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Review

Unique functions of mucosa-associated lymphoid tissues as targets of mucosal vaccines

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ABSTRACT

At various mucosal sites, such as in the eye and the respiratory and intestinal tracts, the immune system uses highly sophisticated and organized lymphoid tissues known as mucosa-associated lymphoid tissues (MALTs). Studies of the molecular and cellular basis of MALT organogenesis have provided evidence that MALTs are key sites for the induction of active immune responses for protection against pathogenic infections. These functions of the mucosal immune system are currently being used to develop mucosal vaccines, and accumulating evidence has recently indicated that targeting MALTs with vaccine antigens efficiently induces mucosal immunity. In this review, we describe the unique immunological functions of MALTs and the current and future directions of the development of mucosal vaccines.

KEYWORDS: mucosa-associated lymphoid tissue, lymphoid tissue development, mucosal vaccine, M cell

INTRODUCTION

The body is separated from the outer environment by the skin and mucosal surfaces, including those of the eye and the respiratory, gastrointestinal, and genital tracts. Because of the villous structure of the mucosa, in particular in the intestine, mucosal tissues possess 200-fold surface area in comparison with the skin. Thus, mucosal surfaces interface with vast areas of the external environment, making them major portals for the entry of infection. Indeed, various types of bacteria and viruses (e.g., *Salmonella*, influenza, and human immunodeficiency virus) use mucosal surfaces for their entry. Therefore, much attention has recently been paid to the importance of the mucosal immune system as the first line of defense against pathogenic infection.

The mucosal immune system uses both innate and acquired immunity. The intestinal mucus layer contains goblet cells, which secrete mucin, and Paneth cells, which secrete antimicrobial peptides (e.g., α-defensins and RegIIIγ) [1]. Epithelial cells (ECs) also secrete antimicrobial peptides such as β-defensins and RegIII family proteins in response to interleukin (IL)-22 produced by the recently discovered innate lymphoid cells [2, 3]. These secretions help to protect the body from bacterial infection. T-cell receptor (TCR)αβ- or TCRγδ-bearing T cells are distributed between the ECs and are commonly termed intraepithelial lymphocytes (IELs). Through their interaction with ECs, the IELs make a kind of "mucosal internet" of reciprocal regulation for activation of IELs and cell growth of ECs through the production of IL-7 by ECs and keratinocyte growth factor by IELs, respectively [4, 5]. In addition, IELs provide CD160-mediated signals to ECs via herpes virus entry mediator, which induces the expression of

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RegIIIγ and IL-22 receptor 1 by ECs, thereby playing a key role in host defenses against bacterial infection [6]. Therefore, the ECs, in communication with innate lymphoid cells and IELs, provide antigen-nonspecific innate immunity at its interface with the environment.

In the induction of acquired immunity in the mucosa, organized lymphoid tissues known as mucosa-associated lymphoid tissues (MALTs) play a key role in the priming of antigen-specific immunity. In contrast, the lamina propria (LP) compartment acts as an effector site through antibody production by plasma cells and through the killer function of T cells. The aim of mucosal vaccines is to induce appropriate acquired, antigen-specific immune responses at mucosal sites through immunization via the mucosal route. In this review, we describe the unique functions of MALTs as organizers of the mucosal immune system.

Various types of MALTs

Secondary lymphoid tissues provide the optimum environment for communication among various immunocompetent cells, thus promoting the initiation of antigen-specific immune responses [7]. In various mucosal tissues, there are several types of MALTs (Figure 1), including Peyer's patches (PPs) and isolated lymphoid follicles (ILFs) in the small intestine, cecal patches in the cecum, and colonic patches in the large intestine (collectively known as gut-associated lymphoid tissue, or GALT) [8, 9]. PPs are the best-characterized MALTs in the gastrointestinal tract. PPs are always found on the anti-mesentery side of the small intestine; there are generally 8 to 10 PPs in mice and hundreds in humans [10]. One PP possesses several B-cell-rich follicle areas and T-cell-rich interfollicular regions (IFRs), as well as dendritic-cell (DC)-rich subepithelial dome (SED) regions [11]. Generally, mice possess hundreds of ILFs as single aggregations of B cells, but they lack T-cell-rich IFRs [9]. As the gut is exposed to large numbers of commensal flora, germinal center formation with expression of activation-induced cytidine deaminase (AID), an enzyme essential for immunoglobulin class-switch recombination (Ig-CSR) and somatic hypermutation (SHM), is observed in both the PPs and the ILFs of naive mice [12]. However, AID is lost in the PPs, but retained in the ILFs, of

