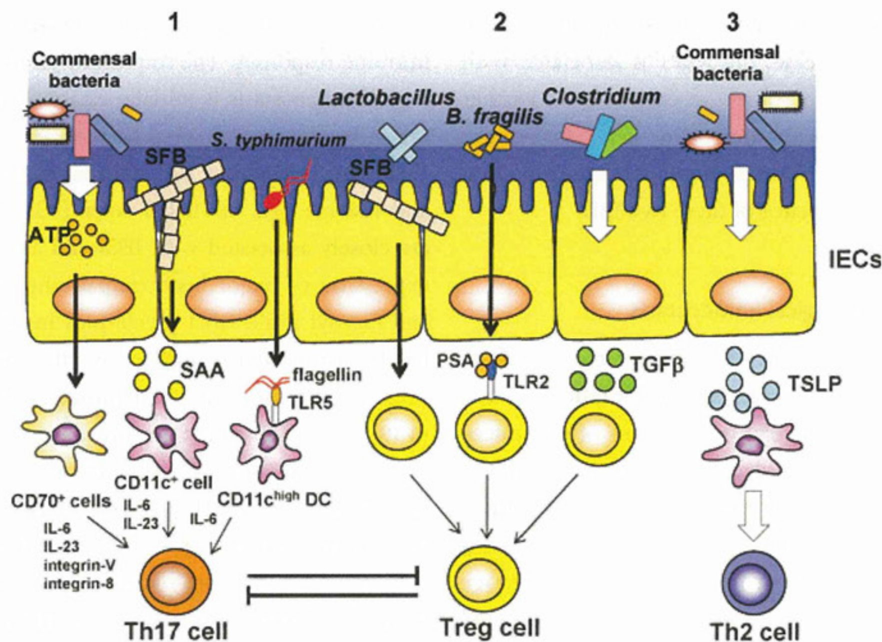

Fig. 4. Gut microbiota–epithelium interaction regulates the homeostasis of the gut immune system. Colonic *lamina propria* $CD70^+$ cells detect adenosine 5′-triphosphate (ATP) produced by commensal bacteria and promote the differentiation of Th17 cells. Segmented filamentous bacteria (SFB) induce the production of serum amyloid A (SAA) from ileal IECs. Epithelial derived SAA stimulates *lamina propria* $CD11c^+$ cells and induces the differentiation of Th17 cells. Flagellin of pathogenic bacteria is detected by Toll-like receptor (TLR) 5 on $CD11c^{high}$ dendritic cells (DCs), leading to the differentiation of Th17 cells. *Clostridium* clusters IV and XIVa induce TGF-β production in intestinal epithelial cells (IECs), which promotes Treg differentiation. Polysaccharide A (PSA) produced by *Bacteroides fragilis* is detected by TLR2 on T cells and induces Tregs. *Lactobacillus reuteri* and SFB induce Tregs as well. Commensal bacteria induce thymic stromal lymphopoietin (TSLP), educating DCs to enhance Th2 cell differentiation. Thus, commensal bacteria–IEC interactions establish a balance of Treg and Th17 cells as well as effector Th2 cells for the mucosal T-cell-mediated intestinal homeostasis and active immunity.

ment of the intestinal dynamic and flexible environments that involves in the formation of mucosal homeostatic balance between quiescent and active immunity (*Fig.* 5). Among the three arms of this biological communication network, IECs might play a central role because they are physically and biologically located between commensal flora and the mucosal immune system, and thus affect both outside and inside biological worlds of the microbiota and host immune responses, respectively. Meanwhile, commensal bacteria influence multiple epithelial functions both directly and indirectly through MAMPs and metabolites. Mucosal immune cells educated by commensal bacteria affect the functions of the epithelium by producing cytokines such as TNF. Some of the mucosal inflammatory cytokines (e.g. TNF, lymphotoxin, and IL-6) contribute to the formation of secondary lymphoid tissues and the homeostasis of mucosal immune systems such as IgA production and T-cell differentiation, which are thus involved in the creation of 'physiological inflammation'. Thus, this tripartite relationship maintains the steady-state homeostasis of the gut microenvironment and at same time, the system is still capable of immediately responding to invasion of undesired antigens.

