GM1 Receptor ELISA

GM1 receptor ELISA was carried out using the modified method of Dertzbaugh and Elson (1993). Briefly, wells of a polyvinyl microtiter plate were coated with 100 ng of GM1 ganglioside (Sigma). Wells were blocked with 1% bovine serum albumin in Tris-buffered saline, pH 7.5 (BSA–TBS). Each protein was adjusted to an equimolar concentration and then serially diluted twofold in BSA–TBS. Each dilution was mixed with an equal volume of biotinylated CTB (List Biologics, Campbell, CA) diluted to a concentration of 100 ng/0.1 mL. After incubation for 2 h at room temperature, the plate was washed and horseradish peroxidase conjugated streptavidin (Pierce Biotechnology, Rockford, IL) was added. The plate was incubated for 2 h at room temperature and, after washing, developed at room temperature with 100 µL of chromogenic substrate, 3,3′, 5,5′-tetramethylbenzidine with H_2O_2 (Moss, Pasadena, MD). Reactions were terminated by adding 50 µL of 0.5 M HCl.

Induction and Reduction of Diabetes

Female NOD mice purchased from the Jackson Laboratory (Bar Harbor, ME), spontaneous diabetes starts approximately 11 weeks of age and reached an incidence of 70% by 28 weeks in our colony. Diabetes was characterized by polydipsia, weight loss, glycosuria as assessed by urine Chemstrips (Bayer, Germany), and persistent hyperglycemia, which was determined with blood glucose levels using a Glucometere (Bayers, Germany) (Bergerot et al., 1997). Mice with a blood glucose level above 100 mg/dL were scored as diabetes (Daniel and Wegmann, 1996). To assess the reduction of diabetes, antigen-specific, mucosally induced tolerance was induced by nasal administration of 5 or 25 µg CTB-insulin B peptide hybrid protein in weeks 6, 7, and 8. Mice receiving 5 or 50 µg insulin B peptide alone or insulin B peptide (5 µg) with CTB (25 µg) were used as a control group. Mice were routinely monitored for diabetes by 40 weeks. The Cox-Mantel log rank test was used for statistical analysis of the data.

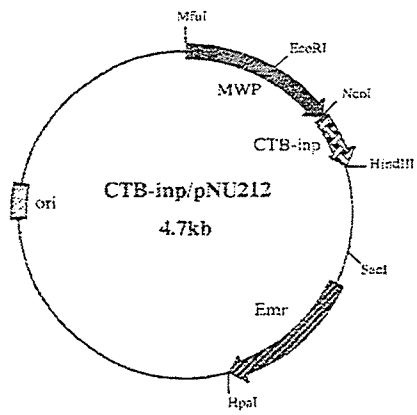
Measurement of DTH Responses

Female NOD mice were immunized with 30 µg of insulin B 9–23 (C19S) in CFA at base of the tail in 9 weeks after nasally immunized in weeks 6, 7, and 8 with 5 µg of the CTB-insulin B peptide hybrid protein, 5 or 50 µg of the insulin B peptide alone, or 5 µg of insulin B peptide together with 25 µg of CTB or non-treatment. In order to induce DTH responses, 1 week after the last systemic immunization, 20 µg of insulin B 9–23 (C19S) in PBS was subcutaneously injected into the right-hind footpad of the mice (Johnson et al., 1998). The left-hind footpad received PBS as a control. Footpad thickness was measured with a gauge before and 24 h after the injection, and the difference in footpad thickness between the right and left footpads was taken as the specific response. The Dunnett's test was used for statistical analysis of the data (Johnson et al., 1998).

RESULTS

Expression and Purification of CTB-Insulin B Peptide Hybrid Protein

The expression vector pNU212 containing CTB-insulin-B-peptide gene is shown in Figure 1. After introduction of



CTB-insulin B peptide(C19S)

CTB | Linker | Insulin B 9-23

AAT GGT CCA GGC TCA CAC CTG GTG GAA GCT CTC TAC CTA GTG TCC GGG GAA CGA GGC TAA GCT TCG ACG
Asn Gly Pro Gly Ser His Leu Val Glu Ala Leu Tyr Leu Val Ser Gly Glu Arg Gly *

Figure 1. Construction of CTB-insulin B 9–23 fusion protein expression-secretion vector CTB-inp/pNU212. The promoter and signal peptide of the *MWP* gene are represented by the hatched bar and the sequence coding for CTB-insulin B 9–23 (C19S) by the filled bar. Arrows indicate the direction of transcription. "Ori" signifies "origin of replication." The nucleotide and amino acid sequences around the linking site with a linker and insulin B 9–23 (C19S) of the fused gene are shown at the bottom.

CTB-inp/pNU212 into *B.choshinensis*, the clones producing CTB-insulin B peptide hybrid protein were identified in culture supernatants by SDS–PAGE followed by coomassie brilliant blue staining. A time course study showed that the rate of CTB insulin B peptide production increased for up to 3 days of incubation and remained constant afterward (data not shown). Finally, secretion level of CTB insulin B peptide could be achieved at up to approximately 250 mg/L. A highly purified CTB-insulin B peptide hybrid protein was obtained from a 3-day culture supernatant of the *B.choshinensis* by using ammonium sulfate precipitation, a DEAE-Sepharose chromatography, a galactose-immobilized affinity chromatography followed by gel filtration on a Sephadex G-100. Using this procedure, 200 mg of purified hybrid protein was obtained from 1-L culture supernatant. The purified CTB-insulin B peptide hybrid protein was more than 95% pure as determined by SDS–PAGE in reducing conditions (data not shown). The purified CTB-insulin B peptide hybrid protein and formerly purified recombinant CTB contained 2.9 and 5.1 pg of lipopolysaccharide (LPS) per 100 μg of protein, respectively.

Characterization of CTB-Insulin B Peptide Hybrid Protein

The SDS–PAGE analysis on non-reducing conditions of purified recombinant CTB and CTB-insulin B peptide hybrid protein revealed a pentamer formation (Fig. 2). The pentameric form of purified recombinant CTB and CTB-insulin B peptide hybrid protein migrated with a molecular size of 40 and 45 kDa, respectively. The N-terminal amino acid sequence of purified CTB-insulin B peptide hybrid protein, TPQNITDLCAEYHNTQI, was identical to that of native CTB (Mekalanos et al., 1983). It was also found a peptide including insulin B 9–23 (C19S) amino acid sequence after the C-terminal of CTB, TPHAIAAIS-MANGPGSHLVEALYLVSGERG, after lysyl endopeptidase digestion of pyridylethylated CTB-insulin B peptide hybrid protein.

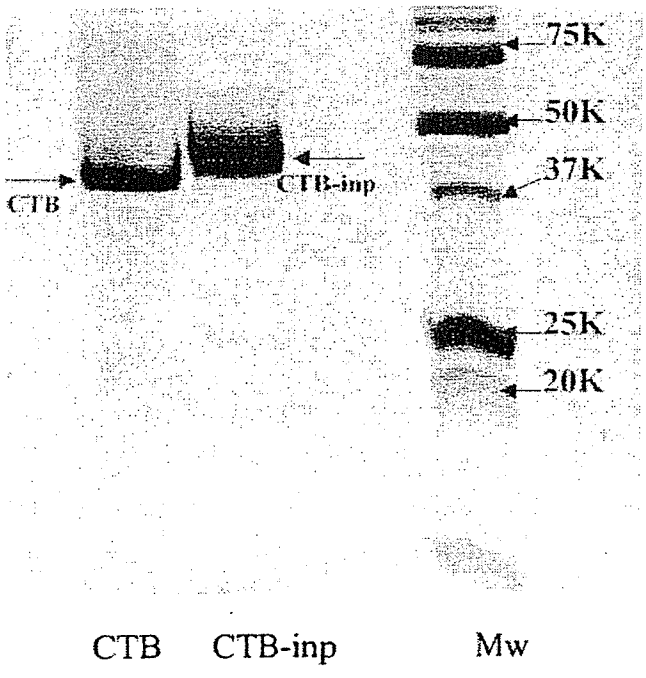


Figure 2. SDS–polyacrylamide gel electrophoresis for the CTB-insulin B 9–23 fusion protein purified CTB and CTB insulin B 9–23 (C19S) fusion protein (CTB-inp) were elcrophoresed in 12% ployacrylaminde gel with 0.1% SDS under non-reducing condition, respectively. The molecular weight maker (Mw) is shown on the right side of the gel.

To verify whether the CTB-insulin B peptide hybrid protein binds to GM1, the affinity of the protein for GM1 was compared with that of native recombinant CTB by using a competitive ELISA. When increasing amounts of the CTB-insulin B peptide hybrid protein were mixed with a constant concentration of biotinylated CTB and then reacted with GM1, the CTB-insulin B peptide hybrid protein was found to bind to GM1 with an equivalent binding affinity as the native form of CTB (Fig. 3). For CTB to bind to GM1 receptors, it must form a pentameric structure composed of identical monomers (Hardy et al., 1988). These findings suggest that the *B.choshinensis*-derived recombinant hybrid protein of CTB-insulin B peptide have a native pentamer form of CTB linked with diabetes inducing insulin peptide B 9–23 (C19S).

Nasal Administration of CTB-Insulin Peptide Hybrid Protein Reduced the Development of Diabetes

The female NOD mice began to develop spontaneously diabetes about 11 weeks of age and reached an incidence of 70% by 30 weeks in the colony used. Diabetes was characterized by polydipsia, weight loss, glycosuria, and persistent hyperglycemia. As shown in Figure 4, when CTB-insulin B

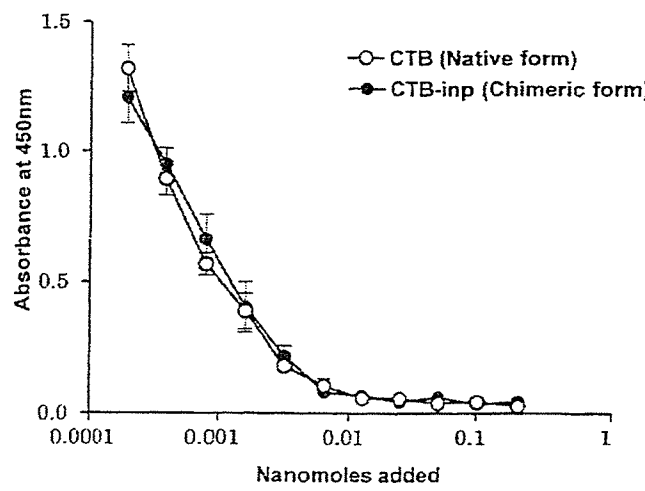


Figure 3. Comparison of the affinities of CTB-insulin B 9–23 fusion protein and the native form of CTB for the GM1 receptor by competive ELISA. Each protein is adjusted to an equimolar concentration. Serially diluted twofold concentrations of individual sample are then mixed with a fixed amount of biotinylated CTB and reacted with GM1 bound on the solid phase. Data are average of five measurements.

