The Mucosal Immune System for Vaccine Development

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Abstract

Mucosal surfaces are continuously exposed to the external environment and therefore represent the largest lymphoid organ of the body. In the mucosal immune system, gut-associated lymphoid tissues (GALTs), including Peyer's patches and isolated lymphoid follicles, play an important role in the induction of antigen-specific immune responses in the gut. GALTs have unique organogenesis characteristics and interact with the network of dendritic cells and T cells for the simultaneous induction and regulation of IgA responses and oral tolerance. In these lymphoid tissues, antigens are up taken by M cells in the epithelial layer, and antigen-specific immune responses are subsequently initiated by GALT cells. Nasopharynx- and tear-duct-associated lymphoid tissues (NALTs and TALTs) are key organized lymphoid structures in the respiratory tract and ocular cavities, respectively, and have been shown to interact with each other. Mucosal surfaces are also characterized by host-microbe interactions that affect the genesis and maturation of mucosa-associated lymphoid tissues and the induction and regulation of innate and acquired mucosal immune responses. Because most harmful pathogens enter the body through mucosal surfaces by ingestion, inhalation, or sexual contact, the mucosa is a candidate site for vaccination. Mucosal vaccination has some physiological and practical advantages, such as decreased costs and reduced risk of needle-stick injuries and transmission of bloodborne diseases, and it is painless. Recently, the application of modern bioengineering and biochemical engineering technologies, including gene transformation and manipulation systems, resulted in the development of systems to express vaccine antigens in transgenic plants and nanogels, which will usher in a new era of delivery systems for mucosal vaccine antigens. In this review, based on some of our research group's thirtyseven years of progress and effort, we highlight the unique features of mucosal immune systems and the application of mucosal immunity to the development of a new generation of vaccines.

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52 **Keywords**

53 MALT, GALT, NALT, M cell, Peyer's patch, and MucoRice

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56 **Abbreviations**

57 APC, antigen presenting cell; cCHP, cationic type of cholesteryl group-bearing pullulan; CCL,
58 CC chemokine ligand; CCR, CC chemokine receptor; CMIS, common mucosal immune system;
59 CT, cholera toxin; CTB, cholera enterotoxin B subunit; CXCL, CXC chemokine ligand; CXCR,
60 CXC chemokine receptor; DC, dendritic cell; Ig, immunoglobulin; IgA⁺ B cell, IgA-committed
61 B cell; FAE, follicle-associated epithelium; GALT, gut-associated lymphoid tissue; GI,
62 gastrointestinal; gp2, glycoprotein 2; Id2, inhibitor of DNA binding 2; IL, Interleukin; ILF,
63 isolated lymphoid follicle; LP, lamina propria, LPS, lipopolysaccharide; LT, heat-labile
64 enterotoxin; LTB, heat-labile enterotoxin B subunit; MALT, mucosa-associated lymphoid
65 tissue; M cell, microfold/membranous cell; NALT, nasopharynx-associated lymphoid tissue; PP,
66 Peyer's patch; PspA, pneumococcal surface protein A; RANKL, receptor activator of nuclear
67 factor κ B ligand; ROR γ t, retinoic-acid-receptor-related orphan receptor- γ ; SIgA, secretory
68 immunoglobulin A; TALT, tear duct-associated lymphoid tissue; TGF, transforming growth
69 factor; Th, T helper; TLR, toll-like receptor; TNFR, tumor necrosis factor receptor; Treg,
70 regulatory T; UEA-1, ulex europaeus agglutinin 1

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73 **1. Introduction**

74 The mucosae of the airways, oral cavities, digestive tracts, ocular cavities, and
75 genitourinary tracts provide the interface for interactions between a host multicellular organism
76 and its external environment. These mucosal sites form one of the largest organs of the body,
77 collectively covering a surface area of more than 400 m², and maintain immunological
78 homeostasis through innate and acquired immunity [1]. Due to its continuous exposure to the
79 outside environment and its vast surface area, the mucosal surface is the major route of entry for
80 numerous pathogens [2]. Therefore, to protect against countless invasions at the mucosal
81 surface, the host is equipped with physical and biological barriers. These include epithelial cells
82 joined firmly by tight junction proteins and a dense layer of mucins [3] as well as antimicrobial
83 peptides, such as defensins produced by epithelial cells [4]. In addition, the mucosal tissues are
84 heavily populated with both innate and acquired immune cells, and their surfaces are the
85 location of the secretory immune system, whose major immunoglobulin is secretory
86 immunoglobulin A (SIgA) [5].

87 Past efforts and recent advances in the field of mucosal immunology have contributed
88 extensively to our understanding of this intricate immune system. In addition, there has been an
89 increasing push to develop vaccines that can be delivered through the mucosal route, because
90 the initiation of immune reactions at mucosal sites can provide both systemic and mucosal
91 protection. In contrast, conventional vaccines injected into the tissues or bloodstream do not
92 usually provide effective mucosal protection [6]. Despite the need, considerable scientific
93 challenges still exist in the development of mucosal vaccines because our understanding of
94 mucosal immunity remains limited.

95 Here, we highlight the unique features of the mucosal immune system and discuss the
96 application of mucosal immunity to the development of a new generation of vaccines.

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2. Unique Characteristics of the Mucosal Immune System in the Aerodigestive Tract

The mucosal immune system consists of an integrated network of tissues, lymphoid and nonlymphoid cells and effector molecules, including antibodies, chemokines, and cytokines, all of which are responsible for orchestrating innate and adaptive immune responses by responding to invading pathogens during infection and to antigens delivered during vaccination [1]. Moreover, most mucosa-associated tissues and mucosae are home to numerous endogenous microorganisms, most of which harmlessly coexist with the host [7]. The mucosal immune system is thus responsible for mediating the symbiotic relationship between the host and these microorganisms and at the same time functioning as the first line of defense against harmful invading pathogens [2].

The mucosal immune system is functionally and anatomically divided into two main components: the organized mucosa-associated lymphoid tissues (MALTs), where antigen-specific immune responses are initiated, and the diffuse lamina propria (LP) region and glandular tissues, which are effector sites for antibody production and immune cell-mediated responses (Fig. 1) [2, 8]. There is constant migration of antigen-primed immune cells from inductive sites to effector sites, and this forms the cellular basis for the common mucosal immune system (CMIS) [2, 9]. MALTs, which share many immunological features with secondary lymphoid organs, are characterized by their unique mucosa-associated features. Because they lack an afferent lymphatic system, they have a unique antigen-sampling system, which is represented by the specialized microfold or membranous cells (M cells) that lie within the epithelial layer of the mucosal tissues (e.g., MALT) [10].