IECs construct a critical defensive barrier system against foreign bacteria, both commensal and pathogenic. Temporary disruption of the epithelial barrier results in invasion by commensal bacteria, and recruitment and activation of pro-inflammatory mucosal immune cells for the initiation of acute inflammation. However, permanent disruption of the epithelial barrier as a result of genetic abnormalities or sustained commensal stimulation leads to the formation of chronic inflammation. Further studies are needed to understand in more detail the molecular and cellular mechanisms of how permanent disruption of the epithelial barrier or physiological inflammation is provoked. The inflammatory cytokine family is one of the critical factors involved in the development and maintenance of the mucosal immune system and the regulation of IECs, including the induction of epithelial apoptosis (94). Although prevention of the constitutive overproduction of TNF is a target for the treatment of Crohn's disease (151, 152), the mechanism of TNF induction and how TNF is involved in the development of Crohn's disease is not yet fully understood. In addition, recent, powerful GWASs have provided critical information about the susceptibility genes of Crohn's disease and ulcerative colitis

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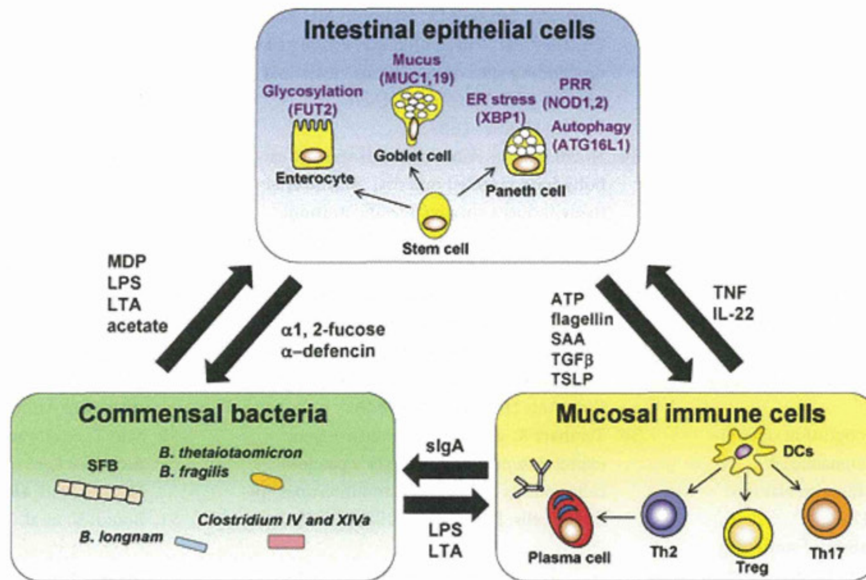

Fig. 5. Tripartite interaction controls intestinal homeostasis. Commensal bacteria modulate mucosal immune responses directly or indirectly through intestinal epithelial cells (IECs). The mucosal immune system (MIS) regulates the homeostasis of gut microbiota, which is mediated by IECs through the cellular axis of MIS–IEC. Therefore, IECs are a key interfacing factor for the tripartite network of commensal microbiota, IEC and MIS in maintaining the homeostasis of the intestinal environment. ER, endoplasmic reticulum; PRR, pattern recognition receptor; DCs, dendritic cells; TNF, tumor necrosis factor; SAA, serum amyloid A; TGF, transforming growth factor; TSLP, thymic stromal lymphopoietin.

(9, 153). From this information, epithelial functions are assumed to be affected by bacterial recognition receptors (NOD2), autophagy (ATG16L1, IRGM), ER stress (XBP1, ORMDL3), mucus production (MUC1, 19), and glycosylation (FUT2) (Fig. 5). Future studies will target the relationships between commensal bacteria and the functions of these genes in the IECs, especially in Paneth cells and stem cells. These studies will contribute to the discovery of novel biological events and the development of innovative therapeutic approaches for mucosa-associated diseases (e.g. IBDs). Furthermore, it will also contribute for the screening of new target molecule for activation of antigen-specific immune responses leading to the development of new generation of mucosal adjuvant.

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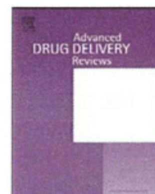

Gut-associated lymphoid tissues for the development of oral vaccines☆

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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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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.

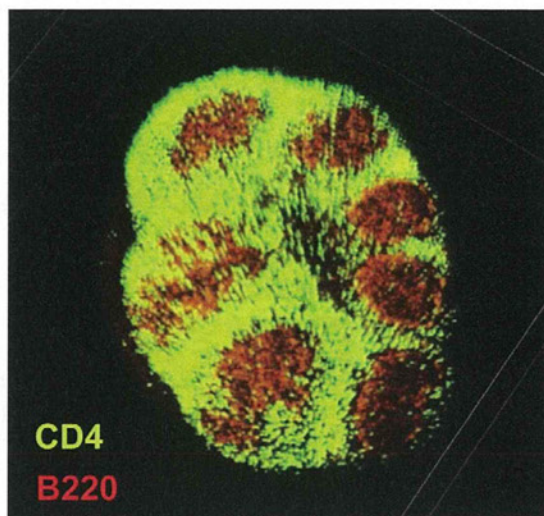

Fig. 1. Microarchitecture of murine Peyer's patches. Purified T cells (green) and B cells (red) were chemically labeled with carboxyfluorescein succinimidyl ester and arboxy SNARF-1, respectively, and adoptively transferred into mice. Fifteen hours after the transfer, cell distribution in the Peyer's 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, CD8α^{+} 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 α4β7 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