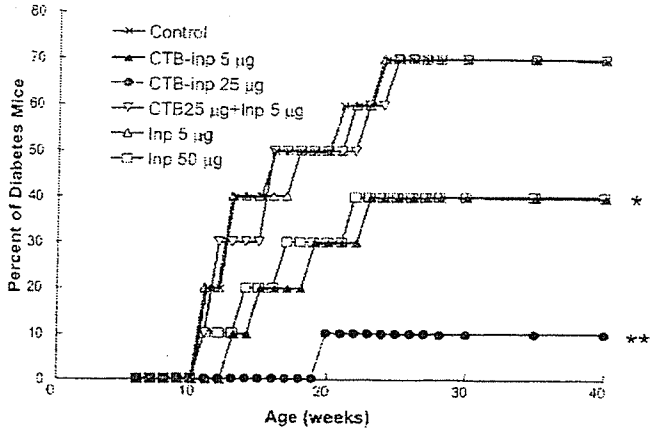


Figure 4. Suppression of spontaneous autoimmune diabetes development in NOD mice after nasal immunization with CTB-insulin B 9–23 fusion protein. The immunogens were nasally administered three times on 1 day in 6, 7, and 8 weeks. Mice (10 animals per group) were routinely monitored for diabetes by 40 weeks. Diabetes was characterized by polydipsia, weight loss, glycosuria as assessed by urine Chemstrips, and persistent hyperglycemia, which was determined with blood glucose levels using a Glucometere. Mice with a blood glucose level above 100 mg/dL were scored as diabetes. *: $P < 0.05$ Cox-Mantel log rank test. **: $P < 0.01$ Cox-Mantel log rank test.

peptide hybrid protein was administered via the nasal route, mice receiving three doses of 5 or of 25 μg of the fusion protein dose-dependently showed a reduction of percent of onset of diabetes. In addition, mice were nasally administered three times with 5 or 50 μg of insulin B 9–23 (C19S) peptide alone (Fig. 4). The nasal administration of 50 μg insulin B 9–23 (C19S) peptide alone show almost the same level reduction of onset of diabetes with that of 5 μg of the CTB-fusion protein. Because 5 μg of the CTB-insulin B peptide fusion protein contained only 0.6 μg of insulin B 9–23 (C19S) peptide from a ratio of their amino acid residues (15/121), the result demonstrate that coupling of autoantigen to CTB is increased its healing potential after nasal administration by up 100-fold on molar base of autoantigen peptide. For comparison purposes, mice were nasally administered three times with 5 μg of insulin B 9–23 (C19S) peptide mixed with 25 μg of the recombinant CTB. The control nasal treatment did not provide the inhibitory effects necessary for reduction of the percent of onset of diabetes (Fig. 4). The result also showed non-conjugated form of CTB did not enhance the induction of immune tolerance after oral insulin peptide administration. These results show that the CTB-insulin B peptide hybrid protein is one of the most effective molecules for the inhibition of the diabetes via the nasal route.

Nasal Administration of CTB-Insulin Peptide Hybrid Protein Reduced Insulin-Peptide Specific DTH Responses in the Diabetes Mice

Our next experiment was aimed at investigating whether nasally induced tolerance was established by insulin B 9–23 (C19S)-peptide specific cell-mediated immune responses because nasal immunization with small amount of the hybrid protein of CTB insulin B peptide led to the reduction of diabetes development. In order to this aim, the NOD mice were subcutaneously immunized with the insulin peptide together with CFA after nasal administration with CTB-insulin B peptide hybrid protein or insulin peptide alone or the insulin peptide plus CTB. The insulin B 9–23 (C19S) peptide-specific DTH responses were measured in 24 weeks after challenge with the insulin B 9–23 (C19S) peptide. As in Figure 5, DTH responses were reduced at almost the same level after nasal administrations of 5 μg CTB-insulin B peptide hybrid protein or 50 μg insulin B 9–23 (C19S) peptide alone subsequent to immunization with the insulin B peptide in CFA. On the other hand, the mice receiving nasal treatment with 5 μg the peptide, either alone or mixed with 25 μg CTB, did not lead to the reduction of peptide specific DTH responses (Fig. 5). These findings demonstrate that tolerance can be nasally induced with the use of hybrid protein of CTB and insulin B peptide.

DISCUSSION

IDDM results from immune-mediated destruction of β cells. Insulin is the only known islet autoantigen produced by β cells (Tisch and McDevitt, 1996). Thus, loss of tolerance to insulin is considered to direct affect β cells-specific destruction. Mucosally induced tolerance including both oral and nasal tolerance to autoantigen has been considered as an attractive strategy for preventing or reducing autoimmune diseases such as IDDM (Wu and Weiner, 2003). Although there were no major side effects, induction of oral-tolerance requires repeated administration of large amount of auto-antigen (Chen et al., 1996). It was shown that a small amount of oral CTB conjugated chemically to insulin prevented spontaneous autoimmune diabetes in NOD mice (Bergerot et al., 1997; Petersen et al., 2003). However, this chemical coupling procedures may affect the immunogenecity of the chemically modified antigen and may lead to the generation of a heterogeneous population of CTB conjugates (McGhee

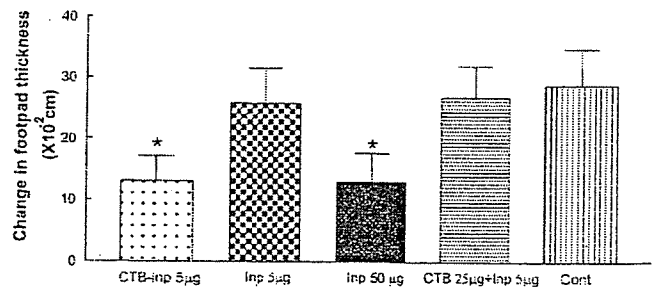


Figure 5. Nasally immunized CTB-insulin B 9–23 fusion protein inhibits insulin peptide-specific DTH responses. The immunogen were nasally administered three times on 1 day in 6, 7, and 8 weeks before systemic challenge for the induction of DTH. After 1 week, all nasally treated mice (10 animals per group) were given 20 μg of insulin B 9–23 (C19S) or saline subcutaneously in the right and left footpads, respectively. Footpad thickness was measured before and 24 h after challenge. The differences in footpad swelling between the two footpads were taken as DTH responses. *: $P < 0.05$ versus control group by Dunnett's test.

et al., 1992). In as much as NOD mouse is considered to be a good model of IDDM (Tisch and McDevitt, 1996), we used the NOD-mouse system to examine the effectiveness of chimeric molecule of CTB and autoantigen T cell epitope of insulin. Thus, we produced the hybrid protein of CTB and insulin B chain peptide 9–23, which is a dominant T cell epitope of the mouse (Daniel and Wegmann, 1996; Devendra et al., 2004), as an effective nasal vaccine against spontaneous autoimmune diabetes using *B.choshinensis* expression system to generate a safe and uniformed immunogen for clinical use.

The use of *B.choshinensis* as a recombinant hybrid protein expression system has many of advantages. First, the *B.choshinensis* system produces high levels of recombinant proteins in culture medium with little contamination of lipopolysaccharide (Inoue et al., 1997). When *B.choshinensis* 47 K is used as a host, CTB-insulin B peptide fusion protein that highly expressed and extracellularly secreted are stably maintained. In fact, 200 mg of the CTB fusion protein per liter could be recovered from the culture medium. In addition, the purified CTB-insulin B peptide fusion protein contained only 2.9 pg of LPS per 100 μg. The range LPS (e.g., less than 50 pg/100 μg) was been shown to have no biological effect on the immune system (Ultrich et al., 1991). Furthermore, sequence analysis confirmed the fusion protein has full sequence of CTB and insulin B peptide 9–23 (C19S). Second, the secreted recombinant protein produced in *B.choshinensis* has shown to form correctly folded structure with appropriate biological activity. *B.choshinensis* has the very low level of extracelular protease activity, which promotes stability of the secreted recombinant proteins (Shiroza et al., 2003). The *B.choshinensis* derived CTB-insulin B peptide fusion protein is secreted as pentamer (Fig. 2) and GM-1 receptor assay showed the fusion protein binds to GM-1 receptor an almost equivalent degree as the native form of CTB (Fig. 3). CTB pentamer functions as a tolerogen based on its affinity for cell surface receptor GM-1 gangliosides expressed by epithelium in mucosal inductive sites such as the gut-associated lymphoreticular tissues (GALT) (Frey et al., 1996). Third, *B.choshinensis* is considered a safe microorganism for production of recombinant proteins that will be administered to animals or humans because the bacteria are known to be harmless resident of soil (Udaka and Yamagata, 1993). These unique characteristics of the *B.choshinensis* recombinant protein expression system result in production of uniform CTB-insulin B peptide fusion protein.

In this study, we produced a recombinant CTB fusion protein linked with insulin B chain peptide 9–23 (C19S) to examine its inhibitory effect on the development of spontaneous autoimmune diabetes. When CTB-insulin peptide hybrid protein was administered via the nasal route, mice receiving three doses of 5 or of 25 μg of the fusion protein showed dose-dependently a reduction of percent of onset of diabetes (Fig. 4). The nasal administration of 50 μg insulin B peptide 9–23 (C19S) alone show almost the same level reduction of onset of diabetes with that of 5 μg of the CTB-fusion protein (Fig. 4). The recombinant hybrid of insulin peptide and CTB is effective at doses 100-fold less than the autoantigen peptide (Fig. 4). The pentametric structure of the CTB fusion protein not only facilitate GM1 ganglioside specific delivery and presentation of conjugated peptide to the GALT, it also increases the molar concentration of autoantigen peptide per molecule of CTB pentamer (Arakawa et al., 1998). The increase in antigen peptide concentration in GALT may reduce the requirement for high concentration of autoantigen synthetic peptide in CTB fusion protein.

In this study, the nasal administration of CTB-insulin B peptide hybrid protein was shown to induce a significant reduction of insulin B peptide-specific DTH responses (Fig. 5). In addition, the recombinant hybrid of insulin peptide and CTB also increased its tolerogenic potential for nasal administration by up 100-fold on molar base of autoantigen peptide (Fig. 5). Because DTH responses are thought to be regulated by $CD4^+$ Th1 cells (Stevens et al., 1998), the reduction of T-cell responses observed suggested that insulin B peptide-specific $CD4^+$ Th1 cells were down-regulated by the nasal administration of CTB-insulin B peptide hybrid proteins. It was reported that oral CTB conjugated chemically to insulin suppressed the development of diabetes on NOD mice by regulatory $CD4^+$ Th2 type T cells via the inhibition of pro-inflammatory Th-1 type response (Ploix et al., 1999; Aspord and Thivolet, 2002). The ability of $CD4^+$ T cells to suppress the adoptive transfer of diabetes was explained as bystander suppression mediated by Th2 anti-inflammatory cytokines (Ploix et al., 1999; Aspord and Thivolet, 2002). The insulin peptide-specific $CD4^+$ Th1 cells on present study may be downregulated by the regulatory $CD4^+$ Th2 type T cells.

It was reported that transgenic potatoes that synthesize human insulin and CTB-insulin fusion protein at levels up to 0.05% and 0.1% of total soluble protein, respectively (Arakawa et al., 1998). Feeding transgenic potato tissues producing CTB-insulin fusion protein but not insulin provided a significant reduction in insulitis or diabetic symptoms. It was considered that the plant-synthesized CTB-insulin fusion protein is effective at doses 100-fold less than generally reported for unconjugated autoantigens (Arakawa et al., 1998). In present study, we produced a recombinant CTB fusion protein linked with insulin B chain peptide 9–23 (C19S) at levels up to 200 mg/L culture media in *B.choshinensis* secretion-expression system. Nasal administration of the recombinant *B.choshinensis*-derived hybrid protein of CTB and insulin B peptide suppressed the development of autoimmune diabetes on NOD mice. The recombinant hybrid of CTB and insulin B peptide increased its tolerogenic potential for nasal administration by up 100-fold on molar base of autoantigen peptide. Taken together with the present study, these findings provided new evidence that a hybrid molecule developed from molecule recombinant CTB and insulin or insulin T-cell epitope may be safe and useful in mucosal immune therapy allowing for the induction of mucosally induced

tolerance to auto-reactive T cells in patients with IDDM.