MALTs also contain all of the immunocompetent cells, such as dendritic cells (DCs), macrophages, T cells, and B cells, that are required for the generation of an antigen-specific immune response [11]. The first step in a typical immune response is when an ingested antigen is taken up by M cells to be presented to antigen-presenting cells (APCs), such as DCs, which process antigen into peptides and transport the peptides on their MHC class I or II molecules to

underlying T-cell zones [12, 13]. Chemokine–chemokine receptor interactions (e.g., those between CC chemokine ligand [CCL]19 and CC chemokine receptor [CCR]7 and between CCL20 and CCR6) play an important role in the transport of DCs to the T-cell zones. [14]. In the T-cell zones, peptide antigen is presented to naïve T cells for the generation of antigen-specific T cells, including Th1, Th2, Th17, and cytotoxic T cells. As a consequence of antigen-specific interactions, antigen-primed T cells support IgA class switching and somatic hypermutation of B cells in the germinal centers and B-cell zones [15]. Molecular interactions, such as those of CD40/CD40 ligand, and the action of members of the IgA-associated cytokine family (e.g., transforming growth factor [TGF]- β , interleukin [IL]-2, IL-4, IL-5, IL-6, and IL-10) also play important roles in T-cell help for inducing IgA-producing B cells and their differentiation into plasma cells [16-18] (Fig. 1).

After IgA class switching and affinity maturation, IgA-committed B cells (IgA⁺ B cells) migrate from inductive sites, such as Peyer's patches (PPs) and nasopharynx-associated lymphoid tissue (NALT), to the regional lymph nodes through the efferent lymph vessels, and antigen-specific CD4⁺ T cells and IgA⁺ B cells then migrate to the effector sites, such as LP regions [8]. For their migration from the inductive to effector sites, mucosal DCs produce retinoic acids, which induce the expression of $\alpha 4\beta 7$ and CCR9 on both B and T cells and augment the IgA-switching process and migration capacity in PPs, resulting in selective trafficking to the effector sites of the intestinal LP [15]. Under the influence of the mucosal homing and imprinting system, antigen-specific IgA⁺ B cells that have migrated undergo final differentiation into plasma cells under the influence of Th2-type cytokines (e.g., IL-5 and IL-6) for the production of the dimeric or polymeric form of IgA [19, 20]. A basolateral polymeric Ig receptor expressed by epithelial cells at LP regions binds to and endocytoses polymeric IgA. The endocytosed IgA then is transported via intracellular vesicular compartments to the apical surfaces, where the IgA complex composed of the polymeric IgA and Ig receptor is cleaved to

become SIgA and is secreted externally [21] (Fig. 1).

1) Gut-Associated Lymphoid Tissue as the ‘Command Center’ for Mucosal Immunity

The primary inductive sites on the gut-associated lymphoid tissues (GALTs), where most of the IgA responses are initiated, include organized follicular structures such as the PPs, cryptopatches, and isolated lymphoid follicles (ILFs) [2]. PPs, the most extensively studied mucosal inductive tissues, are considered to be one of the largest organized lymphoid tissues in the gastrointestinal (GI) immune system [2]. Organogenesis of PPs is initiated during embryogenesis. In embryonic mice, the expression of vascular cell adhesion molecule 1 and intercellular adhesion molecule 1 on stromal cells of the gut, termed PP organizer cells, is the signal that initiates PP formation [22]. This signal is followed immediately by the appearance of PP inducer cells, which are a component of lymphoid tissue inducer cells that have a unique pattern of cell surface markers: IL-7R⁺CD3⁻CD4⁺CD45⁺ [22, 23] (Fig. 2). PP inducer cells express lymphotoxins α and β , which form a heterotrimer and interact with the lymphotoxin β receptor, a member of the tumor necrosis factor receptor (TNFR) family, on stromal cells to provide the next wave of signals to induce the organization of PPs [22, 24]. The importance of these factors in PP organogenesis is demonstrated by the finding that mice lacking lymphotoxin- α , lymphotoxin- β , or lymphotoxin- β receptor lack PPs [25-27]. The blockade of IL-7R or lymphotoxin- β receptor signaling (or both) during a limited period during gestation also leads to the selective loss of PPs [22, 28]. In addition, the reciprocal interaction between inducer and organizer cells through chemokine and cytokine receptors is essential for the formation of PPs. In this regard, organizer cells produce CXC chemokine ligand (CXCL)13 and CCL19, which stimulate PP inducer cells through CXC chemokine receptor (CXCR)5 and CCR7 [29]. Moreover, lymphoid tissue inducer cells express the key transcription factors inhibitor of DNA binding 2 (Id2) and retinoic-acid-receptor-related orphan receptor γ (ROR γ t),

and the genetic deletion of these molecules leads to the absence of PPs and peripheral lymph nodes [30-32] (Fig. 2).

PBs are the primary inductive sites where most of the IgA immune responses are initiated. Enzymatic digestion methods were used to show that PPs are equipped with all the necessary cellular components for the initiation of antigen-specific IgA antibody responses [33, 34]. In particular, the use of the enzymatic dissociation protocol to discover APCs in PPs has enabled further characterization of PPs as the initiation site for the induction of antigen-specific immune responses to orally encountered antigens. Anatomically, the PPs are covered with follicle-associated epithelium (FAE), which harbors M cells [35]. The subepithelial dome region, which lies under the FAE, contains abundant APCs, including DCs. DCs take up antigens retrieved by M cells from the lumen of the digestive tract and then process and present the antigenic peptides to mucosal T and B cells for the initiation of antigen-specific immune responses [35]. T and B cells reside in the follicle regions and represent a majority of PPs (B cells and T cells are approximately 75% and 20% of the PP cells, respectively) [35]. In addition, germinal centers occur in the PPs under homeostatic conditions, and these create the molecular and cellular environment for the class switching of B cells from IgM to IgA [35].

In addition to PPs, ILFs serve as an additional inductive tissue for IgA production in the small intestine [36]. ILFs share many structural similarities with PPs but are developmentally distinct from PPs at several points; for example, unlike PPs, ILFs appear after birth and function independently of lymphotoxin signaling [36]. ILFs mainly contain B cells, DCs, and rare T cells, and the overlying epithelium contains M cells [37]. In contrast to PPs, ILFs lack T-cell-rich intrafollicular regions, and they are proposed to be the sites for T-cell-independent IgA production [37].

Therefore, just like the lymph nodes and spleen in the peripheral immune system, the GALTs bring together antigens and immune cells for the initiation of antigen-driven immune responses, making them a ‘command center’ of the mucosal immune system.