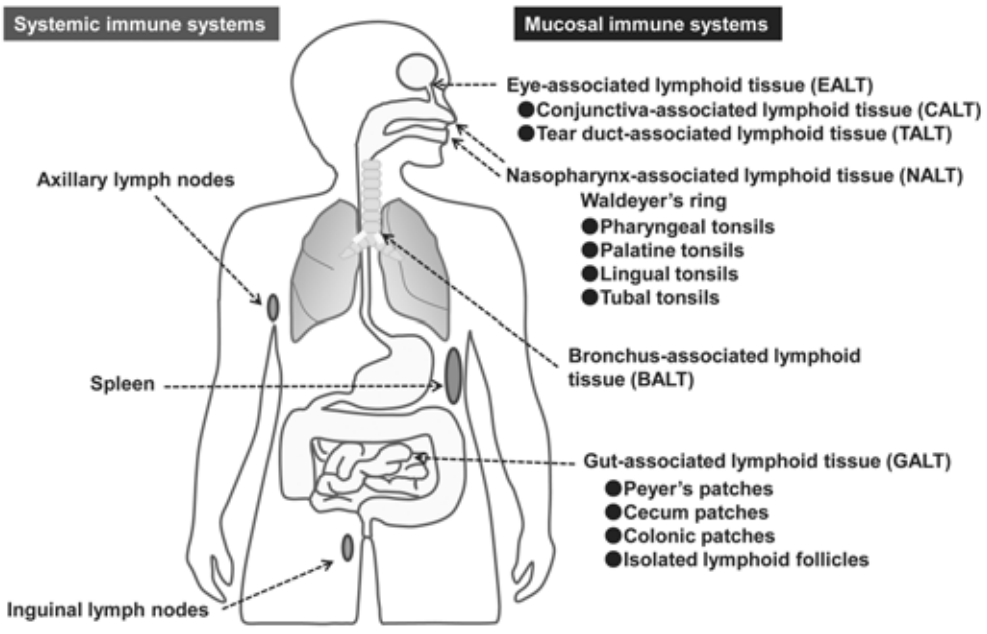

Figure 1. Various secondary lymphoid tissues in the systemic and mucosal compartments. The systemic immune system uses the spleen and peripheral lymph nodes (e.g., the inguinal and axillary lymph nodes), whereas mucosa-associated lymphoid tissues develop in various parts of the mucosal compartment.

Tcrβ$^{-/-}$*Tcrδ*$^{-/-}$ mice [13]. These findings indicate that the PPs and ILFs are respectively responsible for T-cell-dependent and T-cell-independent IgA antibody production [13].

Tear flow begins in the lacrimal glands and carries ocularly encountered antigens that eventually reach the nasal cavity via the lacrimal sac and nasolacrimal duct. This tear pathway forms an interface with the outside environment. Tear duct-associated lymphoid tissue (TALT) develops in the lacrimal sac in humans and mice and takes up luminal antigens that are carried by the tear flow [14, 15]. TALT and CALT (conjunctiva-associated lymphoid tissue) form a functional unit as EALT (eye-associated lymphoid tissue) and play a critical role in ocular defense [15-17].

Nasopharynx-associated lymphoid tissue (NALT), which has been found at the bottom of the rodent nasal cavity, corresponds to Waldeyer's ring (a network of pharyngeal, palatine, lingual, and tubal tonsillar tissues) in humans [18]. NALT is responsible for protective immunity against inhaled antigens [18]. In contrast to PPs, the NALT and TALT generate germinal centers with AID-mediated Ig-CSR and SHM only when they are stimulated by danger signals from, for example, microbial infection or administration of toxins [14, 19].