In summary, we produced a uniformed form of hybrid molecule of CTB and insulin B chain peptide at highly expression level by use of *B.choshinensis* recombinant protein expression system. Further, nasal immunization with low doses of the hybrid protein effectively reduced the development of on-going autoimmune diabetes. These results suggest that nasal tolerance produced by administration of the recombinant *B.choshinensis*-hybrid protein of CTB and autoantigen T-cell-epitope could be useful in the control of T-cell-mediated autoimmune diseases such as autoimmune diabetes.

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Mucosa-Associated Lymphoid Tissues in the Aerodigestive Tract: Their Shared and Divergent Traits and Their Importance to the Orchestration of the Mucosal Immune System

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Abstract: As inductive tissues for the initiation of antigen-specific T and B cell responses, the various mucosa-associated lymphoid tissues (MALT) of the aerodigestive tract, which include gut-associated lymphoid tissue (GALT), nasopharynx-associated lymphoid tissue (NALT) and bronchus-associated lymphoid tissue (BALT), share many histological and immunological characteristics. However, recent advances in our molecular and cellular understanding of immunological development have revealed that the various types of MALT also exhibit different molecular and cellular interactions for their organogenesis. In this review, we delineate the distinctive features of GALT, NALT and BALT and seek to show the role played by those features in the regulation of mucosal tissue organogenesis, the mucosal immune system, and mucosal homeostasis, all in an attempt to provide insights which might lead to a prospective mucosal vaccine.

INTRODUCTION

Mucosal surfaces of the gastrointestinal and respiratory tracts represent the principal entry site of a multitude of viral and microbial pathogens. To protect mucosal sites from these invasions, the aerodigestive tract is equipped with multiple physical, biochemical and immunological barriers. The immunological barrier consists of both innate and acquired immunity, with the latter characterized by the initiation of antigen-specific immune response in mucosa-associated lymphoid tissues (MALTs) including the gut-associated lymphoid tissue (GALT), the nasopharynx-associated lymphoid tissue (NALT), and the bronchus-associated lymphoid tissue (BALT) [1, 2]. In particular, Peyer's patches (PPs) and NALT are thought to be representative MALT in the gastrointestinal and respiratory tract, respectively. Additionally, isolated lymphoid follicles (ILFs), which are located throughout the intestine, have been identified and characterized as an additional organized lymphoid tissue in the digestive tract [3]. These tissues contain an interfollicular area that is abundant in T lymphocytes and in high endothelial venules (HEVs), as well as a germinal center (GC), characterized by a dense network of follicular dendritic cells, providing a source of antigen-primed IgA-committed B cells. They also are overlaid by a follicle-associated epithelium (FAE), which contains antigen-sampling M (microfold) cells, allowing selective transport of antigen from the lumen to underlying antigen-presenting cells (APCs) such as dendritic cells (DCs) and macrophages. Based on these anatomical and histological characteristics of MALT, it has been generally considered that MALTs act as inductive tissues for the generation and priming of antigen-specific T and B cell responses, and that they communicate with effector tissues (e.g., intestinal lamina propria and nasal passages) via an immunological intranet known as the common mucosal immune system (CMIS) [4].

However alike in terms of anatomy and histology, the MALTs of the respiratory and intestinal immune system differ in their organogenesis. Recent studies revealed several key cytokines regulating MALT organogenesis including tumor necrosis factor (TNF) family, lymphotoxin (LT) $\alpha 1\beta 2$/LTβR, and IL-7 receptor α (IL-7Rα) [5]. Additionally, transcriptional regulators, retinoic acid-related orphan receptor γ (RORγ) and inhibitor of DNA binding/differentiation 2 (ID2), shown to play a role in the MALT organogenesis [6, 7]. These discoveries enabled us to paint a detailed picture of the early steps of the development of MALT, especially for PPs and NALT [1, 5]. Surprisingly, though PPs and NALT showed many structural and immunological similarities, they proved to have distinguishable molecular pathways for organogenesis. For example, NALT formation was detected in mice lacking PPs [8]. Additionally, experiments using MALT-deficient mice have revealed the presence of a MALT-independent (an presumably, a CMIS-independent) pathway for the initiation of antigen-specific immune responses [9].

Prompted by these recent findings, we set out to explore whether this new understanding of MALT biology requires us to revisit our assumptions about

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the roles of the various MALTs in the induction and regulation of mucosal homeostasis, including both active and quiescent immune responses. This review attempts to delineate both the traits which link and those which distinguish GALT, NALT and BALT and to explore how the differences may help determine their specific anatomical, developmental and immunological contributions to the mucosal immune system. We will show how this recent progress in our understanding of the structure and function of the various MALTs might facilitate the development of a prospective mucosal vaccine.

THE STRUCTURE AND COMPOSITION OF MALT

The secondary lymphoid tissues are located at immunologically and anatomically important sites where foreign antigens can be presented efficiently to the immune system for the generation of antigen-specific cell-mediated and/or humoral immunity. Systemic immune responses are governed by the anatomical compartment which includes spleen and lymph node (LN), and mucosal immune responses by the compartment including MALT (NALT, BALT, and GALT). These lymphoid compartments share striking similarities in cellular organization, such as discrete T and B cell areas, yet each also has unique characteristics.

Located in the small intestinal anti-mesentery site which extends along the entirety of the small intestine, PPs act as one of the representatives of GALT. They are large enough to be observed upon gross examination, and usually number 8-10 in murine small intestine. PPs are unique in that they contain efferent lymphatics but no afferent lymphatics, reducing the possibility that an antigen will be encountered via the afferent lymphatics. Instead, they take up antigen via a specialized dome region covered with FAE containing M cells [10, 11]. M cells, which are scattered among absorptive epithelial cells (ECs), are distinguished by features such as poorly developed brush borders, reduced degradation activity, and a thin overlying glycocalyx. Additionally, M cells are characterized by a pocket structure (M cell pocket) containing DCs, macrophages, and lymphocytes [12]. Thus, M cells act as a gateway to the outside environment, delivering antigenic substrate to the underlying immune-competent cells for the subsequent induction of antigen-specific immune responses. DCs are present not only in the M cell pocket, but also in the region beneath of FAE (known as the subepithelial dome, SED) [13]. Recent studies demonstrated that $CD11b^+$ DCs were recruited by CCL20 and CCL9 highly expressed by FAE [14, 15]. PPs also contain all of the cellular and microarchitectural elements necessary for the generation of IgA-committed B cells and antigen-specific T cells. At the center of the dome region of PPs lie the B cell follicles, which surround the germinal center (GC) lying at the base of the dome. Unlike the other systemic lymphoid tissues that require infection or local immunization for GC formation, GC is always present in PPs. Because germ-free mice present PPs but lack GCs, it has been postulated that the continuous exposure of the PPs to the commensal bacteria and/or viruses of the outer environment cause the constant GC formation in PPs [16]. At the outside of the dome region, there are interfollicular regions populated by many T cells, and mature DCs but by only a few B cells. These interfollicular regions contain HEV, a main entry site for cells into PPs. The HEV express mucosal vascular addressin cell adhesion molecule 1 (MAdCAM-1), a ligand for the $\alpha4\beta7$ integrin, which determines a selective migration of gut-tropism lymphocytes [17]. The T cell-rich interfollicular regions overlap with the B cell follicles in some areas, providing an important place for initial T-B interaction [18].

Recently, an additional lymphoid structure resembling PP in composition and architecture has been identified as 100-200 clusters on the antimesenteric wall of the murine small intestine, which is now known as ILF [3]. However, ILF is much smaller than PP (e.g., ILF is about 200 μm and PP is about 3 mm in diameter). They possess an overlying M cells-containing FAE and a large B cell area including GCs. Although lacking a T cell-rich interfollicular region, they do still contain some T cells. These data suggest that ILFs are alternative inductive sites for mucosal immune responses [3].

Another recently discovered feature of the murine intestinal compartment is the Cryptopatch (CP) [19]. CPs are tiny but numerous lymphoid tissues (1500-2000 CPs/intestine) in the cryptal region of the small and large intestine. $c\text{-}kit^+IL\text{-}7R^+Thy1^+Lin^-$ ($CD3$, $B220$, $Mac\text{-}1$, $Gr\text{-}1$ and $TER\text{-}119$) lympho-hematopoietic progenitors have been shown to accumulate in CP. CP has also been considered as a site where extrathymic differentiation of T cells occurs for the intraepithelial lymphocytes (IELs). To this end, it has been shown that T cell receptor (TCR) $\alpha\beta$ and TCR$\gamma\delta$ IELs were developed by in vivo transfer of CP progenitor into severe combined immunodeficient (SCID) mice [20].

The respiratory tract is another site characterized by mucosa-associated lymphoid structures. In rodents, NALT is found on both sides of the nasopharyngeal duct dorsal to the cartilaginous soft palate and is considered analogous to the Waldeyer's ring in humans [21]. Furthermore, a NALT-like structure of lymphocyte aggregation with follicle formation was identified in human nasal mucosa, especially that of the middle concha in children less than two years old [22]. Like PPs in the intestine, NALT has been considered as an inductive tissue for the nose and exhibits FAE where antigen-sampling M cells are present [23]. NALT is also composed of follicles and interfollicular regions which are B cell- and T cell-enriched sites, respectively. Characterization of messenger RNA encoding helper

T (Th) 1 and Th2 cytokines in $CD4^+$ T cells isolated from murine NALT revealed a dominant Th0 cytokine profile, indicating that these T cells are capable of becoming Th1 or Th2 cells immediately after antigen exposure through the nasal tract [24, 25]. In the interfollicular regions, NALT-associated HEVs express peripheral node addressin (PNAd), which is regulated by the LTβR- NF-κB-inducing kinase (NIK)-IKKα signaling pathway [26]. APCs including DCs and macrophages are also found in NALT [27]. Thus, NALT contains all of the lymphoid cells necessary for the induction and regulation of mucosal immune responses to nasally exposed antigens.

In 1973, Bienenstock *et al.* described that BALT was classically defined as an aggregated lymphoid structure separated from the bronchial lumen by a specialized lymphoepithelium [28, 29]. Although the presence of murine and human BALT as an aggregated lymphoid follicle remains a subject of debate, BALT has been described in several species including rats, rabbits, and sheep [30, 31]. Similar to the PPs and NALT, BALT does not draw upon afferent lymphatics for antigen retrieval. Instead, it samples antigen directly from the lung lumen through M cells [32], with HEV being the only entry site for lymphocytes into BALT [33]. The migration of the lymphocytes from the blood through HEV is possibly mediated in part by the L-selectin ligand, PNAd [34]. This lymphoid architecture was characterized by the presence of CXCL13 and CCL21 in and around germinal centers, respectively, for IgA antibody production [35]. Another study indicated that lung $CD11c^{bright}$ DCs were the principal APCs to uptake antigens in lung following intranasal immunization, resulting in the direct priming of $CD4^+$ T cells that did not require their migration into draining LNs [36]. These immunological and histological features of BALT in experimental animals suggest that it resembles the other mucosal organized lymphoid structures that induce protection against respiratory infections.