2) Development and Function of NALT

Similar to GALT, NALT is a key secondary lymphoid structure for the upper respiratory tract. Major differences among gut PPs and NALT are their genesis and development. Whereas PP development is initiated embryonically, NALT formation is initiated postnatally [38]. The interaction of lymphotoxin- α 1 β 2 with lymphotoxin- β receptor is the most important step in PP genesis, whereas NALT development appears to be independent of this molecular signaling [38] (Fig. 2): NALT structures have been observed in both lymphotoxin- α -deficient mice and lymphotoxin- β receptor-deficient mice [38]. In addition, deficiency of nuclear factor kappa B inducing kinase, the signaling molecule downstream of the lymphotoxin- β receptor, does not affect NALT development, in contrast to PP development [38]. Moreover, normal NALT development is also observed in IL-7R α -, ROR γ t-, CCL13-, CCL19-, and CCL21-deficient mice, all of which show disruption or impairment of PP development [38-40]. Although these molecules are dispensable for NALT organogenesis, both Id2 and lymphotoxin- α signaling are important for NALT organization [38, 41]. The impaired NALT structure in Id2-deficient mice is due to the lack of CD3⁻CD4⁻CD45⁺ inducers, because NALT organogenesis was initiated after the adoptive transfer of CD3⁻CD4⁻CD45⁺ cells into Id2^{-/-} mice [38]. The uniqueness of NALT organogenesis may therefore stem from these unique inducer cells.

In general, just as the PP-initiated antigen-specific immune response conveys protective immunity in the GI tract, NALT-initiated immune responses provide antigen-specific protection in the respiratory tract [2, 9]. Interestingly, nasal immunization can induce an effective antigen-specific mucosal immune response in salivary glands and reproductive tissue, in addition to the respiratory tract [42, 43]. Nasal immunization with mucosal antigen, cholera toxin B subunit (CTB), induces CTB-specific IgA responses in the vagina and cervix in humans

[44]. Therefore, NALT has been considered to be an attractive site for the delivery of vaccine antigens in order to induce antigen-specific immunity in the oral cavity and reproductive tissues in addition to the respiratory tract.

3) Unique Organogenesis and Function in Tear Duct-associated Lymphoid Tissue

In addition to the aerodigestive tract, the ocular mucosae, composed of the lacrimal sac and nasolacrimal duct, are directly exposed to the outside environment. Tears act as a first line of defense against exogenous antigens and contain inflammatory cytokines (e.g. IL-1, IL-6, IL-8 and TGF- β) [45, 46], antimicrobial defense peptides (e.g., lysozyme, lactoferrin and lipocalin) [47], and SIgA [48], which play important roles in providing appropriate mucosal innate and acquired immunity in the ocular region. In addition, the tear duct, which directly bridges the ocular and nasal cavities, has its origin in lymphoid tissue, called tear duct-associated lymphoid tissue (TALT), that induces and regulates the antigen-specific immune response against antigens deposited on the ocular surface [49, 50]. As with the other well-characterized mucosal inductive tissues, GALT (or PPs) and NALT, TALT also possesses FAE, which contains M cells, and houses B cells, T cells and DCs underneath the epithelium regions [49].

Although TALT, which is also called conjunctiva- or lacrimal drainage-associated lymphoid tissue, had been identified in human ocular tissues [51, 52], its organogenesis and detailed function were unclear for a long time. In mice, TALT develops postnatally, like NALT and ILFs but unlike PPs [49]. TALT develops in B cell- or T cell-deficient mice and germ-free mice, suggesting that B and T lymphocytes and microbial stimulation are dispensable for the development of TALT in mice [49]. Moreover, TALT genesis is initiated independently of Id2-, ROR γ t-, and lymphotoxin-mediated signaling cascades [49], all of which are involved in the development of other lymphoid tissues, such as NALT, lymph nodes, and PPs [8] (Fig. 2).

The immunological function of TALT as a mucosal inductive site is similar to that of

NALT and PPs. For example, M cells localized in the TALT epithelium can take up ocularly encountered antigens and subsequently induce antigen-specific immune responses [49]. Interestingly, ocular immunization with cholera toxin (CT) induces not only the formation of a germinal center for IgA⁺ B cell responses and antigen-specific Th cells in TALT but also antigen-specific B and T cell responses in NALT [49]. It is most likely that antigens deposited in the eye reach the NALT epithelium via the tear duct bridges between the ocular and nasal cavities. Conversely, nasal immunization with microencapsulated antigens elicits ocular immune responses, such as induction of antigen-specific tear IgA antibodies and antibody-secreting cells in lacrimal glands [53]. Therefore, TALT has characteristics that reflect its unique organogenesis, including uptake and induction sites for the ocular mucosal immune responses and cross-talk with NALT.

4) Discovery of Isotype-Specific Th Cells Contributes to the Concept of Th Cell Subsets

Production and secretion of SIgA due to the cellular cooperation between IgA-producing B cells and epithelial cells are prominent features of the mucosal immune system. As mentioned above, IgA dimers or polymers are secreted across the mucosal epithelium by an active transport mechanism involving the polymeric Ig receptor [21, 54]. SIgA is resistant to protease activity and can therefore bind and neutralize pathogens or toxins in the harsh environment of the intestine, thus providing the first barrier to invasion of pathogens [55]. Findings in the 1970s and early 1980s provided substantial evidence that mucosal induction sites such as the PPs are the major source of precursors of IgA-secreting plasma cells [56-58], and later findings revealed that T cells have a major role in the induction and differentiation of IgA⁺ B cells into plasma cells secreting the dimeric or polymeric form of IgA [58, 59]. As discussed above, MALT is the primary site for IgA induction, and the LP serves as the effector site (Fig. 1). Both of these sites contain several distinct T-cell subsets that possess unique immunobiological characteristics for the induction and regulation of antigen-specific IgA immune responses [2, 9]. Early studies

employed enzymatic dissociation procedures to isolate antigen-specific T cells, as well as other lymphoreticular cell subsets from PPs, and discovered two distinct modes of T-cell regulation of IgA responses [60, 61]. The first involved class switching of IgM⁺ B cells to IgA⁺ B cells, a process driven by a subset of T cells termed “switch T cells” [62, 63]. The next step was mediated by isotype-specific T cells that preferentially induce post-switch IgA⁺ B cells to proliferate and differentiate into IgA-producing plasma cells [33, 60]. These PP-residing T cells that preferentially support IgA⁺ B cell differentiation were labeled IgA-isotype specific T helper (Th) cells and were found to express Fc receptors for IgA [33].

These findings preceded the discovery of Th1 and Th2 cells. However, in conjunction with the knowledge derived subsequently, it was postulated that switch T cells were TGF- β -producing T cells (or Th3 cells) and that isotype-specific Th cells were Th2 cells [2, 64]. Now it is established that IL-4 derived from Th2 cells stimulates the class switch of the heavy chain on B cells to γ subclasses (e.g. IgG1) and the ϵ class (IgE) [65]. In addition, Th2-derived IL-5, IL-6, and IL-10 are important IgA-enhancing cytokines for the preferential activation and clonal expansion of IgA⁺ B cells and their terminal differentiation into IgA plasma cells [66].