Bronchus-associated lymphoid tissue (BALT) is found at the second to fourth branchings of the bronchial tree in older (32 to 40 weeks old), but not young (8 to 10 weeks old) mice [20]. Although TALT and NALT develop independently of microbial stimulation [14], BALT tissue formation requires antigen stimulation, for example by viral infection or inflammatory disease [21-23]. Therefore, BALT is generally categorized as a tertiary lymphoid tissue, i.e., inducible BALT (iBALT). It is reported that iBALT formation is a key event in the protection of mice from influenza infection [21].

Features of the intestinal immune system associated with the production of secretory IgA antibodies

Accumulating evidence indicates that PPs play a key role as intestinal inductive sites for the IgA antibody response. They achieve this function through the harboring of unique cell populations through restricted cell distribution and trafficking, and through immunological crosstalk. The route of antigen entry differs between the systemic and the mucosal immune systems. In the systemic immune system, antigen-presenting cells such as DCs carry antigens via the blood to the spleen or via the lymphatics to the peripheral lymph nodes [24, 25]. In contrast, MALTs do not contain afferent lymphatics; instead they take up luminal antigens via the follicle-associated epithelium (FAE). In the FAE, microfold or membranous cells (M cells) are present as specialized antigen-sampling cells [25]. The M cells efficiently do transcytosis of engrafted antigens, thus transferring them from the lumen to the SED region of the MALTs, where DCs take up the antigens. The DCs then migrate into the IFRs to present the antigens and prime T cells for subsequent antigen-specific immune responses.

One major unique feature is that the intestinal immune system preferentially produces IgA antibodies; this is in sharp contrast to the systemic immune system, where IgG is dominant. As one possible mechanism, expression of inducible nitric oxide synthase (iNOS) has been detected in the DCs of PPs but not in the spleen [26]. iNOS promotes T-cell-dependent IgA-CSR by inducing the expression of transforming growth factor (TGF)β receptor on B cells and it also promotes T-cell-independent IgA-CSR through the production of IgA-inducing cytokines (a proliferation-inducing ligand [APRIL] and a B-cell-activating factor belonging to the tumor necrosis factor family [BAFF]) on DCs [26].

The primed T and B cells migrate out of the PPs and then into the intestinal LP. The former step is mediated by sphingosine-1-phosphate (S1P), a lipid mediator [27]. The expression level of type 1 S1P receptor ($S1P_1$) is elevated at the final step of B-cell differentiation in the PPs; the B cells recognize the S1P gradient between the PPs and the lymph ducts and consequently emigrate from the PPs [27]. Indeed, an increase in the number of IgA^+ cells in the PPs and a consequent reduction in the number of these cells in the intestinal LP occur when mice are treated with FTY720 (an $S1P_1$ agonist that induces internalization of the receptor, thereby causing a loss of responsiveness to S1P) [27]. The latter step of migration into the intestinal LP requires the expression of gut-homing

molecules, namely C-C motif chemokine receptor (CCR)9 and α4β7 integrins. DCs in the PPs preferentially express retinal dehydrogenase, a key enzyme for converting vitamin A into retinoic acid; DC-derived retinoic acid is crucial for the induction of CCR9 and α4β7 integrin expression on T and B cells [28, 29]. $CD4^+$ and $CD8^+$ T cells, as well as IgA^+ plasma cells, disappear from the small intestinal LP if vitamin A is depleted from the diet [28, 29].

IgA antibodies produced by plasma cells in the LP form dimeric or polymeric IgA connected by a J (joining) chain. This is then efficiently transported into the lumen by the poly-Ig receptor, which is expressed on the basolateral side of the EC [30]. Expression of the poly-Ig receptor is enhanced by cytokines such as interferon γ, tumor necrosis factor α, IL-4, and IL-1 [31-34]. In the absence of poly-Ig receptor expression, there is a severe defect in the export of dimeric or polymeric IgA into the lumen [35, 36]. Thus, mice deficient in the poly-Ig receptor are more susceptible to infections with influenza virus [37] or with *Streptococcus pneumoniae* [38] or *Mycobacterium tuberculosis* [39].

