of the tight-junction proteins claudin-1 or claudin-2 (fig. S5, A to D), nor did they exhibit histological signs of intestinal inflammation (fig. S5E). Further, the mLNs of control and anti-CD90.2 mAb-treated mice contained equivalent frequencies of macrophages and dendritic cells, whereas anti-CD90.2 mAb-treated mice contained significantly higher frequencies of neutrophils in the mLNs (fig. S5, F to H), suggesting that depletion of ILCs does not result in a global impairment of intestinal epithelial barrier integrity and that disseminating bacteria may not originate from the intestinal lumen.

Metabolic profiling (*22*) of bacterial colonies from the liver or spleen of anti-CD90.2 mAb-treated $Rag1^{-/-}$ mice identified that the disseminating bacteria were *Alcaligenes* spp. (fig. S6A), a genus of Gram-negative bacteria that reside within the Peyer's patches (PPs) and mLNs of healthy humans, nonhuman primates, and mice (*4*, *23*). 16S-directed polymerase chain reaction confirmed the presence of *Alcaligenes* spp. in liver and spleen from anti-CD90.2 mAb-treated, but not isotype-treated, $Rag1^{-/-}$ mice (fig. S6B), and pyrosequencing of 16S recombinant DNA tags demonstrated that samples from the intestinal lumen of untreated $Rag1^{-/-}$ mice contained multiple phylogenetic groups of commensal bacteria, whereas cultures from the liver and spleen of ILC-depleted $Rag1^{-/-}$ mice exhibited a homogeneous population of *Alcaligenaceae* (Fig. 3A). Analysis of these sequences identified the species as *Alcaligenes xylosoxidians* (also referred to as *Achromobacter xylosoxidans*). To interrogate the origins of the *Alcaligenes* spp., tissues from naïve mice were analyzed by fluorescent in situ hybridization (FISH) using *Alcaligenes*-specific probes. Consistent with a previous report (*4*), we found *Alcaligenes* spp. in the interior of PPs and mLNs of healthy mice (Fig. 3, B and C, and fig. S7). Collectively, these results indicate that the loss of ILCs results in selective dissemination of lymphoid-resident *Alcaligenes* spp. to peripheral tissues.

To determine whether *Alcaligenes* spp. were sufficient to promote inflammation, *Alcaligenes* was administered systemically to $Rag1^{-/-}$ mice. In comparison to isotype-treated mice, both anti-CD90.2 mAb-treated $Rag1^{-/-}$ mice and $Rag1^{-/-}$ mice that received systemic *Alcaligenes* spp. exhibited significantly increased hepatic LPS and systemic inflammation (fig. S8, A to F). Furthermore, whereas germ-free $Rag1^{-/-}$ mice exhibited no increases in hepatic LPS or systemic inflammation after administration of anti-CD90.2 mAb, germ-free $Rag1^{-/-}$ mice that were monoassociated with *Alcaligenes* and treated with anti-CD90.2 mAb exhibited increased hepatic LPS, increased spleen weight, and elevated levels of serum IL-6 and TNF-α compared with isotype-treated monoassociated mice (Fig. 3, D to G).

To examine whether decreased expression of IL-22–regulated antimicrobial peptides (Fig. 2, H to K) affects *Alcaligenes*, we added rS100A8/S100A9 (calprotectin) (*24*) to cultures and found that it inhibits the growth of *Alcaligenes* and limits colony formation in a dose-dependent manner (Fig. 3H and fig. S9). Furthermore, delivery of rS100A8/S100A9 in vivo significantly reduced burdens of *Alcaligenes* in the spleen and liver of anti-CD90.2 mAb-treated $Rag1^{-/-}$ mice (Fig. 3, I and J). Collectively, these results suggest that in healthy mice, ILCs promote anatomical containment of *Alcaligenes* spp., in part through promoting expression of calprotectin to limit disruption of systemic immune homeostasis.

To test whether ILCs prevent dissemination of *Alcaligenes* in lymphocyte-replete mice, we generated CD90-disparate $Rag1^{-/-}$ chimeric mice that permit the selective depletion of $CD90.2^{+}$ ILCs without depleting $CD90.1^{+}$ lymphocytes (fig. S10A) (*13*). Administration of anti-CD90.2 mAb to CD90-disparate $Rag1^{-/-}$ chimeric mice resulted in peripheral dissemination of *Alcaligenes* to the spleen and liver at day 3 postdepletion (Fig. 4, A and B, and fig. S10B). Chimeric mice exhibited elevated levels of hepatic LPS and inflammation, increased spleen size, and elevated levels of serum IL-6 and TNF-α at days 3, 14, and 28 postdepletion (Fig. 4, C to G), as well as significantly higher frequencies of splenic $Ki\text{-}67^{+}$ $CD4^{+}$ T cells, $Ki\text{-}67^{+}$ $CD8^{+}$ T cells, and $Ki\text{-}67^{+}$ $CD19^{+}$ B cells (fig. S10, C to E). Splenocyte cultures were restimulated with *Alcaligenes*-derived antigens, and significantly higher frequencies of $IL\text{-}6^{+}$ $CD4^{+}$ T cells and $TNF\text{-}\alpha^{+}$ $CD4^{+}$ T cells were observed in ILC-depleted chimeric mice (fig. S10F). Anti-CD90.2 mAb-treated chimeric mice also exhibited significantly elevated serum immunoglobulin G (IgG) responses specific for *Alcaligenes*-derived antigens, but not luminal-resident *Escherichia coli*–derived antigens (fig. S10G) or opportunistic viruses (table S1). The inability to culture *Alcaligenes* at days 14 and 28 was associated with the development of systemic IgG specific for *Alcaligenes* spp. (Fig. 4H), indicating that despite persistent systemic

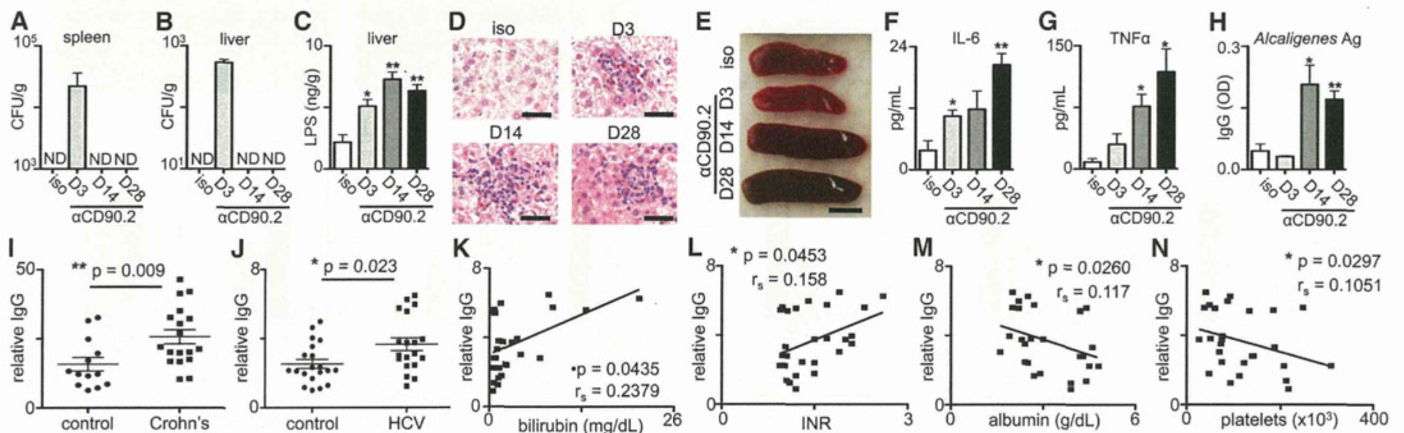

Fig. 4. ILCs regulate anatomical containment of *Alcaligenes* in lymphocyte-replete hosts, and *Alcaligenes*-specific responses are associated with chronic human disease. (**A** to **H**) Naïve CD90-disparate chimeric mice were administered an isotype control or anti-CD90.2 mAb starting on day 0 and were sacrificed on day 3, 14, or 28. CFUs present in homogenates from the (A) spleen and (B) liver of antibody-treated chimeric mice. (C) LPS concentrations in homogenates from the liver of antibody-treated mice. (D) H&E-stained histological sections of the liver of antibody-treated chimeric mice. Scale bar, 5 μm. (E) Spleen size from antibody-treated chimeric mice. Scale bars, 5 mm. Serum concentrations of (F) IL-6 and (G) TNF-α from antibody-treated chimeric mice. (H) Relative optical density (OD) values of serum IgG specific to *Alcaligenes* crude antigens in treated chimeric mice. All data are representative of three independent experiments with a minimum of three to five mice per group. Statistics compare days postdepletion versus isotype using the Student's *t* test. (**I** to **N**) Relative serum IgG (OD values normalized to total serum IgG) specific to *Alcaligenes* crude antigens in (I) control (n = 13) versus pediatric Crohn's disease patients (n = 18) or (J) control (n = 20) versus cirrhotic HCV-infected patients awaiting orthotopic liver transplantation (n = 19). Statistics compare disease status using the Mann-Whitney test. Relative serum IgG specific for *Alcaligenes* crude antigen in chronically HCV-infected individuals (n = 27) was correlated with levels of serum (K) bilirubin, (L) INR of prothrombin time, (M) albumin, and (N) platelets. The association between *Alcaligenes*-specific IgG levels and clinical parameters was compared by nonparametric Spearman's rank correlation coefficient (r_s).

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inflammation, the adaptive immune system can limit the presence of live bacteria in the periphery. Collectively, these data suggest that ILCs are essential to promote anatomical containment of *Alcaligenes* to lymphoid tissues and limit the induction of systemic inflammation in lymphocyte-replete hosts.

Loss of containment of commensal bacteria and chronic systemic inflammation is associated with several chronic human diseases (*6*–*8*). To determine whether these diseases were also associated with a loss of containment of *Alcaligenes* spp., we analyzed serum samples from cohorts of pediatric Crohn's disease patients or chronically hepatitis C virus (HCV)–infected adults for the presence of *Alcaligenes*-specific IgG. In comparison to age-matched controls, serum from pediatric Crohn's disease patients and plasma from cirrhotic HCV-infected individuals awaiting liver transplantation exhibited significantly elevated levels of relative IgG specific for *Alcaligenes* spp. (Fig. 4, I and J). Although further analysis of HCV-infected individuals with and without cirrhosis demonstrated no correlations between *Alcaligenes*-specific IgG levels and patient age or serum alanine transaminase (fig. S11, A and B), there were significant correlations between plasma levels of *Alcaligenes*-specific IgG and laboratory measures of liver disease, including increased serum bilirubin and international normalized ratio (INR) of prothrombin time as well as decreased serum albumin and platelets (Fig. 4, K to N).