DISTINCT PATHWAY FOR MALT ORGANOGENESIS

The Molecular and Cellular Mechanisms of GALT Organogenesis

Recently, a model describing the development of PP was proposed based on the molecular and cellular characterization of IL-7R, LTβR and chemokine receptor-mediated tissue genesis (Fig. **1**) [37]. It was shown that lymphoid-lineage $IL\text{-}7R^+CD3^-CD4^+CD45^+$ cells are considered to be PP inducers expressing CXC chemokine receptor 5 (CXCR5) and can produce membrane-associated $LT\alpha1\beta2$ heterotrimer, whereas mesenchymal-lineage $VCAM\text{-}1^+$ and intercellular adhesion molecule (ICAM)-1^+ PP organizers express LTβR [38, 39]. The profile of receptor expression suggests the presence of reciprocal signaling cascades between the inducer and organizer cells. Following stimulation signals provided through IL-7R, PP inducers express $LT\alpha1\beta2$ to activate PP organizers through LTβR, and PP organizers produce chemokines such as CXCL13 and CCL19 to stimulate PP inducers through CXCR5 and CCR7 [40]. The reciprocal interaction between inducer and organizer cells through the chemokine and cytokine receptors is essential for the initiation of PP formation, and the loss of any part of the signaling program is sufficient to disrupt secondary lymphoid tissue development, as evidenced by the loss of PP in $LT\beta R^{-/-}$ and $IL\text{-}7R\alpha^{-/-}$ mice [41, 42]. Furthermore, deletion of the gene encoding CXCR5 resulted in a partial reduction in the formation and number of PP [43]. The lack of PP and lymph nodes in *aly/aly* (or alymphoplasia) mice, which have defective NIK function, also fits this paradigm, as recent analyses have established that NIK is essential for the transduction of signals through the TNFR family, including those through LTβR [44, 45]. Thus, *aly/aly* mice lack PP as the NIK mutation inhibits the reciprocal interaction between PP inducers and organizers through $LT\alpha1\beta2$ and LTβR [46]. Further evidence in support of this model comes from studies showing that mice lacking the $CD3^-CD4^+CD45^+IL\text{-}7R^+$ inducer cells due to genetic deletion of the transcription regulators Id2 or RORγ also completely lack the formation of PP and LN [7, 47].

The formation of ILF also requires LT- and LTβR-dependent events [3]. However, unlike PP formation, the LT- and LTβR-dependent events required for ILF formation can occur postnatally and require LT-expressing B lymphocytes [48]. Consistent with this fact, treatment with LTβR-Ig fusion protein during the postnatal period results suppresses ILFs but not PPs [49]. It was also demonstrated that immature ILFs with clusters of $B220^+$ cells were present in the intestine of germ-free mice, but exogenous stimuli including bacterial antigens/mitogens are required for the completion of the lymphoid organization of ILFs including GC formation [48]. Consistent data was obtained in the mice lacking activation-induced cystidine deaminase (AID) that is an important molecule for class switch recombination (CSR) as mentioned below [50]. In the AID-deficient mice, the ILFs developed hyperplasia. A recent study demonstrated that the lack of hypermutated IgA production into the intestinal lumen resulted in the expansion of segmented filamentous bacteria in the small intestine of AID-deficient mice. Because antibiotic treatment of AID-deficient mice abolished the hyperplasia, the authors of that study proposed that the anaerobic expansion induced ILF hyperplasia [50, 51]. Taken together, these findings suggest that postnatal and physiological inflammatory signals are essential for the formulation of ILFs.

Membrane-bound $LT\alpha1\beta2$ interactions with LTβR are also essential for CP development. Thus, $LT\alpha^{-/-}$ or $LT\beta R^{-/-}$ mice lack CP [52]. Like ILFs, CP

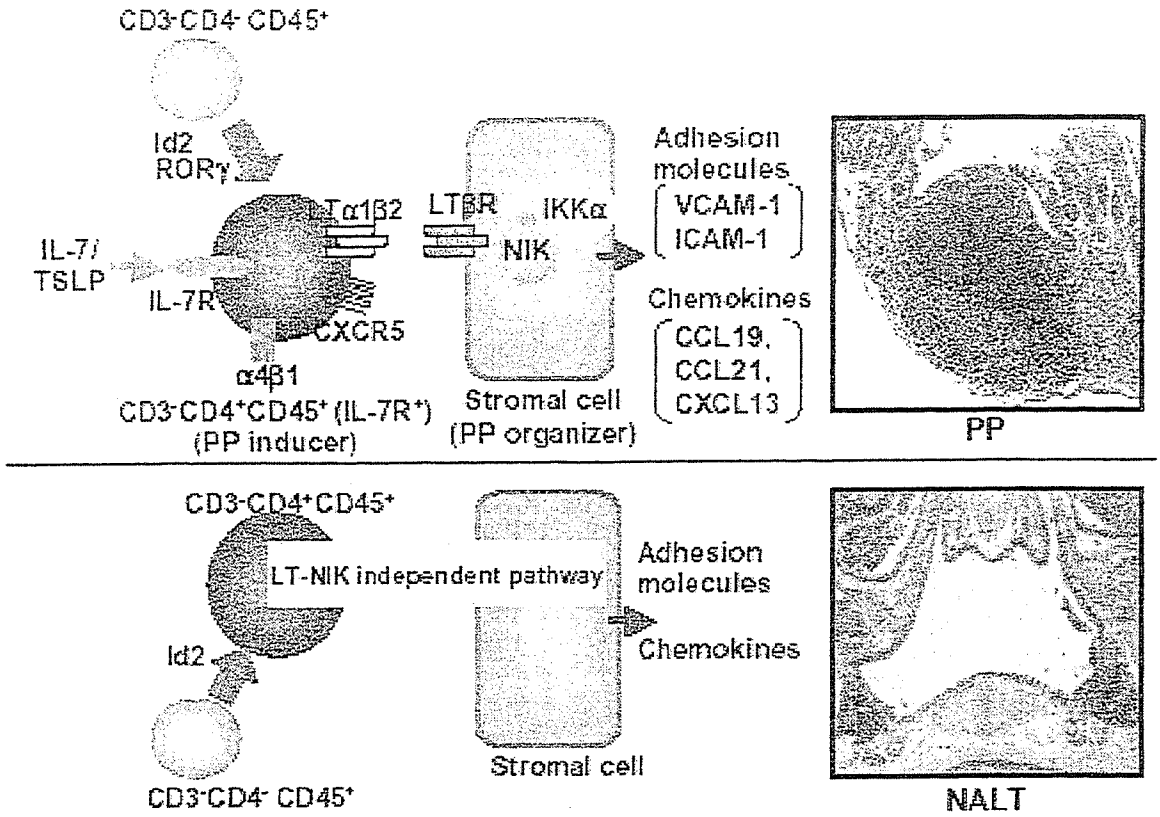

Figure 1. Comparison of the organogenesis programs of NALT and PPs. $CD3^-CD4^+CD45^+$ cells are considered to be the common inducers of secondary lymphoid tissue. Id2 is indispensable for the induction and differentiation of these inducer cells from their fetal liver precursors ($CD3^-CD4^-CD45^+$). In the case of PPs, IL-7 and/or TSLP and IL-7R interaction induces the expression of the LTα1β2 heterotrimer on these $CD3^-CD4^+CD45^+$ PP inducer cells. LTα1β2 heterotrimer binds to LTβR on stromal cells as a PP organizer and induces signal transduction through NIK-IKKα for the expression of necessary adhesion molecules and/or chemokines. CXCL13 produced by a PP organizer activates the form of α4β1 expressed on the CXCR5-positive PP inducer. The activated form of α4β1 contributes to the interaction of the PP inducer and the PP organizer. Thus, IL-7R- and LTβR- mediated signals are essential for the tissue genesis of PPs. The development of $CD3^-CD4^+CD45^+$ cells in NALT also requires Id2; however, the initiation of NALT organogenesis is independent of IL-7R, LTα1β2/LTβR and NIK signaling.

development seems to be a postnatal event. Thus, CPs were fist detected at 14-17 days after birth [19]. However, CPs are present in the small intestine of *aly/aly* mice that do not contain PPs and ILFs [52]. These results indicate that an alternative LTβR and NF-κB activation pathway may play a crucial role in the development of CP.

Distinct Features of NALT Organogenesis Compared with PP

In normal mice, NALT is a bell-shaped tissue characterized by the accumulation of a variety of lymphoid cells that are capable of initiating both humoral and cellular immunity. NALT formation has not been observed during embryogenesis or in newborn nasal tissue. $PNAd^+$ HEV is first detected in bilateral nasal tissue one week after birth, and the complete bell-shaped NALT formation with lymphoid cells eight weeks after birth [8]. These findings indicate the presence of a distinct tissue genesis program in the intestinal and respiratory immune systems, where a pre-natal initiation of lymphoid organogenesis occurs for PP and NALT, respectively. An intriguing possibility is that the NALT genesis program is triggered after birth by stimulation signals provided by environmental antigens and mitogens. However, we have observed the formation of NALT in adult mice born and raised under germ-free conditions (unpublished results). A similar situation was also seen in germ-free rats [53]. Thus, one possibility could be that the initiation of NALT genesis is programmed to be activated after birth and the subsequent maturation process is controlled by environmental antigens.

PP formation requires a cytokine-signaling cascade involving IL-7R and LTβR (Fig. 1). However, organized nasal lymphoid tissue was detected in all mouse strains lacking PP or both PP and LN due to a deficiency in the LTβR-mediated inflammatory cytokine cascade, including $LT\alpha^{-/-}$, $LT\beta^{-/-}$, and *aly/aly* mice, and mice treated *in utero* with LTβR-Ig fusion protein [8, 54]. These findings further support the idea that NALT tissue genesis does not follow the "programmed inflammation" model typical of PP. We

next examined NALT development in IL-7R$^{-/-}$ mice, as both the IL-7R-mediated signaling pathway as well as the LTβR cascade have been shown to be requisite for PP development. NALT, but not PP, did develop in IL-7R$^{-/-}$ mice [8]. Taken together, these findings directly demonstrate that NALT formation is independent of IL-7R- and LTβR-mediated tissue genesis.

A unique subset of mononuclear cells characterized as CD3$^-$CD4$^+$CD45$^+$ have been shown to act as inducer cells for the organogenesis of secondary lymphoid tissues including PP in the intestinal wall (Fig. **1**) [40]. Furthermore, Id2 has been identified as one of the genes responsible for the induction of these CD3$^-$CD4$^+$CD45$^+$ inducer cells [7]. Not surprisingly then, deletion of the Id2 gene completely impaired the genesis of all secondary lymphoid tissues including both NALT and PP [7, 8]. In normal and LT?$^{-/-}$ mice, these CD3$^-$CD4$^+$CD45$^+$ cells accumulate to form the NALT anlagen. Adoptive transfer of fetal liver cells or CD3$^-$CD4$^+$CD45$^+$ cells into Id2$^{-/-}$ newborn mice could generate a NALT-like structure in these mice (Fig. **1**). [8]. The transcriptional regulator ROR? has also been shown to be required for the development of CD3$^-$CD4$^+$CD45$^+$ inducer cells [47]. When the gene encoding RORγ was deleted, PP and LN organogenesis was suppressed [47]. However, NALT development has been reported in RORγ-deficient mice [54]. These findings suggest that although NALT and PP share the same phenotype of inducer cells, CD3$^-$CD4$^+$CD45$^+$, those inducer cells can be categorized into those dependent on Id2 alone (like NALT) and those dependent on both Id2 and RORγ (like PP)[1].