3. Role of the Microflora in the Development of the Mucosal Immune System

The mucosal surfaces are characterized by host–microbe interactions. Mucosal surfaces are adapted to bidirectional host–flora exchange and harbor a diverse bacterial community separated by the epithelial layer [67]. The microbiota, whose constituents are also called commensals, is composed of hundreds of species that are reported to be present in numbers on the order of 10^{13} to 10^{14} in the intestinal tract alone [67]. They benefit the host by supplying nutrients, metabolizing otherwise indigestible foods, and by preventing colonization by harmful pathogens [67]. Studies with germ-free animals, which are bred and maintained in entirely sterile conditions in positive-pressure isolators, highlight the importance of the intestinal

microflora for the development of secondary lymphoid structures in the mucosal immune system [67, 68] (Fig. 3). This is particularly evident in the GI tract, because several studies have demonstrated that germ-free animals show defects in the development and maturation of GALTs, including reduced number of PPs, and in antibody production, with fewer IgA-producing plasma cells and reduced numbers of CD4⁺ T cells [36, 69-72]. Moreover, commensals also induce the development of tertiary lymphoid structures such as ILFs and cryptopatches [73, 74] and promote epithelial cell maturation and angiogenesis, thus contributing to the fortification of the intestinal barrier [75, 76]. Peptidoglycan from gram-negative bacteria induces the genesis of ILFs through recognition by NOD1 (nucleotide-binding oligomerization domain containing 1) and maturation of ILFs through Toll-like receptors (TLRs) and NOD2 [73]. In addition, commensals contribute to the induction of IL22-producing ROR γ ⁺ lymphocytes in cryptopatches, that control the epithelial integrity [77]. These findings suggest that the intestinal microflora provides the most potent environmental stimulation for the postnatal phase of physiological and immunological intestinal development. In fact, the introduction of bacteria into germ-free mice allows their histologically and anatomically immature PPs to develop normally [78]. Oral exposure of germ-free mice to a single pathogenic or nonpathogenic microorganism results in the maturation of PPs and an increase in their numbers [79]. Similarly, the oral administration of lipopolysaccharide (LPS) or a monoinfection of *Escherichia coli* in germ-free mice resulted accelerated the immunologic and functional development of PPs [80]. These findings suggest that intestinal microbial stimulation can lead to the generation of an optimal inflammatory signaling environment (or physiological inflammation) necessary for the maturation and maintenance of the GALT-orchestrated immune system.

Gut commensals are critical and active inducers of regulatory immune responses for the creation of mucosal unresponsiveness (that is, oral tolerance) (Fig. 3). Oral tolerance has been defined as the specific suppression of cellular and humoral immune responses to orally

administered antigens [81] and is important for the creation of unresponsiveness to antigen proteins that are beneficial to the host and for the maintenance of the gut immune system under resting condition. It is now well known that various T-cell networks, including regulatory T (Treg) cells [82, 83] and PPs [84], are involved in the induction and maintenance of oral tolerance. Initial research demonstrated that oral tolerance could not be induced in the absence of signals derived from the gut flora [85-88]. One of the principal mechanisms of tolerance induction is the generation of T cells possessing inhibitory activity, initially and phenomenologically called suppressor T cells and currently well characterized as Treg cells. Oral tolerance is not induced either in mice that cannot respond to LPS or in germ-free mice, thus indicating the important role of the intestinal microbiota and its derived biological molecules in the induction of suppressor T cells [85-87]. Recently, the creation of environments that can induce Treg cells in the GI tract and enable them to thrive has been attributed to specific commensal bacterial species. For example, *Bacteroides fragilis* produces polysaccharide A, which induces and expands IL-10-producing Treg cells [89, 90], and *Clostridium* clusters IV and XIV promote Treg cell accumulation in the colon by creating a TGF- β -rich environment [91]. In contrast, segmented filamentous bacteria, spore-forming gram-positive anaerobic bacteria that colonize the terminal ileum of mice, have been reported to promote the accumulation of both proinflammatory Th1 and Th17 cells in the small intestine and to drive the production of IgA [92-94].

In addition, commensal bacteria contribute to the development of regulatory immune systems through other intermediate pathways (Fig. 3). For instance, metabolites such as butyrate, which is a short-chain fatty acid produced by commensal bacteria in the gut, are involved in the differentiation of colonic Treg cells via T-cell-intrinsic epigenetic upregulation of the *Foxp3* gene [95] and in the generation of peripheral Treg cells via the extrathymic intronic enhancer CNS1 (conserved non-coding sequence 1) [96]. Supporting this mechanism, *Bacteroidetes* and *Lachnospiraceae*, which produce butyrate in the human gut, are greatly depleted in patients with

inflammatory bowel disease [97].

Commensals are involved in the regulation of both systemic and mucosal lymphocyte populations [98, 99]. As mentioned above, some T cell subsets are regulated by commensal bacteria. Similarly, among B cell subsets, marginal zone B cells are reduced in restricted-flora mice compared with germ-free mice [98]. In addition, the adoptive transfer of CD8⁺ T cells from restricted-flora mice to germ-free mice reduces the number of marginal zone B cells, indicating that the reduction occurs via activation of CD8⁺ T cells [98]. Moreover, in the intestinal mucosa, primary B-cell development occurs via the microbiota [99]. The gut microbiota increases the proportion of pro-B cells in the gut LP and thereafter leads to increased Ig editing specifically in LP B cells by increasing the expression of *Rag1* and *Rag2* [99].

Although most commensal bacteria localize in the mucous layer and on the surface of the intestinal epithelium, a unique indigenous bacteria genus, *Alcaligenes*, colonizes the interior of murine PPs [100]. Mucosal, *Alcaligenes*-specific, IgA antibody-producing B cells are induced in PPs, but not in spleen, in a T-cell-dependent manner; CD11c⁺ DCs augment the production of IgA-inducing and enhancing molecules such as IL-6, TGF- β , and B-cell activating factor belonging to the TNF family, with the result that *Alcaligenes*-specific IgA enables *Alcaligenes* to cohabitate with the host in PPs [100]. *Alcaligenes* is usually restricted to its unique intracellular location in PPs by IL-22 produced in innate lymphoid cells in intestinal tissues, but these organisms can trigger systemic immune responses associated with pediatric Crohn's disease and progression of hepatitis C virus infection if they escape this compartmentalization [101].

Therefore, it is evident that commensal bacteria, their cellular elements (e.g., LPS), and their associated metabolites [e.g., butyrate, acetate and propionate [102]], as well as the commensal microbiota located within tissues, provide important environmental and immunological influences on the formation and maintenance of anatomical and functional structures of the mucosal immune system and on the induction and regulation of both active

(e.g., antigen-specific IgA with protective activity) and quiescent (e.g., oral tolerance) immune responses. As much as commensals are important for the development and maintenance of homeostasis of the mucosal immune system, their influence on the effectiveness of mucosal vaccines needs to be further identified, because manipulation of intestinal microbes may alter the relationship among lymphocyte populations in the intestine [55].