Mammals have evolved multiple immunologic and physiologic mechanisms to promote the anatomical containment of commensal bacteria to intestinal sites, including promoting physical barriers (via epithelial cell tight junctions), biochemical barriers (via production of mucus layers and antimicrobial peptides), and immunologic barriers (via IgA-mediated immune exclusion; intraepithelial lymphocytes; and innate pathways involving phagocytosis, Toll-like receptor–mediated sensing, and oxidative bursts) (*1*, *2*, *18*, *19*, *25*). The demonstration that depletion of ILCs results in the selective dissemination and survival of *Alcaligenes* spp. in peripheral tissues of mice indicates that, in addition to established pathways that nonselectively maintain intestinal barrier function, more discriminatory processes may have evolved to promote the selective anatomical containment of phylogenetically defined communities of lymphoid-resident commensal bacteria (fig. S12). It is notable that *Alcaligenes* spp. has recently been identified as a dominant lymphoid-resident commensal species colonizing the PPs and mLNs of mammals (*4*). Moreover, peripheral dissemination of *Alcaligenes* spp. has been reported in patients with HIV infection, cancer, and cystic fibrosis (*26*–*29*). The identification of a pathway through which IL-22–producing ILCs can prevent dissemination of lymphoid-resident *Alcaligenes* spp. and limit systemic inflammation highlights the selectivity of immune-mediated containment of defined commensal bacterial species and could offer therapeutic strategies to limit inflammation associated with multiple debilitating chronic human diseases.

References and Notes

1. D. A. Hill, D. Artis, *Annu. Rev. Immunol.* **28**, 623 (2010).
2. L. V. Hooper, A. J. Macpherson, *Nat. Rev. Immunol.* **10**, 159 (2010).
3. R. E. Ley, D. A. Peterson, J. I. Gordon, *Cell* **124**, 837 (2006).
4. T. Obata *et al.*, *Proc. Natl. Acad. Sci. U.S.A.* **107**, 7419 (2010).
5. A. J. Macpherson, T. Uhr, *Science* **303**, 1662 (2004).
6. J. M. Brenchley, D. C. Douek, *Annu. Rev. Immunol.* **30**, 149 (2012).
7. N. G. Sandler *et al.*, *Gastroenterology* **141**, 1220, 1230, e1 (2011).
8. M. A. McGuckin, R. Eri, L. A. Simms, T. H. Florin, G. Radford-Smith, *Inflamm. Bowel Dis.* **15**, 100 (2009).
9. D. Lescut *et al.*, *Gastroenterol. Clin. Biol.* **14**, 811 (1990).
10. A. Parlesak, C. Schäfer, T. Schütz, J. C. Bode, C. Bode, *J. Hepatol.* **32**, 742 (2000).
11. G. F. Sonnenberg, L. A. Fouser, D. Artis, *Nat. Immunol.* **12**, 383 (2011).
12. W. Ouyang, J. K. Kolls, Y. Zheng, *Immunity* **28**, 454 (2008).
13. G. F. Sonnenberg, L. A. Monticelli, M. M. Elloso, L. A. Fouser, D. Artis, *Immunity* **34**, 122 (2011).
14. H. Spits, T. Cupedo, *Annu. Rev. Immunol.* **30**, 647 (2012).
15. D. A. Hill *et al.*, *Nat. Med.* **18**, 538 (2012).
16. Y. Zheng *et al.*, *Nat. Med.* **14**, 282 (2008).
17. S. J. Aujla *et al.*, *Nat. Med.* **14**, 275 (2008).
18. S. Vaishnava *et al.*, *Science* **334**, 255 (2011).
19. E. Slack *et al.*, *Science* **325**, 617 (2009).
20. A. J. Macpherson, N. L. Harris, *Nat. Rev. Immunol.* **4**, 478 (2004).
21. K. S. Bergstrom *et al.*, *PLoS Pathog.* **6**, e1000902 (2010).
22. G. Funke, D. Monnet, C. deBernardis, A. von Graevenitz, J. Freney, *J. Clin. Microbiol.* **36**, 1948 (1998).
23. H. J. Busse, A. Stolz, in *Prokaryotes*, M. E. A. Dworkin, Ed. (Springer, New York, 2006), pp. 675–700.
24. C. Ryckman *et al.*, *J. Immunol.* **169**, 3307 (2002).
25. R. E. Ley, C. A. Lozupone, M. Hamady, R. Knight, J. I. Gordon, *Nat. Rev. Microbiol.* **6**, 776 (2008).
26. G. Aisenberg, K. V. Rolston, A. Safdar, *Cancer* **101**, 2134 (2004).
27. F. Espinoza-Gómez, O. A. Newton-Sánchez, V. Melnikov, O. Virgen-González, J. Unrau, *Braz. J. Infect. Dis.* **11**, 603 (2007).
28. J. M. Duggan, S. J. Goldstein, C. E. Chenoweth, C. A. Kauffman, S. F. Bradley, *Clin. Infect. Dis.* **23**, 569 (1996).
29. L. Liu *et al.*, *J. Clin. Microbiol.* **40**, 1210 (2002).

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Supplementary Materials

www.sciencemag.org/cgi/content/full/336/6086/1321/DC1
Materials and Methods
Figs. S1 to S12
Tables S1 and S2
Reference (*30*)

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Regulated Virulence Controls the Ability of a Pathogen to Compete with the Gut Microbiota

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The virulence mechanisms that allow pathogens to colonize the intestine remain unclear. Here, we show that germ-free animals are unable to eradicate *Citrobacter rodentium*, a model for human infections with attaching and effacing bacteria. Early in infection, virulence genes were expressed and required for pathogen growth in conventionally raised mice but not germ-free mice. Virulence gene expression was down-regulated during the late phase of infection, which led to relocation of the pathogen to the intestinal lumen where it was outcompeted by commensals. The ability of commensals to outcompete *C. rodentium* was determined, at least in part, by the capacity of the pathogen and commensals to grow on structurally similar carbohydrates. Thus, pathogen colonization is controlled by bacterial virulence and through competition with metabolically related commensals.

Enterohemorrhagic *Escherichia coli* (EHEC) and enteropathogenic *E. coli* (EPEC) are important causes of diarrhea and mortality worldwide (*1*, *2*). These Gram-negative bacteria attach to and colonize the intestinal tract by inducing attaching and effacing (AE) lesions on the intestinal epithelium (*1*, *2*). The genomes of AE pathogens harbor the locus of enterocyte

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EXPERT REVIEWS

Novel vaccine development strategies for inducing mucosal immunity

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To develop protective immune responses against mucosal pathogens, the delivery route and adjuvants for vaccination are important. The host, however, strives to maintain mucosal homeostasis by responding to mucosal antigens with tolerance, instead of immune activation. Thus, induction of mucosal immunity through vaccination is a rather difficult task, and potent mucosal adjuvants, vectors or other special delivery systems are often used, especially in the elderly. By taking advantage of the common mucosal immune system, the targeting of mucosal dendritic cells and microfold epithelial cells may facilitate the induction of effective mucosal immunity. Thus, novel routes of immunization and antigen delivery systems also show great potential for the development of effective and safe mucosal vaccines against various pathogens. The purpose of this review is to introduce several recent approaches to induce mucosal immunity to vaccines, with an emphasis on mucosal tissue targeting, new immunization routes and delivery systems. Defining the mechanisms of mucosal vaccines is as important as their efficacy and safety, and in this article, examples of recent approaches, which will likely accelerate progress in mucosal vaccine development, are discussed.

Keywords: delivery system • mucosal adjuvant • secretory IgA

Mucosal immune system

The mucosal immune system can be separated into inductive and effector sites based on the anatomical and functional properties. The migration of immune cells from mucosal inductive to effector tissues is the cellular basis for the common mucosal immune system (CMIS) (Figure 1). Thus, mucosal vaccination elicits immune responses in distant, multiple mucosal effector sites [1–5]. Mucosal inductive sites, including gut-associated lymphoreticular tissue (GALT) and nasopharyngeal-associated lymphoreticular tissue (NALT), collectively comprise a mucosa-associated lymphoreticular tissue (MALT) network for provision of a continuous source of memory B and T cells to mucosal effector sites [1,3–5]. The MALT contains T-cell zones, B cell-enriched areas containing a high frequency of surface IgA-positive (sIgA+) B cells and a subepithelial area with APCs for the initiation of specific immune responses. The MALT is covered by a follicle-associated epithelium that consists of a subset of differentiated microfold (M) epithelial cells, columnar epithelial cells and lymphoid cells, which play a central role in the initiation of mucosal immune responses. M cells take up antigens (Ags) from the lumen of the intestinal and nasal mucosa and transport them to the underlying APCs, including dendritic cells (DCs). In addition, recent studies have now identified isolated lymphoid follicles (ILFs) in the mouse small intestine. The ILFs have been identified as a part of GALT and as such are a mucosal inductive tissue [6,7]. These ILFs mainly contain B cells, DCs and M cells in the overlying epithelium. In addition, most recent studies showed that tear duct-associated lymphoreticular tissue (TALT) and conjunctiva-associated lymphoreticular tissue (CALT) play a role as mucosal inductive tissues [8,9]. Mucosal effector sites, including the lamina propria regions of the GI, the upper respiratory (UR), and reproductive tracts, secretory glandular tissues and intestinal intraepithelial lymphocytes, contain Ag-specific mucosal effector cells such as IgA-producing plasma cells and B and T cells.

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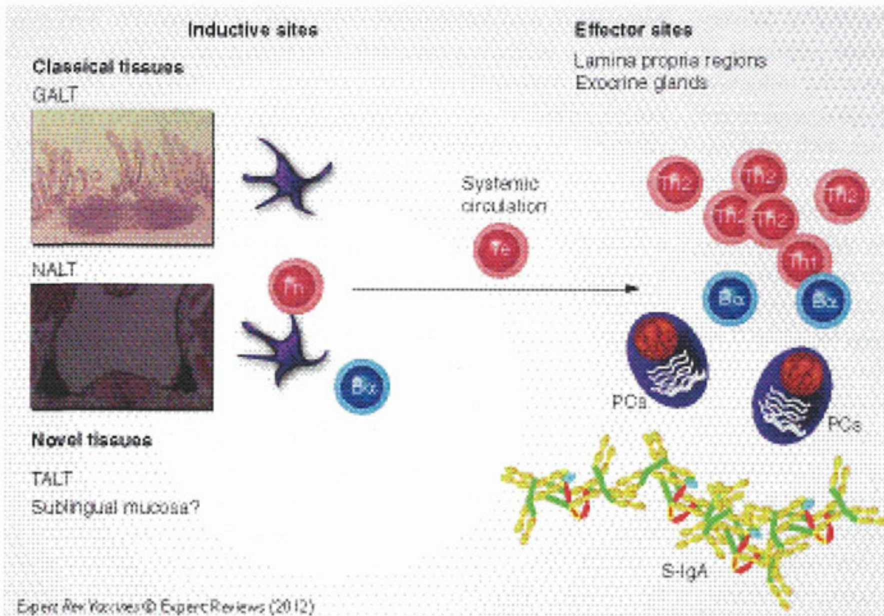

Figure 1. Concept of mucosal inductive and effector sites: when mucosal immunization is initiated, Ags are taken up by mucosal inductive tissues (GALT, NALT and TALT). This is an initial step for eliciting Ag-specific S-IgA Ab responses in mucosal effector tissues. DCs in mucosal inductive tissues play a major role as APCs for the activation of naive CD4+ T cells. In addition, ingested Ags activate IgA-committed B cells. Activated CD4+ T cells and IgA-committed B cells dispatch from mucosal inductive tissues and migrate into the mucosal effector tissues and subsequently interact for the terminal differentiation of IgA-committed B cells into IgA-producing plasma cells. In addition to the classical mucosal inductive tissues, the SL mucosa can initiate mucosal immune responses.
Bα: IgA-committed B cell; GALT: Gut-associated lymphoreticular tissue; MALT: Mucosa-associated lymphoreticular tissue; NALT: Nasopharyngeal-associated lymphoreticular tissue; PC: Plasma cell; Th1: Type 1 helper CD4+ T cell; Th2: Type 2 helper CD4+ T cell; Te: Effector CD4+ T cell; Tn: Naive CD4+ T cell.