This evidence demonstrates that the mucosal immune system is equipped with inductive (i.e., PPs) and effector (i.e., LP) sites for efficient IgA production, and that the two sites are connected by the highly sophisticated machinery of the cellular trafficking system (Figure 2). Unlike in the intestine, however, in the respiratory tract, a vitamin-A-mediated trafficking system is not crucial for cell trafficking to respiratory mucosa, suggesting that the mucosal immune system is regulated differently in the

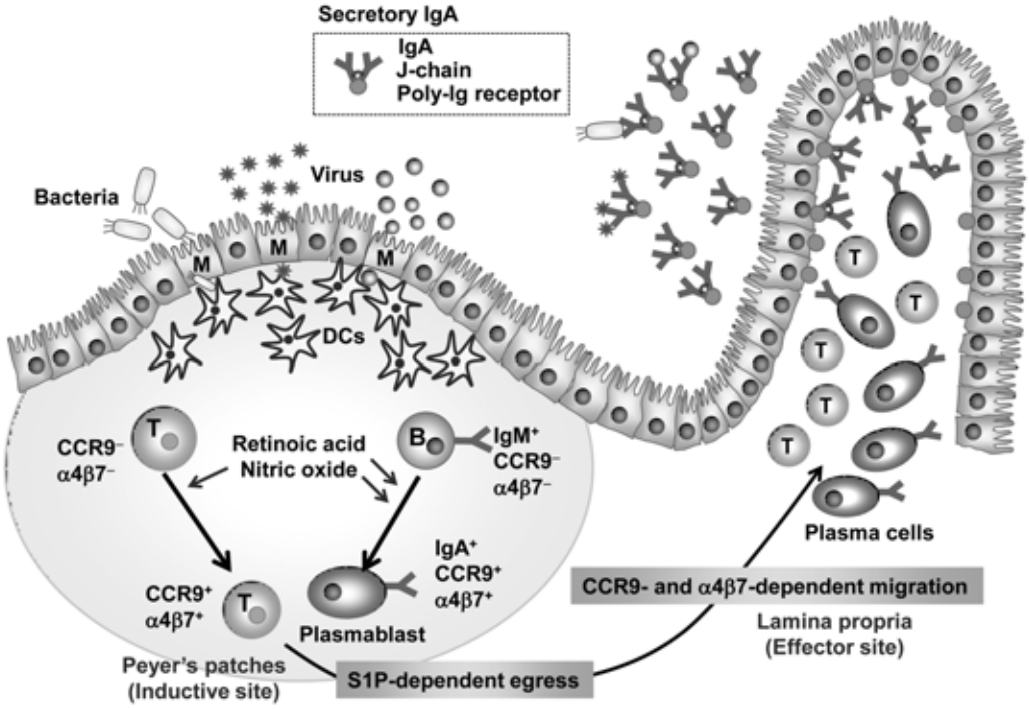

Figure 2. Intestinal immune system for the production of secretory IgA antibodies. In the intestine, PPs and LP are functionally compartmentalized as the inductive and effector sites, respectively. Luminal antigens are efficiently taken up by M cells and transported to DCs in the PPs. DCs produce retinoic acid upon antigen presentation; the retinoic acid induces the expression of CCR9 and α4β7 integrins on T and B cells. Some of the DCs produce nitric oxide, thereby promoting Ig-CSR to IgA. S1P-mediated signals induce the egress of differentiated IgA^+ plasmablasts into the circulation. After circulation, gut-imprinted lymphocytes migrate to the LP compartment, and IgA^+ plasmablasts finally differentiate into plasma cells for the production of secretory IgA antibodies. The J chain and poly-Ig receptor (secretory component) are required for the generation of dimeric or polymeric secretory IgA antibodies. See the text for definitions of abbreviations.

different mucosal compartments [28]. The respiratory mucosa expresses vascular cell adhesion molecule 1 (VCAM-1) rather than mucosal vascular addressin cell adhesion molecule 1, which is expressed on intestinal mucosa. Therefore, induction of α4β1 integrin expression on lymphocytes is likely to be involved in the trafficking system of the respiratory tract, although expression of VCAM-1 is not specific to the respiratory mucosa but occurs also in the bone marrow and skin [40]. The cellular and molecular basis of the trafficking system in the respiratory tract needs to be further investigated.