4. Characterization of the Mucosal Gateway and Antigen-Sampling Cells

To evoke antigen-specific mucosal immune responses, antigens on the mucosal surfaces must be transported across the epithelial barrier into the MALTs, such as PPs, NALT, and TALT. This process, called “antigen transcytosis,” is mediated by specialized antigen-sampling cells located on the FAE, called the M cells (Fig. 4). M cells are specialized for the transcytosis of luminal macromolecules, particulate antigens and even pathogenic or commensal microorganisms [103]. M cells were first observed in rabbit appendix with transmission electron microscopy [104] and were identified in humans in 1974 [10]. In the small intestine under steady-state conditions, M cells make up approximately 10% of the epithelial cells within the FAE in mice and 5% in humans; their morphology is characterized by an irregular brush border and reduced microvilli and glycocalyx [103, 105]. M cells also exhibit an intrapocket structure at their base which houses lymphocytes and APCs, including DCs [106]. This structure enables speedy and effective delivery of antigens in their unmodified form from the gut lumen to the interior of PPs and then to underlying APCs, including macrophages and DCs, for processing and presentation of antigens to lymphocytes [106].

Therefore, because M cells play an important role in antigen sampling, a specific marker that can effectively identify M cells would be useful. One commonly used M cell marker is the $\alpha(1,2)$ fucose binding lectin UEA-1 (ulex europaeus agglutinin 1), which identifies M cells by their unique glycosylation pattern [107]. However, UEA-1 also reacts with goblet cells and the mucus layer covering the intestinal epithelium [108]. To solve this problem, our group

established a murine M cell-specific antibody, NKM 16-2-4, that also recognizes Paneth cells, but not goblet cells or other epithelial cells [109]. Later, we identified another marker for M cells, glycoprotein 2 (Gp2), which is expressed specifically on both human and murine M cells [110, 111]. Gp2 acts as a receptor for FimH, which is a component of type I pili on the outer membranes of bacteria such as *E. coli* and *Salmonella typhimurium* [110] (Fig. 4).

Several key pathways important in the development of M cells have been identified. The development of M cells in the PPs is impaired in mice lacking B cells [112, 113]. The M-cell differentiation program can also be induced by application of the TNF superfamily member RANKL (receptor activator of nuclear factor κ B ligand), which is expressed by non-hematopoietic mesenchymal cells underlying the FAE [114]. RANKL-induced M cell generation has provided us with tools for a detailed transcriptome analysis of functional M cells and has led to the identification of the ETS transcription factor Spi-B as a critical factor driving the M-cell differentiation program [115, 116].

Although PPs play an important role in the induction of antigen-specific immune responses, antigen-specific IgA antibody responses can also be induced in the absence of PPs [28], which implies the existence of a PP-independent mucosal immune pathway for orally encountered antigens. One candidate component of the PP-independent pathway is the intestinal villous M cells, which are morphologically similar to M cells located in the PPs, but which serve as an antigen gateway for the sampling of gut bacteria and the subsequent induction of antigen-specific immune responses in a PP-independent manner [117] (Fig. 4). Apart from the antigen sampling system mediated by PP and villous M cells, dedicated APCs such as DCs have been implicated in direct sampling of intestinal antigens from the gut lumen [118]. DCs in the vicinity of the epithelium express CX3CR1 and are called “intraepithelial DCs.” These intraepithelial DCs have the capacity to extend transepithelial protrusions in the lumen, where they can capture antigens [119, 120] (Fig. 4). Taken together, these findings suggest the existence of a dynamic antigen-sampling system in the digestive tract, a fact that needs to be

considered when developing a strategy for oral vaccines (Fig. 4).

Similar to the M cells in the PPs, M cells located in the NALT FAE can serve as invasion and sampling sites for nasally delivered antigens [8] as well as for respiratory pathogens such as group A *Streptococcus* [121] and for intestinal pathogens such as reovirus [122]. Although NALT exhibits a central role in the generation of antigen-specific Th cells and IgA-committed B cells targeting inhaled antigens, some findings suggest the presence of additional inductive sites or antigen-sampling cells in the upper respiratory tract [123]. For example, Id2-deficient mice, which have impaired NALT development, showed equivalent levels of antigen-specific serum IgG and nasal IgA production in response to nasal administration of *S. typhimurium* or group A *Streptococcus* to those in wild-type mice [123]. This result led us to identify a novel antigen-sampling site for inhaled antigens in the upper respiratory epithelium of the murine nasal cavity; we named these cells “respiratory M cells” because they possess the hallmark characteristics of classic M cells but are anatomically located away from the NALT epithelium (e.g., in the turbinate epithelium of the nasal cavity) [123]. Unlike the NALT M cells, respiratory M cells have no pocket structures [123]. However, CD11c⁺ DCs, which are distributed throughout the nasal passages in the resting state, migrate to the area underneath the respiratory M cells to receive antigens after intranasal group A *Streptococcus* infection [123], indicating that the upper respiratory mucosal immune system consists of NALT-dependent and NALT-independent induction pathways for the generation of antigen-specific mucosal immune responses.

Therefore, accumulated knowledge gained from previous and current research has contributed to the elucidation of the molecular and cellular characteristics of M cells and other gateways. In addition, these findings likely will be applied to the development of new mucosal vaccines, because targeting these mucosal gateways for the delivery of mucosal vaccines could be a logical and attractive strategy for the induction of effective mucosal immune responses.

5. Application of the Mucosal Immune System to New-Generation Vaccine Development

With their vast surface area, the mucosal surfaces of the GI, urogenital, and respiratory tracts and the ocular cavities are major routes of entry into the body for the majority of pathogens. Therefore, new vaccines that effectively protect the mucosal sites are highly desirable [124]. However, the majority of current vaccination methods target the systemic immune system and elicit only a weak or no mucosal immune response [125]. In contrast, mucosal immunization with an appropriate vaccine delivery vehicle induces both protective mucosal and systemic immune responses, leading to a double layer of protection against pathogens [126]. In addition, compared to parenteral routes, mucosal vaccine delivery offers advantages such as ease of administration, decreased costs, avoidance of needle-stick injuries and reduced risk of transmission of blood-borne diseases. Moreover, this needle- and syringe-free delivery helps to reduce medical waste [127]. Despite these numerous advantages, the development of mucosal vaccines has been slow. Only a handful of mucosal vaccines have been approved for clinical use in humans, the best-known example being the Sabin polio vaccine, which is given orally and absorbed in the digestive tract [128]. Others include oral vaccines against *Salmonella typhi*, *Vibrio cholera*, and rotavirus and a nasal vaccine against influenza virus [6, 129, 130]. The reason for the low number of mucosal vaccines is that direct mucosal immunization has proven difficult due to challenges such as identifying the appropriate vaccine formulation, including the adjuvant, to maximize immunogenicity, stability, and delivery [6]. In the following sections, we highlight some of the achievements that have helped to shape the future of mucosal vaccines.