Secretory (S)-IgA antibody (Ab) is a major player in the mucosal immune system and is locally produced in effector tissues [1,2,5,10–12]. The presence of Ag-specific S-IgA Abs at mucosal effector sites other than the inductive sites where initial Ag sampling occurred is definitive evidence for the CMIS. To this end, immunization of GALT or NALT effectively elicits Ag-specific mucosal IgA Ab responses in diverse mucosal effector tissues with some notable differences. Indeed, activated T cells in Peyer's patches (PPs) preferentially express α4β7 and CCR9 as gut-homing receptors for their migration into the intestinal lamina propria [13–16]. In this regard, mucosal addressin cell adhesion molecule-1 (MAdCAM-1), the ligand for α4β7, mediates T-cell recruitment into the intestinal endothelium [17]. Furthermore, small intestinal epithelial cells express the CCR9 ligand, thymus-expressed chemokine. Recent studies demonstrated that retinoic acid-producing DCs in PPs and the mesenteric lymph nodes (MLNs) are key players in the enhancement of α4β7 and CCR9 expression by Ag-specific effector CD4+ T cells, which in turn guides their migration into the intestinal lamina propria [18]. In addition to mucosal T-cell homing, retinoic acid-producing DCs in PPs regulate T cell-independent IgA class switching and gut-homing receptor expression on B cells [19,20]. These findings clearly show that the CMIS exhibits distinct sites for induction and regulation of S-IgA Ab responses in mucosal effector tissues.

Although it has been shown that GALT and NALT share common features, it is also clear that a compartmentalization occurs between the oral and nasal immune systems [21–23]. Thus, oral immunization mainly elicits Ag-specific immune responses in the small intestine, in the proximal part of the large intestine, mammary and salivary glands, whereas nasal immunization induces mucosal immunity in the UR tract, nasal and oral cavities, and the cervicovaginal mucosa [21–23]. Furthermore, the organogenesis, lymphocyte trafficking and progression of immunosenescence in PPs and NALT are distinctly regulated [11,13,15,24–35]. Thus, the PPs develop between embryonic days 14 and 17 in an IL-7-IL-7Rα and LTα1β2-LTβR signaling cascade-dependent manner, whereas NALT organogenesis occurs postnatally in the absence of these cytokine cascades [28,32,34,35]. Furthermore, both Id2 and retinoic acid receptor-related orphan receptor-γt transcripts are essential for PP inducer cell development; however, NALT inducer cells require only Id2 [28,36–38]. In addition, activated T and B cells in PPs preferentially express α4β7 and CCR9 as gut-homing receptors, which help guide their migration back to the intestinal lamina propria [13,15]. In contrast, CD62L, α4β1 and CCR10 preferentially control the migration of T and B cells from NALT into the UR tract effector tissues [24,25,32,33]. The compartmentalization of GI and UR tract immune systems is also evident because distinct differences in mucosal aging occurred between the GI and UR tract immune systems [26,27,29–31]. Thus, age-associated alterations, including a reduction in number of PPs and the level of intestinal Ag-specific S-IgA Abs, occur in mice during aging [26,27]. Furthermore, mice lose oral tolerance, which represents another important mucosal immune regulatory function for maintaining systemic homeostasis to orally administered Ags during the aging process (6–12 months) [26,27,30,31]. In contrast, NALT shows a more intact immune response during aging (1-year-old mice), with signs of immunosenescence noted only in mice older than 2 years [26,27,29].

Because mucosal immunization induces not only Ag-specific mucosal S-IgA Abs but also systemic IgG Abs, developing mucosal vaccines could be used in much the same way as currently available licensed parenteral vaccines. Thus, mucosal vaccine delivery can induce systemic T-cell and Ab responses in peripheral lymphoid tissue, as is seen after parenteral vaccine delivery. However, simultaneous induction of mucosal immunity provides a dual protection

against pathogens. Furthermore, mucosal adjuvants and delivery systems are essential to induce Ag-specific immune responses in both mucosal and systemic compartments by avoiding induction of systemic unresponsiveness. This review focuses on several recent approaches to induce mucosal immunity to vaccines, with emphasis on mucosal tissue targeting, new immunization routes and delivery systems that are both effective and safe. As a mucosal targeting strategy, DCs and M cells are discussed as the two major targeting cell types. Although a large number of DC-targeting components have been studied as mucosal adjuvants, CpG oligodeoxynucleotides (CpG ODN) and Flt3 ligand (FL) are selected based on their effectiveness and safety. Importantly, the cellular and molecular mechanisms for these two DC-targeting mucosal adjuvants and an M cell-targeting vaccine delivery system have been well described. In contrast, the precise mechanisms for sublingual (SL) immunization, eye drops, and rice-based and nanogel delivery systems remain to be elucidated; however, the early results are promising. In summary, these novel strategies are attractive and exhibit high potential from a practical point of view. More extensive reviews, which include additional targeting strategies, adjuvants, and delivery systems, are provided. Some specific details are essential to understand the cellular and molecular mechanisms involved in using these novel vaccine strategies.

Targeting vaccines

Mucosal DCs

DCs play a central role in bridging the innate immune system with the adaptive immune system [39–42]. Thus, DCs are found throughout the body and are especially prominent at mucosal surfaces. Immature type DCs are enriched underneath the epithelium of mucosal inductive sites and are poised to capture Ags. When Ag uptake occurs, these DCs change their phenotype by expressing higher levels of MHC class II and costimulatory molecules and move to T-cell areas of inductive sites for Ag presentation. Thus, DCs and their derived cytokines play key roles in the induction of Ag-specific effector Th-cell responses. In this regard, targeting mucosal DCs is not only an effective strategy to induce mucosal immunity but also a safe approach, especially for nasal application, because vaccines mainly initiate immune responses through DCs in the absence of central nervous system toxicity.

Because of the recent progress in the understanding of innate immunity-associated molecules, toll-like receptor (TLR) ligands are now considered to be candidates as potent mucosal adjuvants. Among these, the TLR9 ligand CpG ODN is known to target professional plasmacytoid DCs for their activation, maturation and subsequent induction of Ag-specific Th1-type responses, including cytotoxic T lymphocytes (CTLs) [43,44]. It has been demonstrated that synthetic CpG ODNs can induce innate immune responses [45–48]. In this regard, CpG ODNs as effective immunomodulators, could target malignant tumors, and reduce allergic responses [49,50]. Furthermore, CpG ODNs have been used as potent adjuvants to elicit Ag-specific Ab and cell-mediated immune responses in mice and rats against both bacterial and viral Ags [51–58]. To this end, mucosal administration of CpG ODN exhibits potent adjuvant activity (Figure 2). Mucosal immunization with CpG ODN plus formalin-inactivated influenza virus, hepatitis B virus surface Ag, or tetanus toxoid effectively elicited vaccine-specific immunity in the mucosal compartment of mice [57–59]. CpG ODN as adjuvant mainly induces Th1-type responses. In this regard, CpG ODN could even switch a predominant Th2 into a Th1-type immune response pathway [60]. Although the detailed mechanisms of adjuvant activity of CpG ODN are still unclear, it has been demonstrated that CpG ODN enhanced MAPK-mediated IL-12 production by APCs [61]. Others also clearly showed that nasal immunization with the recombinant protective Ag of the anthrax lethal toxin and CpG ODN induced protective Ag-specific plasma IgG2a and mucosal S-IgA Ab responses with *in vitro* neutralizing activities [62].

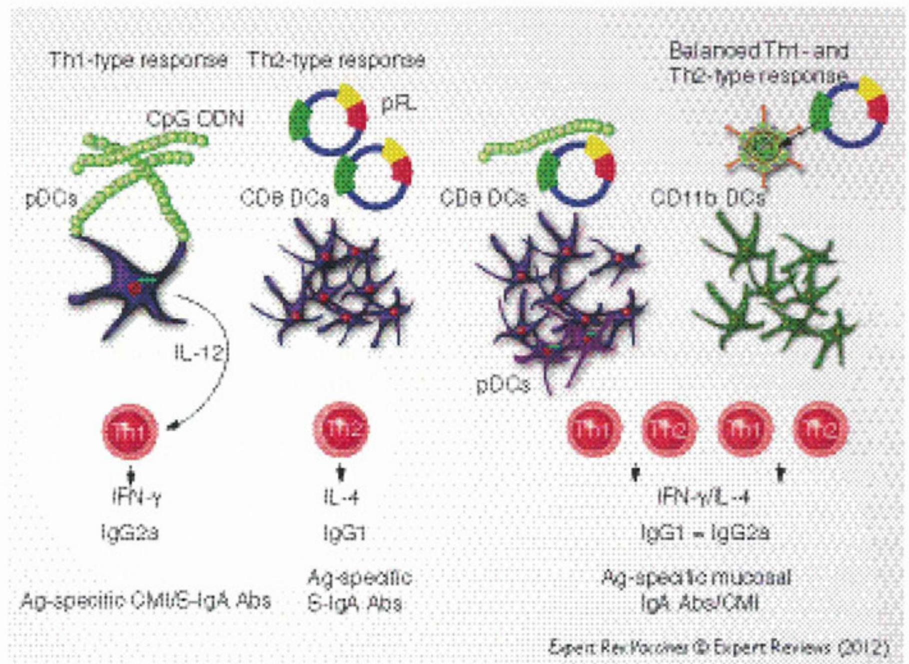

Figure 2. Nasal DC-targeting mucosal vaccines: nasal application of CpG ODN activates plasmacytoid DCs (pDC, B220⁺ DCs) for the induction of Th1-type cytokine responses. Thus, CMI and cytotoxic T lymphocyte (CTL) activity can be elicited in addition to Ag-specific S-IgA Ab responses. In contrast, pFL as nasal adjuvant preferentially expands the CD8⁺ DC subset and subsequently elicits Th2-type cytokine-mediated Ag-specific S-IgA Ab responses. Adenovirus expressing FL (Ad-FL) or a combination of CpG ODN and pFL induces a more balanced Th1- and Th2-type immune response. Ad-FL activates CD11b⁺ CD11c⁺ DCs, whereas a combined nasal CpG ODN and pFL stimulates both CD8⁺ DCs and pDCs for the induction of CMI and S-IgA Ab responses.
Abs: Antibodies; Ag: Antigen; CMI: Common mucosal immune; CPG ODN: CpG oligodeoxynucleotides; DC: Dendritic cell; pDC: Plasmacytoid dendritic cell; pFL: Plasmid-expressing Flt3 ligand; S-IgA: Surface IgA.