Requirement of MALTs for appropriate induction of mucosal immune responses

As exemplified above, rigid regulation of the lymphoid structure and cell trafficking in MALTs play critical roles in protective immunity in the mucosal compartments. Knowledge of organogenesis allows us to obtain MALT-null animals by inhibiting the molecular pathways needed for MALT organogenesis. This has greatly contributed to the analysis of MALT function.

Differentiation, migration, and activation of lymphoid tissue inducer (LTi) cells are key steps in the development of secondary lymphoid tissues [41]. In general, differentiation of LTi cells, categorized by $CD3^{-}CD4^{+}CD45^{+}$ populations, occurs in the fetal liver and is essentially regulated by the transcriptional regulators such as inhibitor of DNA binding 2 (Id2) and retinoic acid-related orphan receptor (ROR)γt and by the transcriptional complex of P1-Runx1 (promoter-1-transcribed Runt-related transcription factor 1) and CBF (core binding factor) β2 [42-45]. When LTi cells pick up the signals from C-X-C motif chemokine ligand (CXCL)13 (which is induced by neuron-derived retinoic acid [46]) via CXCR5, latent α4β1 integrin changes to the active form, thus enabling the LTi cells to stably interact with $VCAM\text{-}1^{+}$ICAM (intercellular adhesion molecule)-1^{+} lymphoid tissue organizer (LTo) cells [47]. The LTi cells are then activated by cytokine receptors, including IL-7Rα or RANK (receptor activator of NF-κB), which induce the expression of lymphotoxin (LT)α1β2 on the surfaces of the LTi cells [48]. LTα1β2 stimulates LTo cells via LTβR-NIK-p52/RelB alternative NF-κB pathways. This results in the production of large amounts of CXCL13, CCL19, and CCL21, as well as VCAM-1 and ICAM-1, by the LTo cells [49, 50]. The interaction of LTi cells with LTo cells via cytokine signals creates a positive feedback loop, which finally induces the migration of T and B cells, as well as DCs, to the lymphoid tissue anlagen [41]. These successive interactions among hematopoietic cells (i.e., LTi cells) and non-hematopoietic cells (i.e., LTo cells) induce the development of the organizing centers of PPs (Figure 3).

As these molecular and cellular axes are essential for the genesis of lymphoid tissues, the use of neutralizing antibody treatment or gene-targeting methods results in lymph node depletion. Treatment with IL-7Rα neutralizing antibodies or antagonistic LTβR-Ig fusion protein during a limited embryonic period leads to the selective failure of PPs to develop in the offspring, without affecting the development of other lymphoid tissues [51, 52]. Studies of PPs-null mice give direct evidence that PPs are inductive sites for mucosal immune responses. These mice show decreased levels of an antigen-specific IgA antibody response against orally administered *Salmonella* or particulate antigens [53, 54].

Several lines of evidence have demonstrated that the organogenesis of NALT and TALT differs from that of PPs and other lymph nodes. Although organogenesis of PPs and peripheral lymph nodes occurs during embryogenesis, NALT and TALT develop at postnatal days 7 to 10 in mice [14, 18]. In addition, the molecular requirements for NALT and TALT development differ from those for PPs and peripheral lymph nodes development. As is the case with other secondary lymphoid tissues, NALT genesis is dependent on Id2 but is uniquely independent of RORγt [55, 56]. Intriguingly, TALT genesis requires neither Id2 nor RORγt, indicating that LTi cells for TALT genesis differentiate in the absence of these transcriptional regulators [14]. Indeed, $CD3^{-}CD4^{+}CD45^{+}$ LTi cells isolated from the TALT anlagen express neither Id2 nor RORγt, whereas cells from the small intestine (namely PPs inducer cells: PPi cells), express both transcription regulators [14]. Moreover, unlike in the case of PPs, CXCL13, IL-7R, and LTα1β2 are