1) Mutant Bacterial Toxins for Mucosal Adjuvants

One of the most significant challenges we need to overcome is the issue of tolerance. The mucosal immune system is naturally built to exhibit immune tolerance to most of the vast quantity of antigens encountered on a daily basis, to prevent excessive and unwanted

inflammatory responses and food allergies [131]. However, this feature also can impede the effective development of mucosal vaccines, because administered antigens become weak immunogens when given through a mucosal route; effective, reliable and safe mucosal adjuvants are therefore necessary in order to realize the full potential of the antigens [55]. However, few mucosal adjuvants have been found to exhibit sufficient potency without being toxic or reactogenic, and only aluminum salts and specific types of emulsions have been approved clinically in the United States [9, 55].

Among the most potent mucosal adjuvants available are toxin-based adjuvants such as heat-labile enterotoxin (LT) and CT, produced by *E. coli* and *V. cholerae*, respectively. These are used widely in experiments. Both LT and CT consist of a single active A subunit and five binding B subunits and can promote mucosal and systemic immunity when co-administered with protein antigen via oral or parenteral routes [9, 132-134]. Despite their potential adjuvant effects, CT and LT can cause severe diarrhea and pose a potential threat to the nervous system after mucosal administration [135]. Therefore, to reduce the harmful enzyme activities of CT and LT, mutants of these native toxins have been generated by site-directed mutagenesis [136-139].

Two mutants of CT, designated S16F and E112K, each contain a single amino-acid substitution in the ADP-ribosyltransferase region in the A subunit. These mutants are considered nontoxic because they lack ADP-ribosyltransferase activity and cAMP formation *in vitro* and do not induce diarrhea *in vivo* [138, 139]. In addition, nasal immunization of mice with the surface protein antigen of *Streptococcus pneumoniae* together with mutant CT elicits antigen-specific serum IgG and mucosal IgA antibodies, which provide protective immunity against *S. pneumoniae* infection [139]. These findings also suggest that both native and mutant CT primarily induce Th2-type immune responses characterized by CD4⁺ T cells producing IL-4, IL-5, IL-6 and IL-10 [138, 133]. However, later findings indicated that CT (and LT) induces Th1-type as well as Th2-type immune responses [140].

A mutant form of LT, LTK63, is another attractive candidate for a new effective and safe adjuvant. LTK63, which contains a single amino-acid substitution in the active site of the LT A subunit, is a synthetic non-toxic AB complex mutant of LT [141]. Intranasal immunization with an inactivated virosomal-subunit influenza vaccine together with an enzymatically active mutant of LT is thought to be associated with the development of Bell's palsy [135]. In contrast, LTK63 is considered nontoxic because of its lack of ADP-ribosyltransferase activity, similar to the S16F and E112K mutant forms of CT [142]. However, a recent human phase I clinical trial suggested that the enzymatically inactivated LTK63 adjuvant was associated with the occurrence of transient peripheral facial nerve paralysis when intranasally administered with subunit vaccines against human immunodeficiency virus and tuberculosis [143]. The LT B subunit (LTB) is expected to be a safer and more promising adjuvant than is the LT adjuvant including the A subunit. In mice, intranasal administration of tetanus toxoid mixed with mutant CTA/LTB, which is a chimeric protein consisting of the CT E112K A subunit and pentameric LTB, induces antigen-specific serum IgG and nasal IgA antibodies and also protects the mice from lethal challenge with tetanus toxin without serious adverse events [144].

Our current knowledge in the area of the TLR and their ligands, host molecules that function in pattern recognition of microbial components, should also be applied to the development of safe and effective mucosal adjuvants [145]. Monophosphoryl lipid A, which signals via TLR4; flagelin, which is a ligand for TLR5; and CpG oligodeoxynucleotide, which works through TLR6 are not licensed but have already been tested as mucosal adjuvants in human clinical studies [145]. In fact, CD11c⁺ DCs at the LP in the small intestine express TLR5 and induce the differentiation of naïve B cells into IgA⁺ B cells through a GALT-independent mechanism; they also drive the differentiation of Th1 cells and IL-17-producing Th cells [146]. Continued efforts in the molecular and genetic manipulation of bacterial toxins and searches for TLR-specific ligands as adjuvants that do not have any undesired biological activity will contribute greatly to the field of mucosal vaccination.

534

535 **2) MucoRice as a Novel System for Mucosal Vaccine Delivery and Protein Expression**

536 Plant-based mucosal vaccines have been considered an attractive strategy for achieving
537 the goal of generating self-administrable vaccines free of the need for a “cold chain,” needles,
538 and syringes. Such vaccines have huge advantages, especially in developing countries, which
539 have difficulty maintaining cold storage systems for vaccines, poor infrastructure to manage
540 medical waste derived from mass vaccination using syringes and needles, and an insufficient
541 number of skilled medical professionals to provide inoculations [129]. Several edible
542 plant-based vaccines have been developed by introducing bacterial and viral antigen genes into
543 plant expression systems. The antigens have included the B subunit of LT, the CTB, and
544 antigens from *Yersinia pestis* and viruses such as hepatitis B virus, rotavirus, and Norwalk
545 virus; these antigens have been introduced into carrot, soybean, tomato, rice, potato, and
546 tobacco [126, 147-153]. Among these plant-based vaccines, we have focused on a rice-based
547 vaccine (MucoRiceTM) because of its practical advantages [126, 154-162] (Fig. 5).

548 An unique protein-storage organelle in the rice seed, named the protein body, provides a
549 suitable vehicle for the expression of vaccine antigens that are not only stable at room
550 temperature for a long time without loss of immunogenicity but also are protected from
551 digestive enzymes in the GI tract [126]. When mice were orally immunized with a rice-based
552 vaccine expressing CTB, named MucoRice-CTB, the antigenic protein was effectively taken up
553 by antigen-sampling M cells on the PP without digestion in the stomach [126]. Moreover,
554 protective CTB-specific serum IgG as well as mucosal SIgA antibodies were induced, but no
555 notable IgG or SIgA response was detected against endogenous rice storage proteins [126] (Fig.
556 5). The effective induction of mucosal immune responses by oral administration of
557 MucoRice-CTB has been observed not only in mice but also in nonhuman primates
558 (cynomolgus macaques) [155]. In addition, oral immunization with MucoRice-CTB induces
559 cross-protective immunity against LT [156]. This cross-reactivity to LT is important, because

enteropathic *E. coli* is a major pathogen associated with severe diarrhea in children, especially in developing countries [163]. The MucoRice system therefore offers a highly practical global strategy for the development of effective, environmentally friendly, cold-chain-free and needleless vaccines that will benefit both developed and developing countries (Fig. 5).