FL is a growth factor that binds to the fms-like tyrosine kinase receptor Flt3/Flk2. *In vivo* FL treatment markedly upregulates the number of DCs but not their activation [63,64]. Mouse FL has been cloned and shown to be a key player in the proliferation and differentiation of early hematopoietic precursor stem cells [63,65–68]. Furthermore, it has been reported that FL could mobilize and stimulate not only DCs [64] but also natural killer cells and B cells [69]. Of interest, it was first reported that systemic FL injection facilitated oral tolerance induction because of its ability to result in significant increases in the number of DCs in several lymphoid tissues, including the intestinal lamina propria, PPs, MLNs, and spleen [70,71]. In contrast to tolerance induction, others showed that FL treatment also upregulated immune responses when delivered via mucosal [71], systemic [72], or cutaneous [73] routes. It has also been reported that when plasmid DNA encoding FL (pFL) was coadministered with plasmids encoding protein Ags or linked to the Ag itself, effective immune responses were induced [74,75]. In this regard, it has been suggested that FL possesses adjuvanticity for both humoral and cell-mediated immune responses and that the FL cDNA system may be a potential alternative approach to using the FL protein system [76–79]. To this end, pFL has been used as a mucosal DC-targeting adjuvant for the induction of Ag-specific protective mucosal immune responses (Figure 2). Nasal administration of pFL as mucosal adjuvant facilitated expansion of CD8$^+$ DCs, which subsequently elicited IL-4-producing CD4$^+$ T-cell- and Ag-specific S-IgA Ab responses [80]. NALT has been the major site for sampling pFL and for producing the FL protein locally, which subsequently induced the expansion and activation of DCs [80]. In this regard, pFL did not show any potential to migrate into the CNS.

Other types of FL-based NALT-DC-targeting immune modulators, including an adenovirus serotype 5 vector expressing FL (Ad-FL), were found to elicit Th1- and Th2-type responses, thereby providing both Ag-specific S-IgA Ab and cell-mediated immune responses [81]. When mice were nasally immunized with ovalbumin (OVA) and Ad-FL, high levels of Ag-specific Ab responses were elicited in both mucosal and systemic compartments. Furthermore, significantly increased levels of Ag-specific IFN-γ and IL-4 production were noted in cervical lymph nodes and spleen [81]. Because of OVA-specific Th1-type cytokine responses, Ag-specific CTL responses were upregulated in mice administered with nasal OVA and Ad-FL. Interestingly, the number of CD11b$^+$ CD11c$^+$ DCs was preferentially increased. This DC subset expressed high levels of costimulatory molecules and migrated from the NALT to mucosal effector tissues [81]. These findings show that nasal administration of Ad-FL facilitated the induction of mature-type CD11b$^+$ CD11c$^+$ DCs and Th1- and Th2-type CD4$^+$ T cells in the NALT for Ag-specific Ab and CTL responses (Figure 2). Balanced Th1- and Th2-type responses have become key issues in mucosal vaccine development because this type of cytokine response would not only provide Ag-specific S-IgA Ab and CTL responses against viral and bacterial infections but also avoid induction of allergic (IgE) and inflammatory-type responses.

CpG ODN has been shown to induce polarized Th1-type cytokine responses in mice [62]. In contrast, pFL preferentially elicits coadministered Ag-specific Th2-type cytokine immunity [80]. To this end, one could hypothesize that an ideal but balanced Th1- and Th2-type cytokine response would be elicited by using a combination of pFL and CpG ODN as DC-targeting nasal adjuvants. Indeed, recent studies clearly showed that pFL and CpG ODN as a combined nasal adjuvant induced the activation and expansion of plasmacytoid DCs and CD8$^+$ DCs in the nasal cavity for the development of Th1- and Th2-type cytokine-producing CD4$^+$ T cells. Thus, these Ag-specific CD4$^+$ T cells successfully upregulated coadministered Ag-specific immunity in both the mucosal and systemic immune compartments (Figure 2) [82,83]. Increased frequencies of mature-type DCs in NALT correlated well with induction of Ag-specific immune responses. Of significance, nasal delivery of pFL and CpG ODN successfully elicited significant levels of Ag-specific S-IgA Ab responses in 2-year-old mice [82,83]. To this end, aged mice given nasal pneumococcal surface protein A and a combination of pFL and CpG ODN showed protective immunity against nasal *Streptococcus pneumoniae* colonization [83]. These results suggest that nasal administration of pFL and CpG ODN as mucosal adjuvants provides an attractive possibility for the development of a vaccine against *S. pneumoniae* in the elderly.

M cells

As discussed earlier, GALT, including PPs, is covered by a specialized follicle-associated epithelium, 10–20% of which is composed of M cells that show a unique topical morphology (microfold/membranous) and form pockets for the inclusion of lymphoid cells, including B and T cells, DCs, and macrophages [84–89]. M cells show significantly different features compared with intestinal epithelial cells. M cells possess relatively short microvilli, small cytoplasmic vesicles and few lysosomes. Thus, M cells are able to capture and transport lumenal Ags, including viruses, bacteria, small parasites, and microspheres [86,87,89,90]. It has been suggested that M cells may also play a role as APCs because M cells express MHC class II molecules and acidic endosomal–lysosomal compartments [91]. In this regard, activation and potential MHC class II expression by M cells may depend on the nature of endocytosed Ag. M cells serve not only for transport of lumenal Ags but also for provision of an entry way for pathogens to invade the host. In particular, it has been shown that invasive but not noninvasive strains of *Salmonella typhimurium* enter the host through PP M cells [92]. In addition to PPs, the ILFs and NALT also contain a lymphoepithelium with M cells. Thus, *Mycobacterium tuberculosis* uses NALT M cells for host entry [93]. In addition, it was reported that M cells are also detected in nonlymphoid follicle-associated epithelium that covers small intestinal villi [94]. Thus, villous M cells in the small intestine were present in several PP-deficient mouse strains, including *in utero* LT-βR-Ig-treated, LT-α$^{-/-}$, TNF/LT-α$^{-/-}$ and inhibition of differentiation 2 (Id2)$^{-/-}$ mice [94]. Importantly, these villous M cells functionally take up bacteria and induce bacterial Ag-specific immune responses [94]. Indeed,

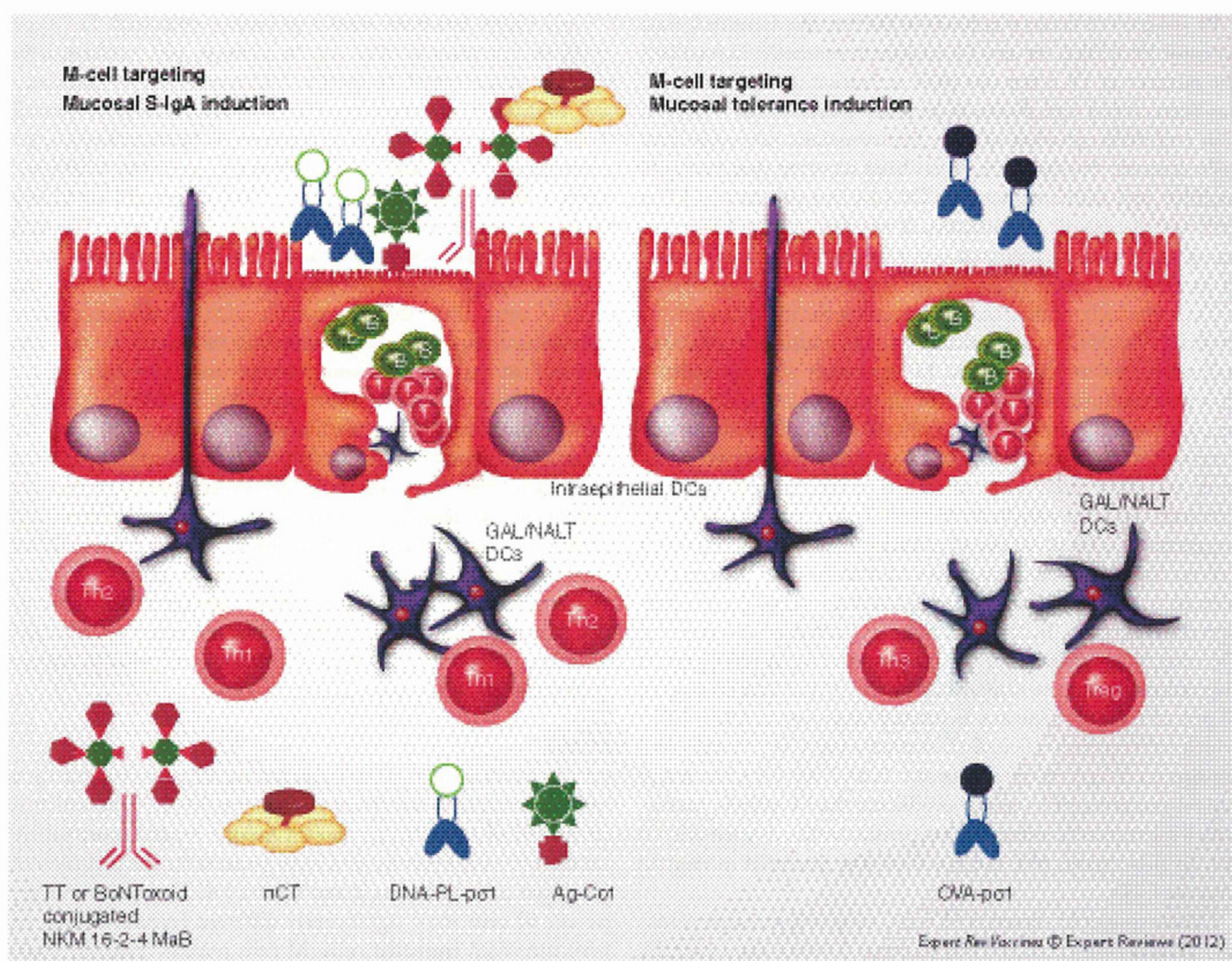

Figure 3. Potential for an M cell-targeting strategy: mucosal M-cell targeting by M cell-specific monoclonal antibody or surface proteins can facilitate Ag delivery for the induction of Ag-specific S-IgA antibody responses to provide effective immunity at the entry site of pathogens. M-cell targeting is achieved by using the protein sigma-1 (pσ1) from reovirus, the ligand for M cell-specific peptide (Co1) or M cell-specific mAb. However, mucosal administration of genetically conjugated OVA protein with pσ1 in the absence of an adjuvant elicits mucosal tolerance.