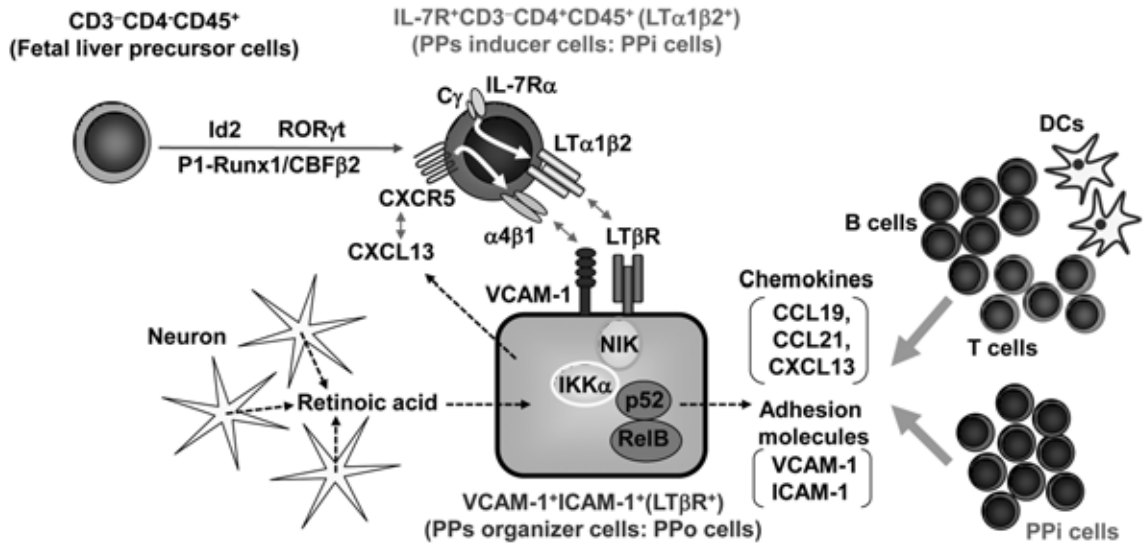

Figure 3. Interaction of hematopoietic and non-hematopoietic cells induces development of organizing centers of PPs. Differentiation of PPi cells requires the expression of Id2, RORγt, and P1-Runx1/CBFβ2 transcriptional regulators. Retinoic acid produced by neuron cells induces the initial expression of CXCL13 by PPo cells. $CXCR5^+$ PPi cells respond to the chemokine, migrate to the PP anlagen, and interact with PPo cells. The IL-7R-mediated cytokine signal activates the PPi cells, leading to the expression of membrane-bound LTα1β2. $LT\beta R^+$ PPo cells are activated by PPi-derived LTα1β2 via a NIK-dependent alternative NF-κB pathway, which results in the production of large amounts of lymphoid chemokines and adhesion molecules. These molecular and cellular interactions make up a positive feedback loop for the migration of PPi cells as well as conventional leukocytes to the PP anlagen; they are therefore critical for tissue genesis and organization of PPs. See the text for definitions of abbreviations.

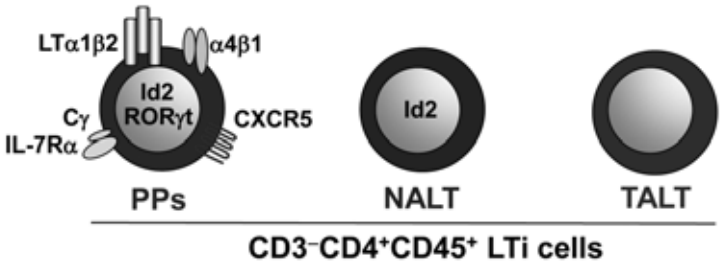

Figure 4. Uniqueness of $CD3^-CD4^+CD45^+$ LTi cells at the MALT anlagen. Expression of Id2 and RORγt is required for the differentiation of PPi cells, whereas that of RORγt is dispensable for NALT development. Expression of neither Id2 nor RORγt is essential for TALT development. CXCR5/IL-7R/LTα1β2-mediated organogenesis-related chemokine or cytokine signals are required for the initiation of PP development but not critical for the initiation of NALT or TALT development. See the text for definitions of abbreviations.

not essential for NALT and TALT development [14, 55]. This evidence indicates that the developmental programs for each MALT (e.g., PPs, NALT, and TALT) are distinctively different and thus might be operated by unique LTi cells (Figure 4). As the molecules specifically required for NALT or TALT development are currently unknown, experimental data from mice lacking specifically TALT or NALT are not yet available, and this issue is a subject of ongoing study.