Development of BALT

As mentioned above, BALT appears in some but not all vertebrate species and the timing of its appearance varies among species as well. Classical BALT appears during the late embryonic development in the rat and the pig, but not in the human and the mouse [30, 31]. However, several lines of evidence suggest that *de novo* organization of lymphoid tissues and GCs are induced in murine and human lung by infection, immunization or inflammation [55, 56]. To distinguish these immunological reaction-induced structures and formation pathway from those of classical BALT, they have been termed inducible BALT (iBALT). A recent study indicated that iBALT structures were formed independently of LTα expression and demonstrated that mice lacking spleen, LNs, and PPs, but having iBALT, exhibited substantial T cell- and B cell-mediated immune responses to clear influenza infection, which seems to be mediated by iBALT [35]. However, it is also possible that NALT may contribute to the original phase of the induction pathway of iBALT, since the inhaled antigens first make contact with NALT prior to their deposition on BALT. Also, since antigen-activated lymphocytes are first observed at the lymph nodes (e.g., NALT and CLN) that drain the respiratory tract and are subsequently found in the lung, it is possible that iBALT is just an accumulation site for the effector cells that are initially primed in conventional lymphoid organs (e.g., NALT) [57]. Thus, both the organogenesis of iBALT and its contribution to the priming and expansion of the T and B cell responses remain obscure, and further study is required to clarify this important issue.

INITIATION OF ANTIGEN-SPECIFIC IMMUNE RESPONSES IN MALT FOR THE COMMON MUCOSAL IMMUNE SYSTEM (CMIS) PATHWAY

The M cell: a Unique Antigen-Sampling Cell

Although the organogenesis pathways for PP, ILF and NALT are quite distinct above, they all act as inductive tissues for the initiation of antigen-specific immune responses via the CMIS-dependent pathway (Fig. **2**). First, epithelia covering the inductive tissues develop an FAE containing scattered professional antigen-sampling M cells to achieve selective transport of foreign antigen from the lumen into the inductive tissues [3, 11, 23]. The M cells can be distinguished from the surrounding ECs by their unique histological and biological features, including a lack of brush borders, a limited mucus production, and a unique basolateral invagination that creates a pocket containing immunocompetent cells such as DCs, macrophages, T cells and B cells [12]. M cells also show lower degradation and higher transcytosis activity than do the neighboring ECs, and they express receptors for some microorganisms [10]. For example, *Yersinia* adheres to M cells via the invasin and the β1 integrin expressed on the *Yersinia* and M cells, respectively, allowing the selective invasion through M cells [58]. Thus, when invasion was mutated, Yersinia could no longer adhere to and invade M cells [58]. Similarly, when reovirus is ingested orally, intestinal proteases modify its outer capsid membranes (sigma 3 and μ1c), thereby inducing the expression of σ1 protein containing a sialic acid-specific, lectin-like domain which is specific for glycoconjugates containing α2-3-linked sialic acid expressed on the apical side of M cells [59]. These characteristics of M cells allow them to selectively transport antigens and microorganisms from the lumen to the APCs in the M cell pocket..

Priming by Mocosal APCs of Naïve T Cells and B Cells in Inductive Tissues

Once APCs in the M cell pocket take antigen up from the intestinal lumen through the mediation of the M cell, they process the antigen and migrate into the interfollicular areas of the PPs, where they present antigenic epitopes with major histocompatibility complex (MHC) molecules (Fig. **2**) [14, 60]. Although it has been already reported that NALT DCs present antigen after intranasal

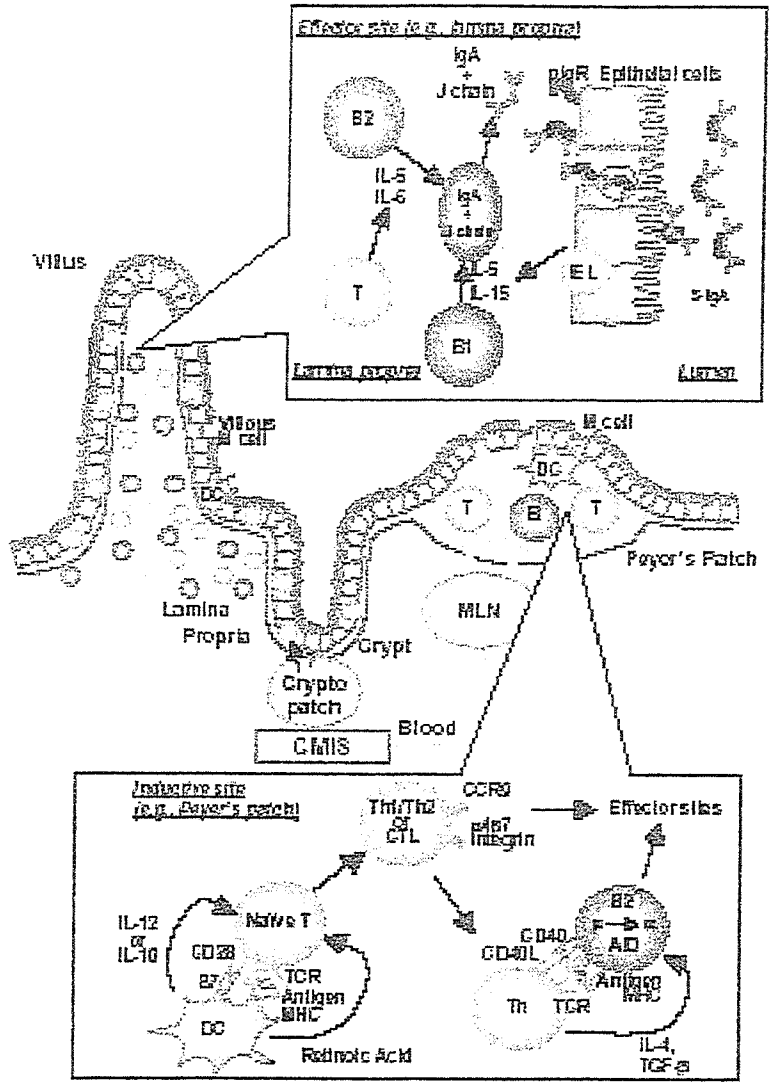

Figure 2. Multi-layered barriers provided by innate and acquired immunity in the intestine. Following antigen transport through M cells, DCs obtain the antigen and present it to naïve T cells, causing them to differentiate into Th or CTL cells in the presence of DC-produced IL-10 or IL-12, respectively. Retinoic acid derived from DCs determines the T cell tropism to migrate to mucosal effector sites by the up-regulation of CCR9 and α4β7 integrin on T cells. Th cells interact with B2 cells to induce CSR, which induces IgA-committed B cells. This step is mediated by the CD40/CD40 ligand interaction, and by IL-4 and TGF-β. These antigen-specific T and B cells migrate preferentially into mucosal effector sites (e.g., lamina propria) through HEV. Mucosal T cell-derived IL-5 and IL-6 promote differentiation of IgA-committed B2 cells into IgA plasma cells, resulting in the production of polymeric IgA (IgA+J chain). The polymeric IgA is associated with the expression of pIgR on the basal side of epithelial cells followed by the production of secretory IgA (S-IgA) into the lumen. At the mucosal effector sites, B1 cells are also present and are activated by EC-derived IL-15 to differentiate into plasma cells for the secretion of IgA against common bacterial products like phosphatidylcholine. Like the IgA originating from B2 cells, the B1 cell-derived IgA is also transported into the lumen as a form of S-IgA. Some populations of IELs located between the ECs recognize non-classical MHC molecules on ECs that are induced by some bacterial and/or viral infections. Thus, B1 cell-derived IgA and IEL have an ability to react to the antigen without any fine specificity for microorganisms, and thus may play a crucial role in the innate aspect of mucosal immunity.

immunization [27], detailed pathway for the antigen uptake and their migration in NALT is still unclear. Also unclear is the mechanism by which the newly identified and characterized ILF in the intestinal tract prime Th cells and B cells via DCs. The antigen presentation by APCs stimulates naïve T cells which predominate in the inductive tissues, resulting in the differentiation of naïve T cells into Th1, Th2 or cytotoxic T lymphocytes (CTLs) [24, 60, 61] (Fig. 2). To this end, three distinct populations of DCs were reported in PPs [14]. $CD11b^+$ DCs are capable of producing IL-10 by the stimulation of the CD40 ligand, whereas $CD8^+$ DCs or $CD11b^-CD8^-$ (double negative) DCs produce IL-12 by microbial stimulation, resulting in the induction of Th1 and Th2, respectively [60].

The cross talking among DCs, T cells, and B cells at inductive tissues promotes the IgA-commitment of B cells, which undergo μ to α isotype CSR [62, 63] (Figs. **2** and **3**). This μ to α isotype CSR is likely dependent on antigen stimulation at GCs, where naïve B cells interact with local $CD4^+$ T cells and with follicular antigen-trapping DCs. Key interactions required for this switch include ligation of CD40 on B cells with the CD40 ligand on Th cells [64], the presence of transforming growth factor (TGF)-β [65, 66], and the interaction between the inducible co-stimulator (ICOS) expressed on activated Th cells and its ligand ICOS-L constitutively expressed by B cells [67]. Stimulated by these interactions, multiple transcription factors are activated and promote the process of CSR (Fig. **3**). For example, transcriptional activation of the $C_H\alpha$ gene requires the TGF-β-mediated activation of Smad3/4 and the *de novo* synthesis of core-binding factor (CBF) α3 that binds to the Smad-binding elements (SBEs) and CBF sites on the intronic $C_H\alpha$ promoter region [68, 69]. A dramatic breakthrough in our understanding of CSR came with the discovery of AID [70, 71]. AID, which exhibits a single-stranded DNA deaminase activity, is expressed in activated B cells, particularly in the GC [72]. It associates with the CSR target chromatin in a germline transcription-coupled manner [73]. As might be expected given the ability of PPs, ILFs, and NALT to induce the generation of IgA-committed B cells, AID expression and Iα-Cμ circle transcripts and their reaction products were always detected at these inductive tissues [63].