Ag: Antigen; DC: Dendritic cell; GAL: Gut-associated lymphoreticular; mAb: Monoclonal antibody; NALT: Nasopharyngeal-associated lymphoreticular tissue; S-IgA: Surface IgA; Th1: Type 1 helper CD4+ T cell; Th2: Type 2 helper CD4+ T cell; Th3: Type 3 helper CD4+ T cell; Treg: T regulatory cell.

the MLNs from PP-deficient mice play a key backup role as a mucosal inductive tissue [95]. It has been suggested that MHC class II+ sIgA+ B cells and lamina propria macrophages may be able to capture Ag through endocytic pathways and process and present peptides to CD4+ Th cells. These findings clearly suggest that the intestinal lamina propria–MLN axis performs a potent mucosal inductive function in addition to the PPs.

If one could identify the key molecules expressed by bacteria and viruses that are needed for their invasion or infection of M cells, it would be a great advantage for designing and constructing effective delivery systems for M-cell targeting of vaccines. Reoviruses initially infect the mouse through M cells [96], by using their surface protein sigma-1 (pσ1) [97,98]. In this regard, an M cell-targeting DNA vaccine complex consisting of plasmid DNA and the reovirus pσ1 covalently attached to poly-L-lysine induced significant mucosal S-IgA Ab responses and systemic immunity (Figure 3) [99]. Furthermore, a newly developed M cell-specific monoclonal Ab (NKM 16-2-4) was used as an M cell-targeting carrier for mucosal vaccines. Thus, oral administration of a chimeric vaccine consisting of NKM 16-2-4 and tetanus toxoid or botulinum neurotoxin type A toxoid (BoNToxoid/A), together with native cholera toxin, elicited increased levels of Ag-specific S-IgA and plasma IgG Ab responses (Figure 3) [100]. Importantly, oral immunization with BoNToxoid/A-NKM 16-2-4 provided protective immunity against lethal challenge with botulinum neurotoxin [100]. In addition, oral immunization of Ag fused with M cell-targeting peptide ligand (Co1) resulted in enhanced Ag-specific immune responses [101]. These studies show that an

M cell-targeting delivery system may be of central importance in developing effective mucosal vaccines. Furthermore, it is likely that M cells are also involved in the induction of oral tolerance. In this latter regard, one must carefully consider the nature of formulation of vaccine (or inclusion of adjuvant) because both nasal and oral administration of pσ1 of reovirus genetically conjugated with OVA (OVA-pσ1) alone induced systemic unresponsiveness instead of mucosal IgA immunity (Figure 3) [102,103]. Thus, mucosally induced tolerance was achieved with doses as low as 10–50 µg of OVA-pσ1 when given by the nasal or oral routes [102,103].

Mucosal delivery systems

MucoRice

In 1997, Curtiss and Cardineau successfully filed for and received a US patent (5686079) describing tobacco leaves expressing *Streptococcus mutans* surface protein Ag as an initial indication of a potential plant-based mucosal vaccine. Furthermore, others have developed edible plant-based vaccines by expressing Ags from enterotoxins, hepatitis B, Norwalk virus and respiratory syncytial virus expressed in tobacco leaves or potato tubers [104–111]. Although these plant-based vaccines exhibited some functional properties in experimental systems, their practical application still remains to be elucidated. To develop practical oral vaccines for global immunization, one should consider that the vaccine must maintain effectiveness despite *in vivo* and *ex vivo* environmental changes. In this regard, several practical merits can be found in a rice-based oral vaccine compared with most traditional and other plant-based vaccines. For example, a rice-based vaccine is a rather safe approach. Because this vaccine can be given in a powder form, one could avoid potential problems by using a food-based delivery system. Although the lot-to-lot quality control of a rice-based vaccine may be challenging, stable vaccine Ag expression could be achieved by the third generation of rice-based vaccine. Furthermore, a rice-based vaccine showed stability at room temperature for 2–3 years [112,113]. Oral administration of this rice-based vaccine did not lose activity when exposed to digestive enzymes and subsequently induced protective, Ag-specific Ab responses in mice and non-human primates [112–115]. Recent studies have provided direct evidence that oral MucoRice-cholera toxin B-subunit (CT-B) induced Ag-specific S-IgA Abs that played a critical role in protection against CT-induced diarrhea (Figure 4) [113]. Importantly, cold chain-free oral MucoRice-CT-B induced long-lasting cross-protective immunity against heat-labile enterotoxin-producing enterotoxigenic *Escherichia coli* in addition to CT-producing *Vibrio cholerae* [113]. These results demonstrate that oral administration of a rice-based vaccine provides a potent practical global strategy for the development of cold chain- and needle-free vaccines that protect from gastrointestinal infection.

Nanogels

The application of biomaterials, such as encapsulating Ags in polymer nanoparticles, microparticles, virosomes and liposomes, shows significant potential in the development of vaccines and immunotherapy [116–123]. Although use of liposomes can enhance Ag delivery across mucosal surfaces, they are rapidly cleared and do not allow for long-term Ag release at the mucosal surface [124–126]. In this regard, it is possible that using a bioadhesive gel one could upregulate the residence time and enhance Ag release and retention onto the epithelial cells themselves. Indeed, it has been shown that surface modifications or coadministration with bioadhesive materials, that is, chitosan, resulted in influenza-specific S-IgA Ab responses in nasal washes [127]. A nanometer-sized (<100 nm) bioadhesive polymer hydrogel (nanogel) system has been developed and used as an attractive drug delivery system [128]. Cholesteryl group-bearing pullulan (CHP) form self-assembly of associating polymers as physically crosslinked nanogels in water [129,130]. In general, hydrophobic interactions between CHP and various proteins revealed a CHP nanogel containing the protein inside. When CHP nanogels capture the proteins inside, they form a hydrated nanogel polymer network (nanomatrix) without aggregation. In this regard, trapped proteins maintained their native form and were slowly released [130]. On the basis of these advantages, a CHP nanogel strategy has been used for the development of adjuvant-free nasal vaccines. It was recently shown that nasal administration of a cationic type of CHP nanogel (cCHP nanogel) containing the C-terminus of the H chain (Hc) of botulinum neurotoxin-type A (BoNT/A; nanogel-Hc-BoNT/A) allowed adherence to the nasal epithelium for a longer period compared with naked Hc-BoNT/A (Figure 4) [131]. In this regard, gradually released Hc-BoNT/A was effectively taken up by mucosal APCs and subsequently elicited protective Ag-specific S-IgA Ab responses against BoNT/A intoxication [131]. In summary, this cCHP nanogel system could represent an ideal and effective mucosal delivery system to enhance pathogen-specific mucosal immune responses at the mucosal surface. Because vaccine Ags are retained for a longer period at mucosal surfaces, it is essential to consider the potential side effects of this delivery system in future applications.

Mucosal immunization routes

SL immunization

Oral and nasal routes have been the preferred ones to induce protective immunity in different mucosal compartments [1,5,21]. However, it has been demonstrated that rectal, vaginal or paramucosal (iliac and inguinal lymph nodes) immunization are also effective strategies for the induction of protective immunity against sexually transmitted infectious diseases, including HIV [132–135]. In addition to these mucosal immunization routes, SL administration of Ags has been used to treat allergic, autoimmune or infection-induced pathologic reactions [21,136], by taking advantage of the induction of oral tolerance [137–140]. It is well known that nasal immunization effectively elicits Ag-specific immunity in both mucosal and systemic compartments; however, one must consider that some nasal immunization strategies risk Ag trafficking into olfactory tissues and the CNS [141–145]. To obviate this potential problem, SL immunization may be an ideal mucosal Ag delivery system that avoids CNS involvement. SL administration is also a noninvasive route that has the advantage of requiring lower doses of Ag than the oral route because of the reduced exposure to proteolytic enzymes and lower pH of the stomach encountered

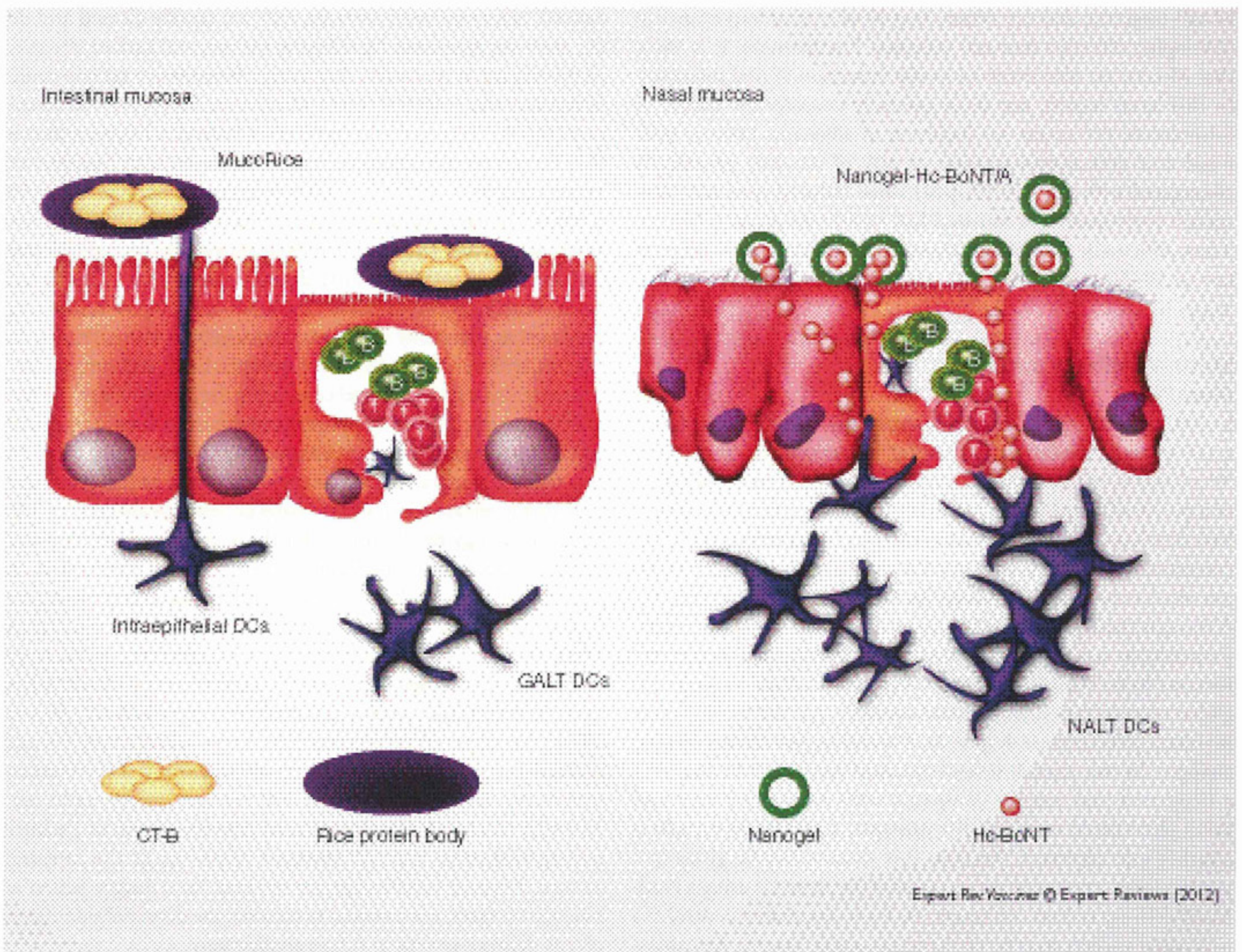

Figure 4. Novel mucosal delivery systems: a plant-based MucoRice-CT-B vaccine effectively induces CT-B-specific protective immunity when orally administered. Because CT-B can be delivered to the small intestine in the rice protein body, MucoRice-CT-B effectively induced CT-B-specific Ab responses in the absence of the CT-A subunit or other potential adjuvants. The cationic nanogel-Hc-BoNT/A is retained for a longer period at the nasal epithelium for slow release of Ag when nasally administered. Thus, nasal APCs, including DCs, can more effectively take up Hc-BoNT/A to initiate Ag-specific immune responses.
DC: Dendritic cell; GALT: Gut-associated lymphoreticular tissue; NALT: Nasopharyngeal-associated lymphoreticular tissue.