Recently, it has been shown that iBALT develops in the absence of Id2 and RORγt, indicating that conventional LTi cells are not essential for tertiary lymphoid organ development in the respiratory

tract [57]. In agreement with the finding that iBALT develops with inflammation [21-23], T-regulatory cells play an important role in the inhibition of iBALT genesis in a CCR7-dependent manner [58]. On the other hand, IL-17 produced by autoreactive T cells contributes at least partially to iBALT formation [57, 59].

MALT targeting is a prospective strategy for the development of mucosal vaccines

The important function of the mucosal immune system in immunosurveillance has led to the development of mucosal vaccines, some of which have already been used clinically (e.g., FluMist, RotaTeq and Rotarix) [60]. The importance of MALTs as inductive sites for mucosal immunity suggests that antigen delivery to MALTs will facilitate the effects of mucosal vaccines. In this aspect, M cells are primary targets for vaccine antigen delivery because of their strong ability to transport antigens from the lumen to DCs in the SED region of MALTs (Figure 5). *Ulex europaeus* agglutinin (UEA)-I is a lectin that is uniquely reactive to murine M cells in the FAE but not to ECs, thus allowing the effective delivery of vaccine antigens to M cells in mice [61, 62]. The reactivity of UEA-I to murine M cells has been experimentally applied to the oral delivery of UEA-I-agglutinated *Helicobacter pylori*; serum IgG and intestinal IgA levels were both significantly greater with this system than when *H. pylori* was given with phosphate-buffered saline or irrelevant lectin [61]. As a similar strategy, M-cell-specific antibodies have been established. One of them is NKM16-2-4, which recognizes α1,2-fucose-containing carbohydrate and can efficiently deliver conjugated vaccine antigens (e.g., tetanus or botulinum toxoid) to MALTs for the effective induction of antigen-specific mucosal IgA and serum IgG antibody responses and the consequent protection of mice from lethal challenge with toxin [63]. Another antibody is anti-glycoprotein