The MucoRice expression system also has the potential to serve as a production, storage, and delivery system for various types of proteins. We have extended the MucoRice expression system to the production of anti-rotavirus antibodies for passive immunotherapy (Fig. 5). First, we introduced RNAi technology to suppress the production of the rice internal storage proteins and enhance the yield of introduced foreign proteins in rice seeds [157]. This system was extended to generate transgenic rice (MucoRice-ARP1) that expressed the neutralizing variable domain of the llama rotavirus-specific heavy-chain antibody fragment [160]. This form of minimum-sized llama antibody is known as a nanoantibody [160]. Immunocompetent and immunodeficient mice infected with rotavirus and given an oral dose of MucoRice-ARP1 dissolved in water had considerably reduced viral load than did control untreated mice [160] (Fig. 5). We further showed that this nanoantibody ARP1 expressed in rice seeds was stable after long-term storage and even after exposure to high heat [160].

Using a system similar to that used for the production of MucoRice-ARP1, we can produce various therapeutic antibodies. For example, TNF is a potent inflammatory cytokine. The production of a llama variable domain of a heavy-chain antibody fragment specific for TNF in the rice seed (MucoRice-mTNF-VHH) suppressed progression of collagen-induced arthritis in mice [162]. The MucoRice system is thus an attractive system for producing vaccine antigens and nanoantibodies because it provides a vehicle for oral delivery that can be preserved for prolonged times without the need for a cold chain.

3) Nanogels for Adjuvant-Free Vaccine Delivery

The airway mucosal immune system is another target for the development of a mucosal

vaccine; in this case, the intranasal route would be used to deliver antigens for protection against respiratory infectious diseases. A nasal spray vaccine against influenza called FluMist, which is composed of live-attenuated and cold-adapted influenza virus, was the first advanced intranasal vaccine to be used in the United States, in 2003 [164]. However, live-attenuated mucosal vaccines occasionally cause side effects because they contain live pathogens [129]. For this reason, subunit intranasal vaccination is expected to be the safest strategy, because it likely has a low risk of causing undesired biological reactions [165]. Just as with oral immunization, inactivated or subunit vaccines need to be co-administered with biologically active mucosal adjuvants due to their poor immunogenicity when used alone. Intranasal immunization with vaccine antigens together with CT or LT has been shown to be effective in inducing protective immunity [137-139]. The drawback of such immunization is the risk of redirection of adjuvants or antigens to the central nervous system [135]. This issue has raised concerns about the safety of intranasal immunization, even when the activity of the toxin is removed from the CT- or LT-based adjuvants, as discussed above [166]. The development of a safe, adjuvant-free nasal vaccine delivery system is needed to address these disadvantages and safety concerns. For this reason, the use of biomaterials, such as polymer nanoparticles and liposomes, has been gathering attention as an alternative strategy for the mucosal delivery of vaccine antigens [167, 168].

To accomplish this goal, and taking particular note of the fact that mucous membranes are anionic, we developed a novel intranasal vaccine-delivery system with a nanometer-sized hydrogel ("nanogel") consisting of a cationic cholesteryl group-bearing pullulan (cCHP) [169] (Fig. 6). A nontoxic subunit fragment of *Clostridium botulinum* type-A neurotoxin, BoHc/A, administered intranasally via the cCHP nanogel (cCHP-BoHc/A) continuously adhered to the nasal epithelium for more than 10 hours and was effectively taken up by mucosal DCs after its release from the cCHP nanogel [169]. Vigorous neutralizing serum IgG and SIgA antibody responses to botulinum neurotoxin type A were induced without co-administration of

mucosal adjuvant [169]. Importantly, intranasally administered cCHP-BoHc/A did not migrate to or accumulate in the olfactory bulbs or brain [169]. Moreover, intranasal immunization with a cCHP nanogel containing tetanus toxoid induced strong antigen-specific systemic and mucosal immune responses [169]. Our findings indicate that the cCHP nanogel can be used as a potential protein-based antigen-delivery vehicle for adjuvant-free nasal vaccination (Fig. 6).

The efficacy of the nanogel-based intranasal immunization was further demonstrated by a pneumococcal vaccine in which pneumococcal surface protein A (PspA) was incorporated into a cCHP nanogel (cCHP-PspA) and tested in murine pneumococcal airway infection models [170]. Nasal vaccination with cCHP-PspA in mice provided protective immunity against lethal challenge with *S. pneumoniae* Xen10 and reduced colonization and invasion of bacteria in the upper and lower respiratory tracts. It also induced both systemic and nasal mucosal Th17 responses, high levels of PspA-specific serum IgG and nasal and bronchial SIgA antibody responses, all of which play key protective roles against *S. pneumoniae* infection [170]. Again, the important thing to note is that there was no sign of PspA delivery by nanogel to either the olfactory bulbs or the central nervous system after intranasal administration [170]. The nanogel antigen delivery system is creating new opportunities for the development of adjuvant-free mucosal vaccines (Fig. 6).

Conclusion

Our accumulated scientific findings have contributed to the elucidation of the immunologically unique mucosal immune system and to the creation of biomedical applications for mucosal immunology. However, we still do not completely understand this system, because of its various unique and unexplored characteristics, as well as the complicated direct and indirect biological interactions among the many components of the host immune system and the surrounding environment at the large mucosal surface. Interestingly, the mucosal immune systems affiliated with various tissues, including the craniofacial, digestive, respiratory, and

reproductive compartments, share common immunological features and are interconnected through the CMIS to provide a physiologically and immunologically important surface barrier. Nonetheless, each also possesses unique immunological features in order to provide appropriate local immunity. MALTs, including GALT (e.g., PPs and IFLs), NALT, and TALT, serve as the inductive tissues for antigens encountered orally, nasally, and ocularly and harbor distinctive characteristics in their genesis and functions. In addition to orchestrated interactions among different mucosal sites and immunocompetent cells via the CMIS, mucosal surfaces continuously interact with distinct commensal microbiota associated with individual local tissues; this feature certainly influences the genesis and maintenance of the corresponding MALTs and the induction and regulation of antigen-specific immune responses against locally delivered mucosal antigens. Deep and increasing understanding of mucosal immunity will contribute to the development of new mucosal vaccines, which is necessary because there are numerous uncontrolled infectious diseases that are acquired through mucosal surfaces. Therefore, we must extend our efforts to understand the molecular and cellular functions of the mucosal immune system and try to apply new technologies and concepts to vaccine expression and delivery systems to accelerate the development of safer and more effective mucosal vaccines.

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Figure legends

Fig. 1 A schematic of the mucosal immune system indicating the inductive and effector tissues for antigen-specific immune responses.