after oral immunization. Furthermore, vaccine uptake may be more efficient based on the number of APCs present at the SL site [138]. Recently, studies have used the SL route for vaccine delivery [146–151]. When plasmid DNA encoding hepatitis B surface Ag was sublingually administered to mice, comparable levels of Ag-specific humoral and CD8$^+$ CTL responses were induced as seen after intradermal injection [147]. The SL delivery of a soluble Ag 2,4-dinitrophenyl bovine serum albumin in starch microparticles in combination with a penetration enhancer resulted in good salivary IgA Ab responses [148]. Finally, SL delivery of lipopeptides induced increased serum Abs and T-cell responses in the spleen and inguinal lymph nodes of mice [146]. Compared with subcutaneous administration of the same vaccine preparation, SL application preferentially induced IFN-γ-producing T cells and IgG2a Ab responses, whereas subcutaneous injection elicited IL-4 and IgG1 Ab responses [146]. More recently, SL immunization with influenza virus successfully elicited influenza-specific immunity and provided protection against lethal viral infection [150]. Furthermore, SL immunization with the outer membrane protein of *Porphyromonas gingivalis* plus the plasmid expressing FL cDNA (pFL) elicited increased frequencies of DCs in submandibular lymph nodes and protective immunity in the oral cavity [151]. In addition, CCR7-expressing DCs in cervical lymph nodes were the key players in the induction of Ag-specific immune responses [149]. These findings show that by using the appropriate quantity and form of Ag with a targeted delivery system, the SL route could be the preferred one for inducing both mucosal and systemic immunity, without induction of T-cell unresponsiveness.

Eye drops

The ocular surface leading to the lacrimal sac and nasolacrimal duct also forms an interface with the outside environment.

In fact, it has been proposed that CALT, together with TALT, organizes eye-associated lymphoreticular tissue to create mucosal surveillance and a barrier in the eye region of humans [152,153]. Although TALT develops in human tear ducts, little information was available on mouse TALT until recently. Thus, it was reported that TALT is located in the murine lacrimal sac covered by an epithelium with M cells for Ag uptake [8]. The administration of Ags using eye drops induced Ag-specific S-IgA Ab responses in both ocular and nasal cavities in addition to serum IgG Abs because of the presence of TALT in the conjunctival sac, located in the tear duct, which bridges the ocular and nasal cavities [8,152–154]. Ocular administered Ags migrate to tear ducts and then to the nasal cavity and thus are taken up by TALT and NALT M cells for the induction of Ag-specific immune responses. Past investigations tended to emphasize the identification and characterization of CALT [153,155–158]. Unlike other mammals (e.g., cat, dog, and human), mice and rats do not possess CALT [155]. However, recent findings showed that eye drop administration of Ag induced CALT development in mice with increased numbers of M cell-like cells [9]. Although it remains unclear whether eye drop immunization induces potential adverse effects, including inflammatory responses, it was reported that the administered Ag did not migrate into the CNS [9]. Taken together, these findings clearly showed that eye drop administration of vaccine would be a novel strategy for the induction of Ag-specific mucosal immune responses, if inflammatory responses could be avoided.

Expert commentary

The CMIS provides both an essential concept and a practical means for the development of mucosal vaccines. Thus, it is essential to effectively activate mucosal inductive tissues or MALT for effective mucosal immunity. For targeting MALT, different routes of mucosal immunization have been developed and shown to successfully elicit protective mucosal immunity against several pathogens. However, one could easily fail to elicit protective mucosal immunity without a better understanding of the cellular and molecular mechanisms that regulate the mucosal immune system. Thus, one must carefully consider the route of immunization, the adjuvant and method of Ag delivery to elicit appropriate and desired mucosal immune responses to a particular pathogen. For example, oral vaccination may have fewer side effects and be the most preferred immunization route from a practical point of view; however, oral vaccines require that one maintain their original quality and efficacy until they reach the GALT, because the GI tract represents a harsh environment. In this regard, the MucoRice delivery system could be potentially beneficial for oral vaccine development. Thus, it is important to test whether this system can be easily adapted to other types of vaccine Ags. Nasal vaccines must be safe and not be taken up by the CNS because the nasal immunization route has an advantage for the induction of Ag-specific S-IgA Ab responses in the elderly. Indeed, targeting DCs or M cells in the MALT not only facilitates Ag uptake but also avoids potential CNS toxicity. Furthermore, SL and eye drop immunization successfully elicit mucosal immunity without serious toxicity or side effects so far. Novel delivery systems significantly enhance Ag uptake by MALT for the induction of Ag- or pathogen-specific mucosal immunity. However, the precise cellular and molecular mechanisms for these immunization systems in the induction of mucosal immunity still remain to be elucidated. Nevertheless, it is possible that a strategy that uses the appropriate combination of mucosal adjuvants and delivery systems and optimizes the immunization schedule by repeating and combining different routes of mucosal immunization as a primary and boosting strategy could lead to development of a new generation of safe and effective mucosal vaccines.

Five-year view

Mucosal vaccination is a needle- and medical waste-free vaccine strategy that provides protective immunity against pathogenic bacteria and viruses in both mucosal and systemic compartments. However, mucosal vaccines must overcome two major hurdles (effectiveness and safety), which are both relatively difficult tasks compared with systemic vaccine development because of the uniqueness of the mucosal environment. Future global warming could introduce unexpected pathogens, such as the malaria parasite, into new areas where they have never been seen causing pandemic infectious diseases. Furthermore, some of the currently available vaccines, including nasal FLuMist, are less effective in the immunocompromized population such as young children and the elderly. These facts indicate that the development of novel mucosal vaccines have the potential to provide a better quality of life. According to current knowledge of mucosal vaccines, an appropriate combination of several mucosal vaccine strategies could facilitate the development of practical vaccines over the next 5 years. However, one must realize that developing licensed products is a time-consuming and difficult task from the point of view of a promising outcome. Furthermore, more intensive vaccine development studies need to be performed using novel approaches such as SL immunization, eye drop delivery, nanomatrix and plant-based delivery systems because recent evidence supports both their effectiveness and safety.

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Key issues

- The concept of a common mucosal immune system with specialized compartments is required for the development of effective mucosal vaccines.
- Mucosal vaccines elicit immune defense in both mucosal and systemic tissue compartments.
- Mucosal adjuvants and delivery systems are needed for the induction of more effective mucosal immune responses.
- Targeting mucosal dendritic cells is an effective and safe strategy for inducing antigen-specific immunity.
- New routes of mucosal immunization and antigen delivery systems should facilitate mucosal vaccine development.
- A combination of appropriate mucosal vaccine strategies is essential for future mucosal vaccine development.

References

Papers of special note have been highlighted as:
• of interest

1 Brandtzaeg P. Induction of secretory immunity and memory at mucosal surfaces. *Vaccine* 25(30), 5467–5484 (2007).

• **Describes the features of the mucosal immune system, including the concept of the common mucosal immune system (CMIS).**

2 Lamm ME. Current concepts in mucosal immunity IV. How epithelial transport of IgA antibodies relates to host defense. *Am. J. Physiol.* 274(4 Pt 1), G614–G617 (1998).

3 Russell MW, Mestecky J. Humoral immune responses to microbial infections in the genital tract. *Microbes Infect.* 4(6), 667–677 (2002).

4 Fujihashi K, Boyaka PN, McGhee JR. Host defenses at mucosal surfaces. In: *Clinical Immunology.* Rich RT *et al.* (Eds). Mosby Elsevier, PA, USA, 287–304 (2008).

5 Kiyono H, Kunisawa J, McGhee JR, Mestecky J. The mucosal imune system. In: *Fundamental Immunology.* Paul WE (Ed.). Lippincott Williams & Wilkins, PA, USA, 983–1030 (2008).

6 Hamada H, Hiroi T, Nishiyama Y *et al.* Identification of multiple isolated lymphoid follicles on the antimesenteric wall of the mouse small intestine. *J. Immunol.* 168(1), 57–64 (2002).

7 Lorenz RG, Chaplin DD, McDonald KG, McDonough JS, Newberry RD. Isolated lymphoid follicle formation is inducible and dependent upon lymphotoxin-sufficient B lymphocytes, lymphotoxin beta receptor, and TNF receptor I function. *J. Immunol.* 170(11), 5475–5482 (2003).

8 Nagatake T, Fukuyama S, Kim DY *et al.* Id2-, RORγt-, and LTβR-independent initiation of lymphoid organogenesis in ocular immunity. *J. Exp. Med.* 206(11), 2351–2364 (2009).

9 Seo KY, Han SJ, Cha HR *et al.* Eye mucosa: an efficient vaccine delivery route for inducing protective immunity. *J. Immunol.* 185(6), 3610–3619 (2010).

10 Lamm ME, Phillips-Quagliata JM. Origin and homing of intestinal IgA antibody-secreting cells. *J. Exp. Med.* 195(2), F5–F8 (2002).

11 Macpherson AJ, McCoy KD, Johansen FE, Brandtzaeg P. The immune geography of IgA induction and function. *Mucosal Immunol.* 1(1), 11–22 (2008).

12 Mestecky J, Lue C, Russell MW. Selective transport of IgA. Cellular and molecular aspects. *Gastroenterol. Clin. North Am.* 20(3), 441–471 (1991).

13 Campbell DJ, Butcher EC. Rapid acquisition of tissue-specific homing phenotypes by CD4+ T cells activated in cutaneous or mucosal lymphoid tissues. *J. Exp. Med.* 195(1), 135–141 (2002).

14 Hamann A, Andrew DP, Jablonski-Westrich D, Holzmann B, Butcher EC. Role of α4-integrins in lymphocyte homing to mucosal tissues *in vivo*. *J. Immunol.* 152(7), 3282–3293 (1994).

15 Kantele A, Zivny J, Hakkinen M, Elson CO, Mestecky J. Differential homing commitments of antigen-specific T cells after oral or parenteral immunization in humans. *J. Immunol.* 162(9), 5173–5177 (1999).

16 Svensson M, Marsal J, Ericsson A *et al.* CCL25 mediates the localization of recently activated CD8αβ+ lymphocytes to the small-intestinal mucosa. *J. Clin. Invest.* 110(8), 1113–1121 (2002).

17 Butcher EC, Williams M, Youngman K, Rott L, Briskin M. Lymphocyte trafficking and regional immunity. *Adv. Immunol.* 72, 209–253 (1999).