In contrast to the dominant class switch to IgA in PPs, B cell differentiation in NALT was shown to lead to the production of both IgA and IgG [74]. It had

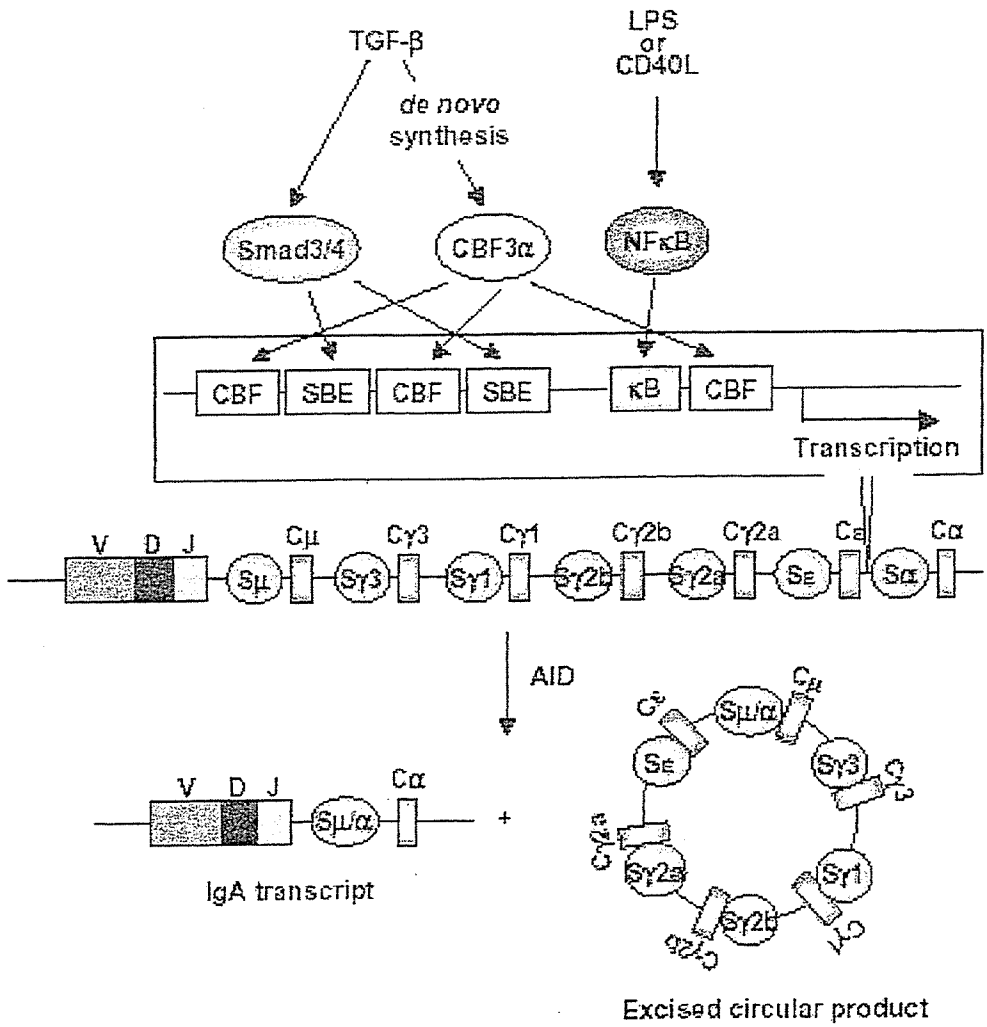

Figure 3. Molecular events for class switching to IgA. A secondary isotype of Ig is produced by CSR, a process for exchange of the constant (C) region of the heavy chain. This pathway requires AID activity for the DNA break in the switch (S) region, which is mediated by DNA deamination. Following DNA repair, it generates rearranged DNA encoding a different isotype of Ig and the deletion of the intervening sequence as a circular DNA. TGF-β-induced Smad3/4 binds to the SBE region on the Cα promoter and the TGF-β-mediated signaling also induces *de novo* synthesis of CBFα3 that is in turn capable of binding to the CBF region on the Cα promoter. These bindings facilitate the transcription of the Cα region, presumably in the presence of the NFκB-mediated κB activation, determining the class switch to IgA.

been previously established that the development of IgA-committed cells in the presence of TGF-β was characterized by sequential CSR from Cμ to Cα via Cγ, a pathway mediated by CD40 engagement [64, 75]. It is also interesting to note that human tonsils have been shown to contain a high frequency of IgG B cells in addition to IgA [76, 77]. These findings may explain the equal commitment of B cells to IgG and to IgA in NALT, but further analysis will be required to reveal the molecular mechanism involved in the generation of mucosal B cells with those two different isotypes.

Egress from Inductive Sites and Migration to Effector Sites

After egressing from PP and NALT, the IgA-committed B cells migrate to mesenteric lymph nodes (MLN) and cervical lymph nodes (CLN), respectively. Accumulating evidence suggests that the egress of B cells and T cells from PP is restricted by sphingosine-1-phosphate receptor type 1 (S1P1) (Fig. **4**) [78, 79]. Thus, treatment of mice with FTY720, an agonist of S1P1, induced dramatic reduction in S1P1 expression, inhibiting lymphocyte egress from PPs and resulting in the accumulation of B and T cells in PPs.

The IgA-committed B cells migrate preferentially into the mucosal effector tissues (e.g., the gut lamina propria and the nasal passage) through the thoracic duct and blood circulation operated by the CMIS (Figs. **2** and **4**). Several lines of evidence suggest that the trafficking of IgA-committed B cells to the gut lamina propria is facilitated by changes in the expression of adhesion molecules and chemokine receptors. Changes in adhesive molecules include α4β7 integrin [80, 81], which is used to MAdCAM-1-mediated traffic into the intestinal effector site [17]. CCR9, a later chemokine receptor is selectively expressed on IgA-, but not IgM- or IgG-, committed B cells and their ligand, CCL25 (also known as thymus-expressed chemokine TECK) is produced predominantly by the intestinal epithelium [82]. These combinations of homing receptors and chemokine receptors conclude the selective homing

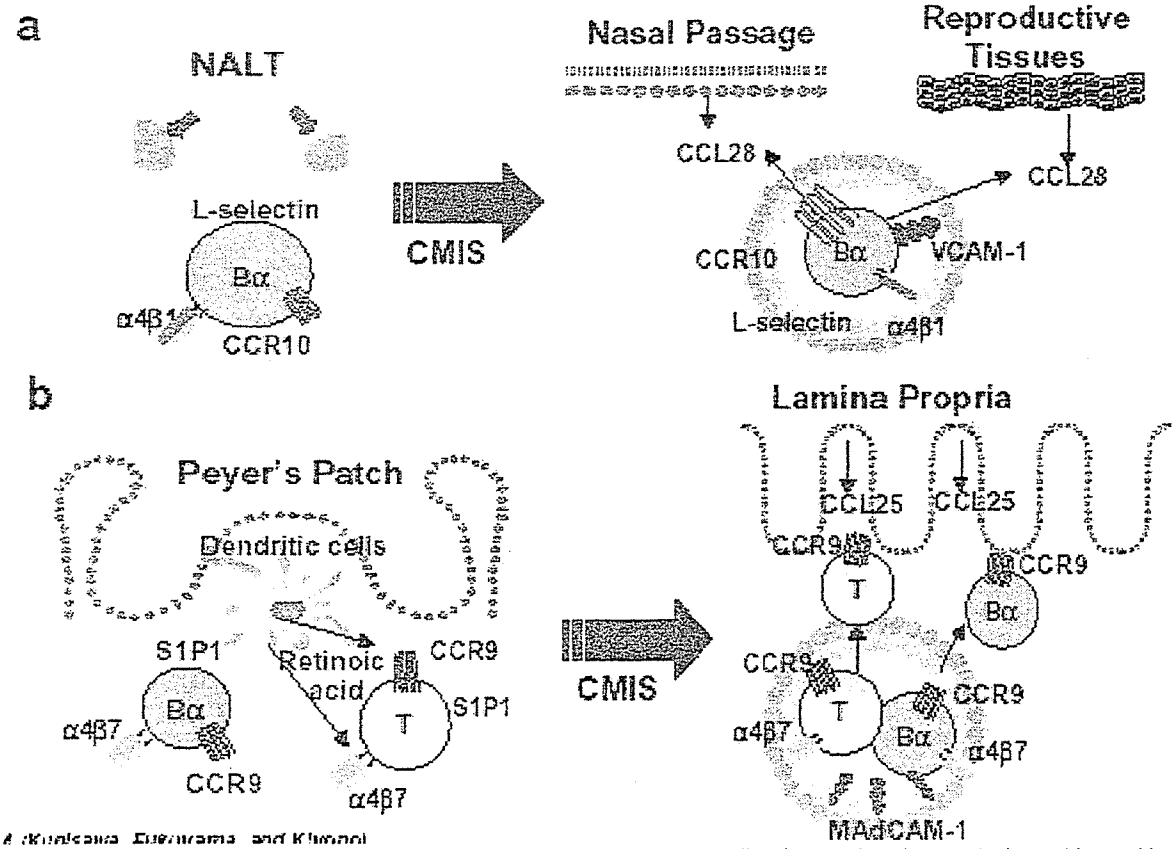

Figure 4. A model of mucosal trafficking of lymphocytes mediated by adhesion molecules and chemokines. Homing of lymphocytes into mucosal effector sites requires and is operated by several adhesion molecules and chemokines. (a) IgA-committed B (Bα) cells developed in NALT express α4β1 integrin and CCR10. Through CMIS, these IgA-committed B cells migrate into nasal passages and reproductive tissues (e.g., vagina) by the interaction of α4β1 integrin/VCAM-1 and CCR10/CCL28. (b) In the case of PP, S1P1 is essential for the egress of T cells and IgA-committed B cells. Retinoic acid produced by DCs induces the expression of α4β7 and CCR9 on antigen-primed T cells. MAdCAM-1$^+$ HEV in intestinal lamina propria substantially contribute to the homing of α4β7$^+$ IgA-committed B cells and T cells from PP. Furthermore, tissue-specific production of CCL25 by intestinal epithelial cells involves the gut-homing specificity of CCR9$^+$ IgA-committed B cells and T cells to the lamina propria region of the intestinal tract.

of IgA^+ B cells from the inductive site into the intestinal lamina propria.

Like IgA-committed B cells in GALT, the preferential migration of T cells activated at intestinal inductive tissues (e.g., PP and MLN) to the lamina propria, an intestinal effector site, is mediated by $\alpha4\beta7$ integrin and CCR9 expression [83, 84]. Several studies have demonstrated that only PP- and MLN-derived, but not spleen-derived, DCs are capable of determining the gut tropism of intestinal T cells by the induction of high levels of $\alpha4\beta7$ integrin and CCR9 expression, resulting in the selective migration to the intestinal lamina propria [85-87]. In this context, a recent study has identified that retinoic acid is dominantly expressed by mucosal DCs determining the mucosal T cell tropism [88]. Thus, mucosal T cells educated by orally inoculated antigen presented by mucosal DCs tend to migrate into distant intestinal effector sites by obtaining the mucosal trafficking molecules (e.g., $\alpha4\beta7$ integrin and CCR9). In this regard, it would be interesting to examine whether the selective homing of intestinal B cells is also operated by intestinal DCs.

It is already known that a similar pathway composed of adhesion molecules and chemokines regulates a restricted distribution of B cells to the upper respiratory tract, especially the nasal cavity. It was previously reported that nasal immunization induced up-regulation of $\alpha4\beta1$ integrin, but not $\alpha4\beta7$ integrin, and CCR10, but not CCR9 expression, allowing the selective trafficking of B cells to nasal passage epithelium expressing their ligands, VCAM1 and CCL28, respectively (Fig. **4**) [89, 90]. It is interesting to note that the same molecules are involved in the trafficking of NALT- stimulated B cells to the genito-urinary tract (Fig. **4**) [91], which may explain why high levels of antigen-specific immune responses are induced in the genital tract after nasal immunization [92, 93]. Additionally, L-selectin expression on B lymphocytes was observed to be elevated after nasal but not oral immunization [94]. Conversely, B cells primed at systemic sites express only very low levels of $\alpha4\beta7$ integrin but high levels of $\alpha4\beta1$ integrin and L-selectin [30], resulting in the up-regulation of responses to the CXCR3 ligands which originate from inflammatory sites. Taken together, the complex interactions between chemokines and tissue-specific adhesion molecules determine the mucosal T and B cell tropism from the inductive tissues to the effector tissues.