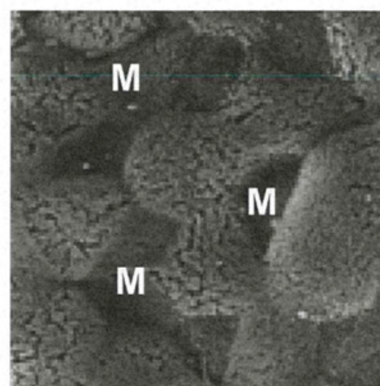

Fig. 2. Scanning electron micrograph of M cells in the Peyer's patches. Scanning electronic microscopy demonstrates that the M cells (indicated as "M") in the Peyer's patches are distinguished from surrounding ECs by their depressed position relative to the ECs, dark brush border, and short microvilli.

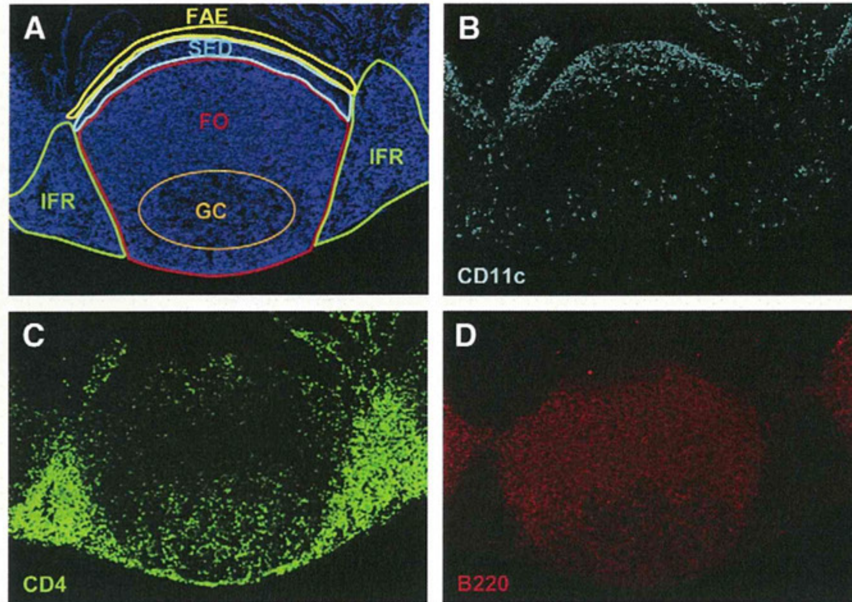

Fig. 3. Distinct cell distribution in the Peyer's patches. Immunohistochemical data on Peyer's patches is shown. (A) Each cell was identified with 4′,6-diamidino-2-phenylindole staining. PP compartments are outlined and labeled as follows: FO, follicle; FAE, follicle-associated epithelium; GC, germinal 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 $[IL\text{-}7R]^+$, $CD3^-$, $CD4^+$, $CD45^+$, lymphotoxin [LT] $\alpha1\beta2$). 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

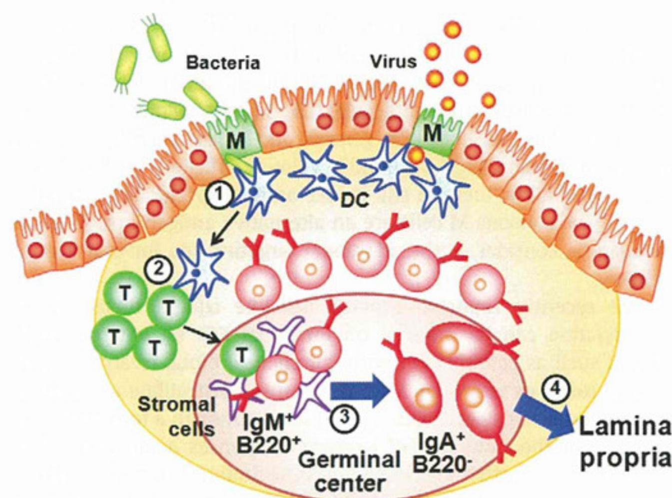

Fig. 4. Sequential processes for initiation of antigen-specific immune responses in Peyer's patches. (1) After transport of antigen by M cells, dendritic cells (DC) take up antigen, and (2) migrate to the T cell region. There, the DCs prime antigen-specific T cells by presenting antigen on MHC molecules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

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

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

Table 1
Tools for M cell targeting.

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

6. Conclusion

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

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