18 Iwata M, Hirakiyama A, Eshima Y, Kagechika H, Kato C, Song SY. Retinoic acid imprints gut-homing specificity on T cells. *Immunity* 21(4), 527–538 (2004).

19 McGhee JR, Kunisawa J, Kiyono H. Gut lymphocyte migration, we are halfway 'home'. *Trends Immunol.* 28(4), 150–153 (2007).

20 Mora JR, Iwata M, Eksteen B *et al.* Generation of gut-homing IgA-secreting B cells by intestinal dendritic cells. *Science* 314(5802), 1157–1160 (2006).

21 Holmgren J, Czerkinsky C. Mucosal immunity and vaccines. *Nat. Med.* 11(Suppl. 4), S45–S53 (2005).

22 Kantele A, Hakkinen M, Moldoveanu Z *et al.* Differences in immune responses induced by oral and rectal immunizations with *Salmonella typhi* Ty21a: evidence for compartmentalization within the common mucosal immune system in humans. *Infect. Immun.* 66(12), 5630–5635 (1998).

23 Wu HY, Russell MW. Nasal lymphoid tissue, intranasal immunization, and compartmentalization of the common mucosal immune system. *Immunol. Res.* 16(2), 187–201 (1997).

24 Campbell JJ, Brightling CE, Symon FA *et al.* Expression of chemokine receptors by lung T cells from normal and asthmatic subjects. *J. Immunol.* 166(4), 2842–2848 (2001).

25 Csencsits KL, Walters N, Pascual DW. Cutting edge: dichotomy of homing receptor dependence by mucosal effector B cells: α(E) versus L-selectin. *J. Immunol.* 167(5), 2441–2445 (2001).

26 Fujihashi K, Kiyono H. Mucosal immunosenescence: new developments and vaccines to control infectious diseases. *Trends Immunol.* 30(7), 334–343 (2009).

- **Reviews mucosal vaccine development and compartmentalization of the mucosal immune system.**

27 Fujihashi K, McGhee JR. Mucosal immunity and tolerance in the elderly. *Mech. Ageing Dev.* 125(12), 889–898 (2004).

28 Fukuyama,S, Hiroi T, Yokota Y *et al.* Initiation of NALT organogenesis is independent of the IL-7R, LTβR, and NIK signaling pathways but requires the Id2 gene and $CD3^-$ $CD4^+CD45^+$ cells. *Immunity* 17(1), 31–40 (2002).

29 Hagiwara Y, McGhee JR, Fujihashi K *et al.* Protective mucosal immunity in aging is associated with functional $CD4^+$ T cells in nasopharyngeal-associated lymphoreticular tissue. *J. Immunol.* 170(4), 1754–1762 (2003).

30 Kato H, Fujihashi K, Kato R *et al.* Lack of oral tolerance in aging is due to sequential loss of Peyer's patch cell interactions. *Int. Immunol.* 15(2), 145–158 (2003).

31 Koga T, McGhee JR, Kato H, Kato R, Kiyono H, Fujihashi K. Evidence for early aging in the mucosal immune system. *J. Immunol.* 165, 5352–5359 (2000).

32 Kunisawa J, Nochi T, Kiyono H. Immunological commonalities and distinctions between airway and digestive immunity. *Trends Immunol.* 29(11), 505–513 (2008).

33 Pascual DW, Riccardi C, Csencsits-Smith K. Distal IgA immunity can be sustained by $\alpha E\beta 7^+$ B cells in L-selectin-/- mice following oral immunization. *Mucosal Immunol.* 1(1), 68–77 (2008).

34 Rennert PD, Browning JL, Mebius R, Mackay F, Hochman PS. Surface lymphotoxin α/β complex is required for the development of peripheral lymphoid organs. *J. Exp. Med.* 184(5), 1999–2006 (1996).

35 Yoshida H, Honda K, Shinkura R *et al.* IL-7 receptor α^+ CD3(-) cells in the embryonic intestine induces the organizing center of Peyer's patches. *Int. Immunol.* 11(5), 643–655 (1999).

36 Kurebayashi S, Ueda E, Sakaue M *et al.* Retinoid-related orphan receptor γ (RORγ) is essential for lymphoid organogenesis and controls apoptosis during thymopoiesis. *Proc. Natl Acad. Sci. USA* 97(18), 10132–10137 (2000).

37 Sun Z, Unutmaz D, Zou YR *et al.* Requirement for RORγ in thymocyte survival and lymphoid organ development. *Science* 288(5475), 2369–2373 (2000).

38 Yokota Y, Mansouri A, Mori S *et al.* Development of peripheral lymphoid organs and natural killer cells depends on the helix-loop-helix inhibitor Id2. *Nature* 397(6721), 702–706 (1999).

39 Banchereau J, Steinman RM. Dendritic cells and the control of immunity. *Nature* 392(6673), 245–252 (1998).

40 Hochrein H, Shortman K, Vremec D, Scott B, Hertzog P, O'Keeffe M. Differential production of IL-12, IFN-α, and IFN-γ by mouse dendritic cell subsets. *J. Immunol.* 166(9), 5448–5455 (2001).

41 Pulendran B, Banchereau J, Maraskovsky E, Maliszewski C. Modulating the immune response with dendritic cells and their growth factors. *Trends Immunol.* 22(1), 41–47 (2001).

42 Steinman RM. The dendritic cell system and its role in immunogenicity. *Annu. Rev. Immunol.* 9, 271–296 (1991).

43 Klinman DM, Currie D, Gursel I, Verthelyi D. Use of CpG oligodeoxynucleotides as immune adjuvants. *Immunol. Rev.* 199, 201–216 (2004).

44 Wagner H. Bacterial CpG DNA activates immune cells to signal infectious danger. *Adv. Immunol.* 73, 329–368 (1999).

45 Klinman DM, Yi AK, Beaucage SL, Conover J, Krieg AM. CpG motifs present in bacteria DNA rapidly induce lymphocytes to secrete interleukin 6, interleukin 12, and interferon gamma. *Proc. Natl Acad. Sci. USA* 93(7), 2879–2883 (1996).

46 Krieg AM, Yi AK, Matson S *et al.* CpG motifs in bacterial DNA trigger direct B-cell activation. *Nature* 374(6522), 546–549 (1995).

47 Yamamoto S, Yamamoto T, Kataoka T, Kuramoto E, Yano O, Tokunaga T. Unique palindromic sequences in synthetic oligonucleotides are required to induce IFN and augment IFN-mediated natural killer activity. *J. Immunol.* 148(12), 4072–4076 (1992).

48 Zimmermann S, Egeter O, Hausmann S *et al.* CpG oligodeoxynucleotides trigger protective and curative Th1 responses in lethal murine leishmaniasis. *J. Immunol.* 160(8), 3627–3630 (1998).

49 Jahrsdorfer B, Weiner GJ. CpG oligodeoxynucleotides for immune stimulation in cancer immunotherapy. *Curr. Opin. Investig. Drugs* 4(6), 686–690 (2003).

50 Kline JN, Waldschmidt TJ, Businga TR *et al.* Modulation of airway inflammation by CpG oligodeoxynucleotides in a murine model of asthma. *J. Immunol.* 160(6), 2555–2559 (1998).

51 Brazolot Millan CL, Weeratna R, Krieg AM, Siegrist CA, Davis HL. CpG DNA can induce strong Th1 humoral and cell-mediated immune responses against hepatitis B surface antigen in young mice. *Proc. Natl Acad. Sci. USA* 95(26), 15553–15558 (1998).

52 Davis HL, Weeratna R, Waldschmidt TJ, Tygrett L, Schorr J, Krieg AM. CpG DNA is a potent enhancer of specific immunity in mice immunized with recombinant hepatitis B surface antigen. *J. Immunol.* 160(2), 870–876 (1998).

53 Eastcott JW, Holmberg CJ, Dewhirst FE, Esch TR, Smith DJ, Taubman MA. Oligonucleotide containing CpG motifs enhances immune response to mucosally or systemically administered tetanus toxoid. *Vaccine* 19(13–14), 1636–1642 (2001).

54 Klinman DM. Therapeutic applications of CpG-containing oligodeoxynucleotides. *Antisense Nucleic Acid Drug Dev.* 8(2), 181–184 (1998).

55 Klinman DM, Barnhart KM, Conover J. CpG motifs as immune adjuvants. *Vaccine* 17(1), 19–25 (1999).

56 Kovarik J, Bozzotti P, Love-Homan L *et al.* CpG oligodeoxynucleotides can circumvent the Th2 polarization of neonatal responses to vaccines but may fail to fully redirect Th2 responses established by neonatal priming. *J. Immunol.* 162(3), 1611–1617 (1999).

57 McCluskie MJ, Davis HL. CpG DNA is a potent enhancer of systemic and mucosal immune responses against hepatitis B surface antigen with intranasal administration to mice. *J. Immunol.* 161(9), 4463–4466 (1998).

58 Moldoveanu Z, Love-Homan L, Huang WQ, Krieg AM. CpG DNA, a novel immune enhancer for systemic and mucosal immunization with influenza virus. *Vaccine* 16(11–12), 1216–1224 (1998).

59 McCluskie MJ, Weeratna RD, Krieg AM, Davis HL. CpG DNA is an effective oral adjuvant to protein antigens in mice. *Vaccine* 19(7–8), 950–957 (2000).

60 Weeratna RD, Brazolot Millan CL, McCluskie MJ, Davis HL. CpG ODN can re-direct the Th bias of established Th2 immune responses in adult and young mice. *FEMS Immunol. Med. Microbiol.* 32(1), 65–71 (2001).

61 Yi AK, Yoon JG, Yeo SJ, Hong SC, English BK, Krieg AM. Role of mitogen-activated protein kinases in CpG DNA-mediated IL-10 and IL-12 production: central role of extracellular signal-regulated kinase in the negative feedback loop of the CpG DNA-mediated Th1 response. *J. Immunol.* 168(9), 4711–4720 (2002).

62 Boyaka PN, Tafaro A, Fischer R, Leppla SH, Fujihashi K, McGhee JR. Effective mucosal immunity to anthrax: neutralizing antibodies and Th cell responses following nasal immunization with protective antigen. *J. Immunol.* 170(11), 5636–5643 (2003).

63 Brasel K, McKenna HJ, Morrissey PJ *et al.* Hematologic effects of flt3 ligand *in vivo* in mice. *Blood* 88, 2004–2012 (1996).

64 Maraskovsky E, Brasel K, Teepe M *et al.* Dramatic increase in the numbers of functionally mature dendritic cells in Flt3 ligand-treated mice: multiple dendritic cell subpopulations identified. *J. Exp. Med.* 184, 1953–1962 (1996).

65 Lyman SD, James L, Johnson L *et al.* Cloning of the human homologue of the murine flt3 ligand: a growth factor for early hematopoietic progenitor cells. *Blood* 83(10), 2795–2801 (1994).

66 Pulendran B, Banchereau J, Burkeholder S *et al.* Flt3-ligand and granulocyte colony-stimulating factor mobilize distinct human dendritic cell subsets *in vivo*. *J. Immunol.* 165(1), 566–572 (2000).