IgA Production into Lumen as a Missile Molecule Against Microorganisms

When IgA-committed B cells migrate into effector sites, they are influenced by IgA-enhancing cytokines such as IL-5 and IL-6 to differentiate into IgA plasma cells (Fig. **2**) [95, 96]. To achieve the transport of IgA across the epithelial layer, the mucosal immune system selectively formulates secretory IgA (S-IgA). In order to form S-IgA antibodies, the J-chain and poly Ig receptor (pIgR) are essential. Produced by B cells, the J chain is a small polypeptide that regulates dimer/polymer formation of IgA and IgM, but not of other types of Ig [97]. pIgR is expressed on the basal membrane of ECs [98]. Mucosal dimeric or polymeric IgA containing J-chain shows a high affinity for the basolaterally expressed epithelial pIgR, thereby accelerating the internalization and transport of the complex to the apical site via transcytosis [99]. In both J-chain knockout and pIgR knockout mice, serum IgA levels rose while fecal IgA levels fell [100, 101]. In order to induce S-IgA at the diffused effector site, a mucosal internet must be formed through the cooperation of three types of cells: 1) dimeric/polymeric IgA-committed B cells originating in the inductive tissues, 2) Th2-type cells producing IgA-enhancing cytokines (IL-5 and IL-6) and 3) pIgR-expressing ECs. These cells must collaborate if S-IgA is to reach the lumen side of the mucosa where it can act as a first line of defense against invading pathogens.

CTL Plays a Pivotal Role in the Protection of Mucosal Sites

Although there is no doubt that S-IgA plays a pivotal role in protecting the mucosal surface from aerodigestive tract infection, the mucosal immune system is equipped with multiple layers of protective immunity. A sizeable body of evidence suggests that mucosal T cells harbor cytolytic activity and thus are capable of killing cells infected with virus or bacteria [102-104]. Like IgA-producing B cells, most of these mucosal T cells are thought to be derived from the CMIS-dependent induction pathway. Peripheral CTLs mainly recognize cytoplasmic antigen presented by MHC class I molecules following cytoplasmic antigen processing [105, 106]. Like the peripheral CTLs, mucosal CTLs in the inductive and effector sites are largely composed of $TCR\alpha\beta^+$ and $CD8^+$ lymphocytes and recognize the antigen derived from the cytosolic antigen as a complex with MHC class I molecules. Interaction with mucosal DCs at the inductive tissues (e.g., NALT and PPs) induces the clonal expansion of antigen-specific T cells and also determines a T cell tropism by the induction of adhesion molecules and chemokine receptors ($\alpha4\beta7$ integrin and CCR9) that allow for a selective migration to mucosal effector sites (e.g., intestinal lamina propria and the nasal passage) (Figs. **2** and **4**) [85-88]. Once mucosal $CD8^+$ T cells have encountered the specific antigen, they express pore-forming protein, perforin, and cytolytic granules containing granzyme proteases and so become capable of cytotoxic activity against pathogenic target cells [107].

CMIS-INDEPENDENT INDUCTION PATHWAY

It has been generally accepted that CMIS is a major pathway for the induction of antigen-specific mucosal immune responses. However, because antigen-specific immune responses have been

induced in PP- and/or ILF-null mice following oral immunization [9, 49, 108], the existence of alternate pathways has been proposed. Indeed, a number of those pathways have been identified, especially in the gastrointestinal tract, where multi-layers of IgA-mediated immunity provide a first line of defense against invading pathogens.

Alternative Gateways for Antigen Sampling from the Intestinal Lumen

As mentioned above, FAE-associated M cells at inductive tissues are thought to be a principal gateway for the uptake of antigen from the lumen and for the initiation of Ag-specific immune responses. Thus, M cells have been thought to be always developed at and associated with the organized mucosal lymphoid tissues. However, alternative antigen-sampling pathways have been reported. At least three different scenarios have been offered regarding this alternative antigen-encountering pathway (Fig. **2**). First, we have recently identified M cells on intestinal villous epithelium (villous M cells) not in proximity to PP [109]. Intestinal villous M cells are developed in various PP-/ILF-null mice and are capable of taking up bacterial antigens. The discovery of villous M cells has not only shed light on a novel gateway for antigen uptake into the intestine but has also suggested the possibility of a previously unsuspected route of pathogenic invasion.

Secondly, a recent study identified a unique DC population among intestinal ECs [110]. These intraepithelial DCs express tight junction-associated proteins (e.g., occuludin, claudin 1 and zona occuludens 1) and thus are capable of extending their dendritical arm between ECs. A previous study had already demonstrated that CD18-expressing phagocytes were involved in an M cell-independent pathway for bacterial invasion [111]. By protruding dendrites into the lumen, mucosal DCs located between ECs take up gut antigens and then present them to T and/or B cells.

The third pathway for antigen uptake is EC itself. There is evidence to suggest that ECs could process and then present antigens to T cells via MHC class I as well as class II molecules [112]. In addition to sampling a wide variety of foreign antigens, the mucosal immune system must contend with the high number of apoptotic ECs that result from the frequency with which the epithelium is replaced. Although most of these apoptotic ECs are ceded by the epithelium to the lumen, some of these apoptotic ECs have been shown to be potentially immunogenic and transportable to T cell areas of MLNs by mucosal DCs [113, 114]. These findings suggest that the intestinal immune system is equipped with at least two distinct gateways, one of which is located in the organized compartment and the other in the diffused compartments which serve as a portal to outside environments. Thus, M cells developed in the FAE of PP and ILF belong to the former gateway, while villous M cells and intraepithelial DCs are affiliated with the latter. It would be intriguing, of course, to examine how these two gateways, located in such distinctively different immunological environments (e.g., organized lymphoid structure and diffused connective tissue structure), cooperate in the induction of antigen-specific immune responses. For pathogens, the second entry site might be the more attractive for infection, since there they will not be directly exposed to organized lymphoid structures.

B1 Cells are Another Source for Mucosal IgA

Two lineages of murine B cells, B1 and B2 cells, have been identified, which can be distinguished by their cell surface markers (e.g., B220, IgM, IgD, CD5, and Mac-1), origins, and growth properties [115]. As we mentioned above, some IgA plasma cells are derived from conventional B cells (B2 cells) originating from the MALTs (e.g., PP, IEL and NALT), while B1 cells are preferentially observed in mucosal effector sites like the lamina propria and the nasal passages as well as the peritoneal and pleural cavity [116]. Our previous results demonstrated that IL-5, a well-known IgA-enhancing cytokine, supported the differentiation of both B1 and B2 cells into IgA plasma cells [116]. In contrast, mucosal EC-derived IL-15 promoted the proliferation and differentiation into IgA-producing cells of B1 but not of B2 cells [117]. Thus, treatment with anti-IL-15 antibody resulted in the severe paucity of B1 cells at effecter sites such as the intestinal lamina propria and the nasal passage, but did not affect B2 cell levels [117]. Further, it was shown that B2 but not B1 cells developed at organized inductive tissues such as PP and ILF, while both B1 and B2 cells were found in the effector tissues such as the intestinal lamina propria. Based on these findings, it seems that intestinal B1 cells migrate from non-inductive tissues, presumably in the peritoneal cavity, to the effector sites, where they further differentiate into IgA plasma cells under the influence of IL-5 and IL-15. Regarding B1 cell migration, a previous study using *aly/aly* mice that carried a point mutation in the NIK demonstrated a complete absence of B cell population in the intestinal lamina propria but elevated B cell levels in the peritoneal cavity [118]. Additionally, *aly/aly* peritoneal B cells exhibit defective *in vitro* chemotatic responses to CCL21 and CXCL13, implying that the NIK-mediated pathway is involved in B1 cell mucosal migration, which might be operated by specific but not yet identified chemokine receptor(s).

Not only do cell surface markers and points of origin distinguish B1 and B2 cells, but also their different antigen recognition patterns. For example, B1 cells are thought to be specialized in responding to T cell-independent antigens conserved on common pathogens like DNA and phosphatidylcholine, whereas B2 cells require activation by DCs and Th cells [119-121]. Thus, IgA production from B1 cells was noted in MHC class II-

deficient mice as well as TCR β- and δ chain-deficient mice [122, 123]. Of note, about 65% of fecal bacteria were reactive with B1-derived IgA and 30% of bacteria were bound with B2-derived IgA, indicating that S-IgA derived from B1 cells recognized a large population of commensal bacteria [124]. Since, as mentioned above, the T cell-independent IgA antibodies originating from B1 cells possess antigen reactivity to conserved bacterial products, they completely prevented the attachment of commensal bacteria. Thus, it is assumed that the specialized role of B1-derived IgA is not to protect from pathogenic bacterial invasion but rather to maintain the mucosal homeostasis by preventing the attachment of commensal bacteria. In contrast, B2 cell-originated IgA is a key protective antibody against pathogenic microorganisms.

IEL, a Unique Mucosal T Cell Population, Bridges Innate and Acquired Immunity

A unique subset of IELs also characterizes CMIS-independent mucosal immunity. IELs are located at every four to nine ECs and are mainly composed of heterogeneous groups of T cells which may be distinguished by their usage of TCR as well as CD4 and CD8 [125]. Most $CD8^+$ IELs are either TCRγδ- or TCRαβ-positive cells with homodimeric CD8αα in addition to the classical TCRαβ positive CD8αβ T cells [126]. Similar to $CD8^+$ T cells at the periphery and lamina propria, CD8αβ IELs are developed at the thymus and migrate specifically into the mucosal compartments by the selective expression of CCR9 and α4β7 integrin [127]. In contrast to the thymus-dependent CD8αβ IEL T cells, at least some populations of CD8αα IEL T cells, such as TCRγδ T cells, are thought to be thymus-independent and thus developed in gut-associated CPs [19]. The main population of CP cells displayed c-kit, IL-7R, and CD44-positive, but lineage marker (CD3, B220, Mac-1, Gr-1 and TER-119)-negative lympho-hematopoietic stem cell phenotypes [20]. Consistent with the IL-7R expression on CP lymphocytes, gut epithelium-derived IL-7 has been shown to be important in the induction of CD8αα IEL T cells and CP maturation, since $IL\text{-}7^{-/-}$ mice do not have TCRγδ IEL and CP [128, 129]. Additionally, an *in vivo* progeny study demonstrated that they can generate both TCRαβ and TCRγδ IELs without thymic influence [20, 130]. However, other studies questioned the thymus-independent nature of IELs and implied that both CD8αα TCRαβ and TCRγδ IEL originated from the thymus [131-133]. Thus, it remains a subject of debate whether IEL develops independently of the thymus. However, still considered as a key member of the GALT network, CP serves as one of the important immunological sites of development for IELs that form the mucosal intranet with ECs.