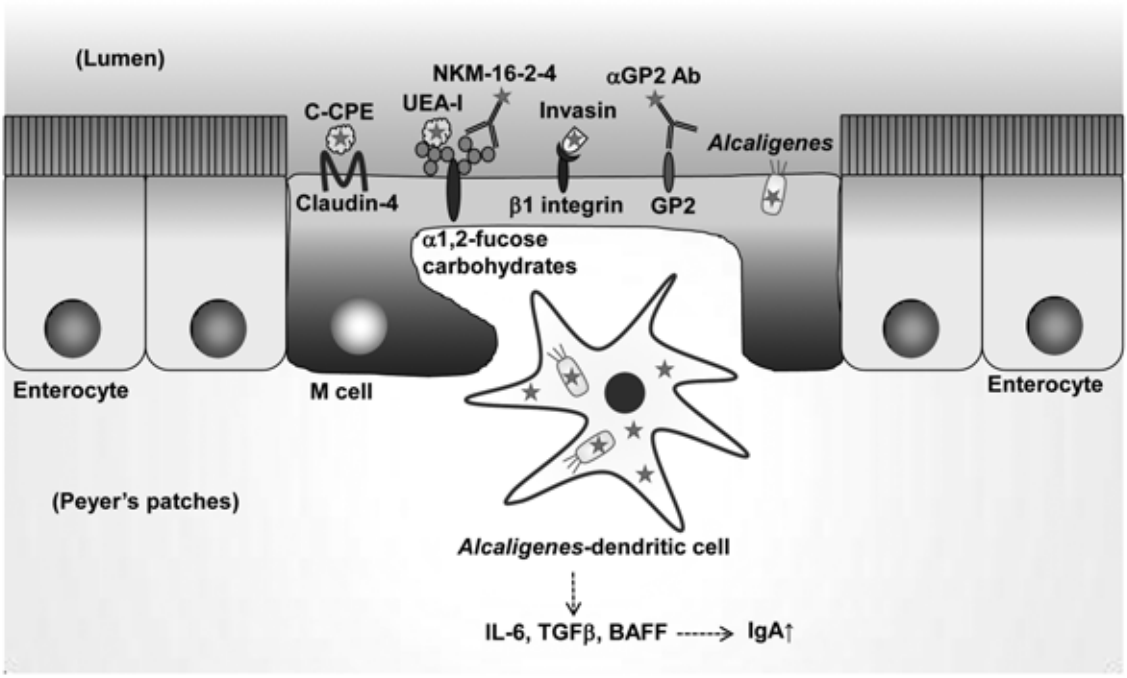

Figure 5. M-cell-targeted mucosal vaccines. Several systems have been tried for the targeting of vaccine antigens selectively to the M cells of PPs. Unlike neighboring enterocytes, M cells express unique molecules such as GP2, β1 integrin, claudin-4, and α1,2-fucose-containing carbohydrates. Vaccine antigens with specific antibodies or ligands can be effectively transported to M cells. *Alcaligenes* are unique commensal bacteria that bind to M cells and reside inside PPs, where *Alcaligenes* promote IgA production through the induction of IL-6, TGFβ and BAFF production by DCs. See the text for definitions of abbreviations.

(GP)-2 antibody [64, 65]. GP-2 is selectively expressed in M cells, and this expression is involved in the transcytosis of type I piliated bacteria such as *Salmonella* [65]. Thus, vaccine antigen delivery with M-cell-specific antibodies provides an efficient tool for the induction of mucosal immune responses.

As some pathogens possess these systems for invading via M cells, another approach is to utilize the systems to deliver vaccine antigens to M cells or MALTs (e.g., *Yersinia*-derived invasion [66, 67] or reovirus-derived σ1 protein [68]). The C-terminal fragment of *Clostridium perfringens* enterotoxin (C-CPE), which binds with high affinity to claudin-4, would be another candidate [69]. Claudin-4 is highly expressed in the FAE of PPs, including in M cells [70, 71]. M cells take up antigens more efficiently when antigens are included in nanoparticles that display C-CPE [72]. Nasal immunization with C-CPE-conjugated antigen, but not with a mixture of C-CPE and antigen, induces antigen-specific mucosal IgA and serum IgG responses [73]. In addition to pathogenic microorganisms, some types of commensal bacteria are uniquely transported into the PPs. Genetic analysis of the commensal bacteria inside PPs has identified commensal bacteria present predominantly inside PPs, but not in the mucus layer or the LP compartment [74]. Subsequent studies have revealed that *Alcaligenes* binds to M cells in the PPs and are transported to the SED region, where they are taken up by PP DCs and induce IL-6, TGFβ and BAFF expression to promote IgA production [74, 75]. These findings suggest that *Alcaligenes* have an intrinsic ability to deliver antigen to the PPs and stimulate IgA production through the activation of DCs. Taken together, the results of these studies reveal the fundamentals of the mucosal immune system and should help to develop effective mucosal vaccines (Figure 5).

CONCLUSION

Mucosal vaccination is an attractive strategy for protection against infectious diseases by inducing the production of both systemic IgG and mucosal IgA antibodies. Studies of lymphoid tissue development have revealed that MALTs are key sites in the induction of mucosal immune systems. Therefore, MALT-targeted vaccine delivery is a promising method of efficient mucosal vaccination. Unlike the case with intestinal immunology, there are a number of unresolved questions in other mucosal compartment, including the eye and respiratory tract with regard to the mechanisms of MALT development and the imprinting of lymphocyte trafficking, as well as the details of cell phenotype and function. Understanding the basis of mucosal immunology will further facilitate the development of effective mucosal vaccines.

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