Mucosally administered antigens are picked up from the lumen of the digestive tract, nasal cavities and tear ducts by dynamic antigen-sampling system including M cells located in the follicle associated epithelium (FAE) of MALTs. The subepithelial dome region, which lies under the FAE, contains antigen-presenting cells such as dendritic cells (DCs), B cells and T cells. DCs capture antigens taken up by M cells and present them to $CD4^+$ T cells and $CD8^+$ T cells for the initiation of antigen-specific immune responses. Consequently, interactions between DCs and $CD4^+$ T cells induce IgA-producing B cells via CD40/CD40 ligand interaction and IgA-associated cytokines such as transforming growth factor (TGF)- β , interleukin (IL)-2, IL-4, IL-5, IL-6, and IL-10 at the inductive site. IgA-committed B cells enter lymph nodes, go through peripheral blood, migrate to the effector sites such as the intestinal lamina propria regions, and then differentiate into plasma cells via the molecular signals of Th2-type cytokines such as IL-5 and IL-6. Plasma cells secrete polymeric IgA antibodies that are endocytosed through binding to the basolateral polymeric Ig receptors (pIgRs) located in the mucosal epithelial cells. The resultant polymeric IgA-pIgR complexes are transported to the apical surfaces and are then cleaved into secretory IgA (SIgA).

Fig. 2 Unique characteristics of tissue genesis of gut-, nasopharynx-, and tear duct-associated lymphoid tissues (GALT, NALT, and TALT).

In the GALT, Peyer's patch inducer cells, which have the unique $IL-7R^+CD3^-CD4^+CD45^+$ pattern of cell surface markers, differentiate into inducer cells that express lymphotoxin (LT)- α and LT- β under the influence of transcription factors such as inhibitor of DNA binding 2 (Id2) and retinoic-acid-receptor-related orphan receptor- γ (ROR γ t). Inducer cells interact via their

heterotrimer protein LT α 1 β 2 with the LT- β receptor (LT β R) on stromal cells, which express vascular cell adhesion molecule (VCAM) and intercellular adhesion molecule (ICAM1) for adhesion to the intestine. Consequently, stromal organizer cells produce CXC chemokine ligand (CXCL)13 and CC chemokine ligand (CCL)19, which stimulate inducer cells through CXC chemokine receptor (CXCR)5 and CC chemokine receptor (CCR)7, resulting in the development of Peyer's patches in the intestinal mucosal surfaces. In contrast, the development of NALT does not involve ROR γ t, interaction of LT α 1 β 2 with LT β R, or downstream signals such as CCL13, CCL19, and CCL21 but is thought to require Id2 and LT- α signaling. The organogenesis of TALT remains unclear, but it is independent of Id2, ROR γ t, and LT-mediated signaling cascades.

Fig. 3 Critical role of the microbiota in the mucosal immune system.

Mucosal surfaces harbor a diverse bacterial community consisting of commensals and their components that is necessary for the development and maintenance of the mucosal immune system. Critical components include the IgA induction and regulation pathway, oral tolerance, and mucosa-associated immune tissues such as Peyer's patches (PPs), cryptopatches, and epithelial cells (ECs). Lipopolysaccharide (LPS) contribute to the development and maintenance of PPs, and peptidoglycan induces the genesis and maturation of ILFs through the NOD1 (nucleotide-binding oligomerization domain containing 1), NOD2 and TLRs (Toll like receptors). Commensals also induce the differentiation to ROR γ t⁺ lymphocytes in cryptopatches, that maintain the epithelial integrity via production of IL22. Some antigens from commensal bacteria taken up from the intestinal lumen via M cells are thought to be captured by dendritic cells (DCs) under the follicle-associated epithelium. Subsequent interactions between DCs and T cells induce regulatory T (Treg) cells that produce IL-10 and TGF- β . IL-10 produced by DCs and Treg cells promotes the accumulation of Treg cells and also inhibits their differentiation

into Th2 cells. Paradoxically, TGF- β also promotes the accumulation of proinflammatory Th17 cells. Metabolites such as butyrate that are produced by commensals also promote the differentiation of colonic Treg cells via T-cell intrinsic epigenetic upregulation of the *Foxp3* gene.

Fig. 4 Dynamics of the mucosal antigen sampling system.

Most antigens on the mucosal surfaces are taken up from the lumen by M cells, which are located on the follicle-associated epithelium (FAE) of mucosa-associated lymphoid tissues (e.g., Peyer's patches). M cells exhibit an intrapocket structure, where lymphocytes and antigen-presenting cells such as dendritic cells (DCs) are housed at their basal sites. Glycoprotein 2 (Gp2) expressed on M cells acts as a receptor for FimH of type I piliated bacteria and assists with the sampling of antigens. Internalized antigens are captured by DCs and are consequently presented to lymphocytes in the Peyer's patches, resulting in the initiation of antigen-specific immune responses. In addition to traditional M cells located in the FAE, intestinal villous M cells serve as an antigen gateway for the sampling of gut bacteria and the subsequent induction of antigen-specific immune responses. Moreover, some DCs (intraepithelial DCs) extend transepithelial protrusions into the gut lumen and directly pick up antigens.

Fig. 5 MucoRice as a novel cold chain- and needle/syringe-free oral vaccine.

A rice-based mucosal vaccine, MucoRice, has unique advantages as a new-generation oral vaccine. In the MucoRice system, vaccine antigens are expressed in the protein body (PB) of the rice seed, which provides a suitable vehicle for stable storage and effective delivery. MucoRice antigens are stable at room temperature without loss of antigen content and immunogenicity. Moreover, antigens expressed in the PB are protected from digestive enzymes in the gastrointestinal tract but are also effectively captured by M cells. MucoRice-CTB is protected

from the digestive enzymes and elicits not only CTB-specific serum IgG antibodies but also fecal IgA antibodies in mice and nonhuman primates. The MucoRice system has now been applied to the production of nanoantibodies for passive immunotherapy. MucoRice-ARP1 is a transgenic rice that expresses a neutralizing rotavirus-specific llama antibody fragment. Oral administration of MucoRice-ARP1 attenuates rhesus rotavirus (RRV)-induced diarrhea in mice.

Fig. 6 The nanogel system as novel adjuvant-free nasal vaccine delivery vehicle.

A novel nasal vaccine delivery system consists of a nanometer-sized hydrogel ('nanogel') containing a cationic type of cholesteryl group-bearing pullulan (cCHP) that is self-assembled from a hydrophilic polymer, hydrophobic cholesteryl group, and cationic amino group. cCHP nanogels can trap vaccine antigens within their nanoscale polymer networks, and their cationic charge assists the effective delivery of vaccine antigens to nasal surfaces. After intranasal administration of a cCHP nanogel-based vaccine containing the A subunit fragment of *Clostridium botulinum* type-A neurotoxin (cCHP-BoHc/A) or pneumococcal surface protein A, these vaccine antigens can be retained in the nasal mucosae for more than two days without dissemination to the central nervous system. Further, the cCHP nanogel-based vaccine is capable of inducing protective immunity without co-administration of mucosal adjuvant.

Fig. 1

Fig. 2

GALT

NALT

TALT

Fig. 3

Fig. 4

Fig. 5

Fig. 6