The experiments using MHC class $I^{-/-}$ mice also demonstrated that CD8αβ IELs were restricted by MHC class I, while CD8αα IELs were not [134]. Based upon these findings, it can be postulated that CD8αβ IELs exhibit a high degree and CD8αα IELs a low degree of cytotoxic activity against MHC class I-associated, non-self cytoplasmic antigen. The number of CD8αα IELs was markedly reduced in β2-microglobulin (β2m)-deficient mice, implying that non-classical MHC molecules might contribute to the antigen presentation to CD8αα IELs [135]. This hypothesis is supported by the fact that intestinal ECs express several non-classical MHC molecules including thymus leukemia antigen (TL), Qa-1, Qa-2, CD1, and MHC class I-related molecules (MICA and MICB), and IELs express their ligands like a $V\gamma1V\delta1^+$, NKG2D, and CD8αα [136]. It has been generally accepted that these responses mediated by non-classical MHC molecules are induced promptly after infection without any peptide specificity for virus or bacteria [137, 138]. IELs then seem to provide a bridge between rapid innate responses, which may be CMIS-independent, and more time-consuming acquired immune responses, which may be CMIS-dependent [139].

DEVELOPMENT OF MUCOSAL VACCINE

Because of several advantages they offer over systemic vaccination, mucosal vaccines (e.g., nasal or oral vaccine) have become in recent decades an increasingly attractive option for the prevention and control of infection by emerging and re-emerging microorganisms [140]. Perhaps because the restricted absorption system and the fluid secretions which characterize mucosal surfaces preclude the efficient uptake of antigen into MALT after oral or nasal immunization with vaccine antigen alone, oral and nasal immunization have thus far failed to induce adequate antigen-specific immune responses. One obvious means to overcome this problem would be the development of an effective antigen delivery vehicle targeted to the MALT, especially to M cells. We outline the various approaches to the development of such an antigen delivery vehicle system below.

Genetically Modified Live Microorganisms for Antigen Delivery

Historically, vaccine has been prepared from microorganisms inactivated by formaldehyde or β-propiolactone. The inactivated vaccine is capable of inducing humoral immune responses but not cell-mediated immune responses such as CTL responses, since this type of vaccine generally induces MHC class II-mediated immune responses following their processing in lysosomal compartments [141]. For CTL responses, in contrast, cytoplasmic antigen processing and subsequent MHC class I-mediated antigen presentation are requisite [105, 106].

Attenuated live microorganisms are also capable of inducing immune responses and have already been utilized for mucosal vaccines (e.g., poliovirus,

Salmonella typhi Ty21a, and *Vibrio cholerae*) [142]. As mentioned above, some microorganisms naturally express ligands for M cells which facilitate their targeted invasion of host cells including M cells [10]. However, since vaccines using live pathogens sometimes result in undesirable disease symptoms, the attenuation of those pathogens must be complete and irreversible. Recent progress in genetic technology has helped to ensure vaccine safety and enabled us to develop recombinant attenuated vaccines expressing heterologous antigen [143, 144]. In the attenuated recombinant vaccines, several genes determining pathogenicity have been mutated and a gene encoding a heterologous antigen has been inserted. Microorganisms considered as candidates for the mucosa-targeted delivery of DNA encoding heterologous vaccine antigens include non-pathogenic *Lactobacilli*, attenuated strains of *S. typhi*, *Vibrio Cholerae*, *Shigella flexneri*, *Y. enterocolitica*, and *Listeria monocytogenes* [143]. Some of these mucosal delivery systems (e.g., *Salmonella typhi* Ty21a expressing *Shigella sonnei* O antigen and *Salmonella typhi* isolate Ty2 expressing hepatitis B antigen) have already been tested in humans [145, 146]. Based upon these findings, the genetically modified microorganisms seem to have proven capable of inducing immune responses without reverting back to a more virulent form capable of triggering disease symptoms, even in the hostile circumstances of the mucosal lumen. They are able to bind to the apical membrane of M cells using the innate ligand for M cells, thereby inducing humoral and cell-mediated immunity without any undesirable side effects. Such a mucosa-targeted vaccine delivery system is not limited to DNA, but can, in a bacteria-based form, also deliver peptide or protein antigen [147]. In such a bacteria-based system, intrinsic secretion systems (e.g., type III secretion system of *Yersinia* and *Salmonella*, and α-hemolysin secretion system of *E. coli*) are used to deliver the antigen into the cytoplasm to induce MHC class I-restricted antigen presentation, eventually leading to antigen-specific CTL induction.

Application of an Inert Synthetic Delivery System and a Hybrid Delivery System to Mucosal Vaccines

A variety of delivery systems have been developed using inert mucosal antigens including lipid-based particles such as liposomes and ISCOMs, as well as biodegradable polymer-based particles [148]. Encapsulation of antigens within particles is widely used to protect them from degradation in the harsh environment of the gastrointestinal lumen. Evidence suggests that several physical factors of the synthetic particles (e.g., size, hydrophobicity, and surface charge) determine the efficiency of the selective delivery of the encapsulated antigen to M cells [149]. Mucosal antigen delivery could be optimized if those particle features were modified by the addition of chemical or biological bioadhesins. For example, chemical mucoadhesive molecules (e.g., carboxy vinyl polymer) were used to elongate particles containing protein antigen, thereby improving their durability in the intestinal lumen [150]. Appropriate lectins, microbial and viral adhesions, and immuloglobulins have also been widely exploited to gain or enhance access to M cells [151]. Intestinal ECs possess a cell surface glycocalyx composed of membrane-anchored glycoconjugates. Several studies have demonstrated that Ulex europaeus 1 (UEA1), a lectin specific for α-L-fucose residues, binds almost exclusively to the apical surface of M cells of murine PPs [152, 153]. In these studies, the unique reactivity of UEA1 to M cells allowed for the selective delivery of microparticles to M cells after oral administration, leading to the successful induction of mucosal and systemic immune responses [154, 155].

Because a diverse range of microorganisms express a ligand for M cells, as noted above, another approach using bioadhesins is to apply microbial adhesins to mediate M cell binding for the targeted delivery of synthetic particles. As expected given the selectivity of ligands, enhanced antigen uptake was attained by coating polystyrene nanoparticles with *Yersinia*-derived invasin, a ligand for β1 integrins on the apical site of M cells [58, 156]. Viral as well as bacterial products can be utilized. Reovirus has been known to invade through M cells using 45-kDa viral haemagglutinin σ1 protein [157]. It was recently demonstrated that mucosal immune responses were significantly increased by coupling a plasmid DNA encoding HIV gp160 with a reovirus-derived σ1 protein capable of targeting M cells [158, 159]. We have also developed a hybrid antigen delivery vehicle using fusogenic liposome, which is composed of synthetic liposome and Sendai virus (also known as a haemagglutinating virus of Japan) [160]. The nasal administration of protein antigen via fusogenic liposomes resulted in its preferential delivery to NALT ECs, including M cells [161]. After nasal administration of fusogenic liposomes containing HIV glycoprotein 160 (gp160), equally striking results were seen, with high levels of gp160-specific serum IgG and mucosal IgA induced in saliva, fecal extracts and nasal and vaginal washes [93]. Also, because nasally administered fusogenic liposome delivers the antigen directly into the cytoplasm, antigen-specific CTL responses are also induced [93, 161, 162]. In addition to fusogenic liposomes, several other virus-like particles (e.g., hepatitis E virus and Sindbis virus) have been developed for nasal and oral vaccination [163, 164]. In addition to such viral systems, bacterial ghosts composed of intact bacterial envelopes may represent another non-living carrier system for mucosal vaccines [165]. By using hybrid delivery systems and bacterial ghosts, antigen can be selectively delivered to M cells without running the risk of the complications posed by live microbial and viral delivery systems. Because it is both effective and safe, the hybrid delivery system could mark an important step towards a feasible mucosal vaccine.

CONCLUDING REMARKS

This review has been aimed at summarizing the unique anatomical, developmental and immunological aspects of MALT, especially those of NALT, BALT, and GALT in the aerodigestive tracts. MALT serves as multi-functional immunological sites for the initiation of the molecular and cellular regulation of mucosal B cell- and T cell-mediated S-IgA and cell-mediated immunity as a first line of defense against invading pathogens. Nasal and/or oral immunization targeted to M cells in the aerodigestive tract induce antigen-specific IgA antibody and/or CTL responses at mucosal surfaces. Although mucosal immunization presents many advantages over systemic immunization, it does not effectively induce protective immunity when the protein antigen alone is delivered via the nasal and/or intestinal mucosa. To fully profit from the potential of the mucosal immune system, nasal and/or oral vaccinations require the co-administration of a mucosal adjuvant or the use of mucosal antigen delivery vehicles. Accordingly, much research has been focused on developing safe and effective mucosal adjuvants and vaccine delivery systems.

Recently, it was learned that PPs and NALT have distinct organogenesis programs. PP genesis requires the IL-7R- and LTβR-mediated signaling cascades and occurs in the prenatal period, while NALT genesis is independent of the IL-7R/LTβR signaling cascades and occurs postnatally. This discovery of two distinct organogenesis programs for PP and NALT could have important ramifications for our understanding of the mucosal immune system. In experiments using MALT-deficient mice (e.g., PP-null mouse), key molecules regulating organogenesis were disrupted, revealing PP-dependent and -independent mucosal induction pathways for the induction of antigen-specific immunity. These surprising observations challenge our conception of the mucosal immune system, revealing that it is more complex and nuanced than our current schemas suggest. A more profound and comprehensive molecular and cellular understanding of the mucosal immune system and mucosal organogenesis could facilitate the design of mucosal immune therapies and mucosal vaccines, thereby proving a real boon to public health.

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LIST OF ABBREVIATIONS

AID	=	Activation-induced cystidine deaminase
APC	=	Antigen- presenting cell
BALT	=	Bronchus-associated lymphoid tissue
CLN	=	Cervical lymph node
CMIS	=	Common mucosal immune system
CP	=	Cryptopatch
CSR	=	Class switch recombination
CTL	=	Cytotoxic T lymphocyte
DC	=	Dendritic cell
EC	=	Epithelial cell
FAE	=	Follicle-associated epithelium
GALT	=	Gut-associated lymphoid tissue
GC	=	Germinal center
HEV	=	High endothelial venule
ICOS	=	Inducible co-stimulator
ID2	=	Inhibitor of DNA binding/ differentiation 2
IEL	=	Intraepithelial lymphocyte
ILF	=	Isolate lymphoid follicle
LN	=	Lymph node
LT	=	Lymphotoxin
MAdCAM-1	=	Mucosal vascular addressin cell adhesion molecule 1
MALT	=	Mucosa-associated lymphoid tissues
MHC	=	Major histocompatibility complex
MLN	=	Mesenteric lymph node
NALT	=	Nasopharynx-associated lymphoid Tissue
NIK	=	Nf-κB-inducing kinase
PNAd	=	Peripheral node addressin
pIgR	=	Poly Ig receptor
PP	=	Peyer's patch
RORγ	=	Retinoic acid-related orphan receptor γ
SCID	=	Severe combined immunodeficient
SED	=	Subepithelial dome
S-IgA	=	Secretory IgA
S1P1	=	Sphingosine-1-phosphate receptor type 1
TCR	=	T cell receptor
Th	=	Helper T cells
TNF	=	Tumor necrosis factor
UEA-1	=	Ulex europaeus 1

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