67 Robinson S, Mosley RL, Parajuli P *et al.* Comparison of the hematopoietic activity of flt-3 ligand and granulocyte-macrophage colony-stimulating factor acting alone or in combination. *J. Hematother. Stem Cell Res.* 9(5), 711–720 (2000).

68 Small D, Levenstein M, Kim E *et al.* STK-1, the human homolog of Flk-2/Flt-3, is selectively expressed in CD34⁺ human bone marrow cells and is involved in the proliferation of early progenitor/stem cells. *Proc. Natl Acad. Sci. USA* 91(2), 459–463 (1994).

69 Lyman SD, James L, Vanden Bos T *et al.* Molecular cloning of a ligand for the flt3/flk-2 tyrosine kinase receptor: a proliferative factor for primitive hematopoietic cells. *Cell* 75(6), 1157–1167 (1993).

70 Viney JL, Mowat AM, O'Malley JM, Williamson E, Fanger NA. Expanding dendritic cells *in vivo* enhances the induction of oral tolerance. *J. Immunol.* 160, 5815–5825 (1998).

71 Williamson E, Westrich GM, Viney JL. Modulating dendritic cells to optimize mucosal immunization protocols. *J. Immunol.* 163, 3668–3675 (1999).

72 Pisarev VM, Parajuli P, Mosley RL *et al.* Flt3 ligand enhances the immunogenicity of a gag-based HIV-1 vaccine. *Int. J. Immunopharmacol.* 22(11), 865–876 (2000).

73 Baca-Estrada ME, Ewen C, Mahony D, Babiuk LA, Wilkie D, Foldvari M. The haemopoietic growth factor, Flt3L, alters the immune response induced by transcutaneous immunization. *Immunology* 107, 69–76 (2002).

74 Hung CF, Hsu KF, Cheng WF *et al.* Enhancement of DNA vaccine potency by linkage of antigen gene to a gene encoding the extracellular domain of Fms-like tyrosine kinase 3-ligand. *Cancer Res.* 61, 1080–1088 (2001).

75 Moore AC, Kong WP, Chakrabarti BK, Nabel GJ. Effects of antigen and genetic adjuvants on immune responses to human immunodeficiency virus DNA vaccines in mice. *J. Virol.* 76, 243–250 (2002).

76 Esche C, Subbotin VM, Maliszewski C, Lotze MT, Shurin MR. FLT3 ligand administration inhibits tumor growth in murine melanoma and lymphoma. *Cancer Res.* 58(3), 380–383 (1998).

77 Lynch DH, Andreasen A, Maraskovsky E, Whitmore J, Miller RE, Schuh JC. Flt3 ligand induces tumor regression and antitumor immune responses *in vivo*. *Nat. Med.* 3(6), 625–631 (1997).

78 Pulendran B, Smith JL, Caspary G *et al.* Distinct dendritic cell subsets differentially regulate the class of immune response *in vivo*. *Proc. Natl Acad. Sci. USA* 96(3), 1036–1041 (1999).

79 Vollstedt S, Franchini M, Hefti HP *et al.* Flt3 ligand-treated neonatal mice have increased innate immunity against intracellular pathogens and efficiently control virus infections. *J. Exp. Med.* 197(5), 575–584 (2003).

80 Kataoka K, McGhee JR, Kobayashi R, Fujihashi K, Shizukuishi S, Fujihashi K. Nasal Flt3 ligand cDNA elicits CD11c⁺CD8⁺ dendritic cells for enhanced mucosal immunity. *J. Immunol.* 172(6), 3612–3619 (2004).

81 Sekine S, Kataoka K, Fukuyama Y *et al.* A novel adenovirus expressing flt3 ligand enhances mucosal immunity by inducing mature nasopharyngeal-associated lymphoreticular tissue dendritic cell migration. *J. Immunol.* 180(12), 8126–8134 (2008).

82 Fukuiwa T, Sekine S, Kobayashi R *et al.* A combination of Flt3 ligand cDNA and CpG ODN as nasal adjuvant elicits NALT dendritic cells for prolonged mucosal immunity. *Vaccine* 26(37), 4849–4859 (2008).

83 Fukuyama Y, King JD, Kataoka K *et al.* A combination of Flt3 ligand cDNA and CpG oligodeoxynucleotide as nasal adjuvant elicits protective secretory-IgA immunity to *Streptococcus pneumoniae* in aged mice. *J. Immunol.* 186(4), 2454–2461 (2011).

84 Bockman DE, Cooper MD. Pinocytosis by epithelium associated with lymphoid follicles in the bursa of Fabricius, appendix, and Peyer's patches. An electron microscopic study. *Am. J. Anat.* 136(4), 455–477 (1973).

85 Farstad IN, Halstensen TS, Fausa O, Brandtzaeg P. Heterogeneity of M-cell-associated B and T cells in human Peyer's patches. *Immunology* 83(3), 457–464 (1994).

86 Gebert A, Rothkotter HJ, Pabst R. M cells in Peyer's patches of the intestine. *Int. Rev. Cytol.* 167, 91–159 (1996).

87 Neutra MR, Frey A, Kraehenbuhl JP. Epithelial M cells, gateways for mucosal infection and immunization. *Cell* 86(3), 345–348 (1996).

- **Focuses on M cells in the mucosal immune system.**

88 Owen RL, Jones AL. Epithelial cell specialization within human Peyer's patches: an ultrastructural study of intestinal lymphoid follicles. *Gastroenterology* 66(2), 189–203 (1974).

89 Wolf JL, Bye WA. The membranous epithelial (M) cell and the mucosal immune system. *Annu. Rev. Med.* 35, 95–112 (1984).

- **Focuses on M cells in the mucosal immune system.**

90 Ermak TH, Dougherty EP, Bhagat HR, Kabok Z, Pappo J. Uptake and transport of copolymer biodegradable microspheres by rabbit Peyer's patch M cells. *Cell Tissue Res.* 279(2), 433–436 (1995).

91 Allan CH, Mendrick DL, Trier JS. Rat intestinal M cells contain acidic endosomal-lysosomal compartments and express class II major histocompatibility complex determinants. *Gastroenterology* 104(3), 698–708 (1993).

92 Jones BD, Ghori N, Falkow S. *Salmonella typhimurium* initiates murine infection by penetrating and destroying the specialized

epithelial M cells of the Peyer's patches. *J. Exp. Med.* 180(1), 15–23 (1994).

93 Teitelbaum R, Schubert W, Gunther L *et al.* The M cell as a portal of entry to the lung for the bacterial pathogen *Mycobacterium tuberculosis. Immunity* 10(6), 641–650 (1999).

94 Jang, MH, Kweon MN, Iwatani K *et al.* Intestinal villous M cells: an antigen entry site in the mucosal epithelium. *Proc. Natl Acad. Sci. USA* 101(16), 6110–6115 (2004).

95 Yamamoto M, Rennert P, McGhee JR *et al.* Alternate mucosal immune system: organized Peyer's patches are not required for IgA responses in the gastrointestinal tract. *J. Immunol.* 164(10), 5184–5189 (2000).

96 Wolf JL, Rubin DH, Finberg R *et al.* Intestinal M cells: a pathway for entry of reovirus into the host. *Science* 212(4493), 471–472 (1981).

97 Nibert ML, Furlong DB, Fields BN. Mechanisms of viral pathogenesis. Distinct forms of reoviruses and their roles during replication in cells and host. *J. Clin. Invest.* 88(3), 727–734 (1991).

98 Wu Y, Boysun MJ, Csencsits KL, Pascual DW. Gene transfer facilitated by a cellular targeting molecule, reovirus protein σ1. *Gene Ther.* 7(1), 61–69 (2000).

99 Wu Y, Wang X, Csencsits KL, Haddad A, Walters N, Pascual DW. M cell-targeted DNA vaccination. *Proc. Natl Acad. Sci. USA* 98(16), 9318–9323 (2001).

100 Nochi T, Yuki Y, Matsumura A *et al.* A novel M cell-specific carbohydrate-targeted mucosal vaccine effectively induces antigen-specific immune responses. *J. Exp. Med.* 204(12), 2789–2796 (2007).

101 Kim SH, Seo KW, Kim J, Lee KY, Jang YS. The M cell-targeting ligand promotes antigen delivery and induces antigen-specific immune responses in mucosal vaccination. *J. Immunol.* 185(10), 5787–5795 (2010).

102 Rynda A, Maddaloni M, Mierzejewska D *et al.* Low-dose tolerance is mediated by the microfold cell ligand, reovirus protein σ1. *J. Immunol.* 180(8), 5187–5200 (2008).

103 Suzuki H, Sekine S, Kataoka K *et al.* Ovalbumin-protein σ1 M-cell targeting facilitates oral tolerance with reduction of antigen-specific CD4+ T cells. *Gastroenterology* 135(3), 917–925 (2008).

104 Arakawa T, Chong DK, Langridge WH. Efficacy of a food plant-based oral cholera toxin B subunit vaccine. *Nat. Biotechnol.* 16(3), 292–297 (1998).

105 Haq TA, Mason HS, Clements JD, Arntzen CJ. Oral immunization with a recombinant bacterial antigen produced in transgenic plants. *Science* 268, 714–716 (1995).

106 Mason HS, Ball JM, Shi JJ, Jiang X, Estes MK, Arntzen CJ. Expression of Norwalk virus capsid protein in transgenic tobacco and potato and its oral immunogenicity in mice. *Proc. Natl Acad. Sci. USA* 93(11), 5335–5340 (1996).

107 Mason HS, Haq TA, Clements JD, Arntzen CJ. Edible vaccine protects mice against *Escherichia coli* heat-labile enterotoxin (LT): potatoes expressing a synthetic LT-B gene. *Vaccine* 16(13), 1336–1343 (1998).

108 Richter LJ, Thanavala Y, Arntzen CJ, Mason HS. Production of hepatitis B surface antigen in transgenic plants for oral immunization. *Nat. Biotechnol.* 18(11), 1167–1171 (2000).

109 Sandhu JS, Krasnyanski SF, Domier LL, Korban SS, Osadjan MD, Buetow DE. Oral immunization of mice with transgenic tomato fruit expressing respiratory syncytial virus-F protein induces a systemic immune response. *Transgenic Res.* 9(2), 127–135 (2000).

110 Streatfield SJ, Jilka JM, Hood EE *et al.* Plant-based vaccines: unique advantages. *Vaccine* 19(17–19), 2742–2748 (2001).

111 Tacket CO, Mason HS, Losonsky G, Clements JD, Levine MM, Arntzen CJ. Immunogenicity in humans of a recombinant bacterial antigen delivered in a transgenic potato. *Nat. Med.* 4(5), 607–609 (1998).

112 Nochi T, Takagi H, Yuki Y *et al.* Rice-based mucosal vaccine as a global strategy for cold-chain- and needle-free vaccination. *Proc. Natl Acad. Sci. USA* 104(26), 10986–10991 (2007).

113 Tokuhara D, Yuki Y, Nochi T *et al.* Secretory IgA-mediated protection against *V. cholerae* and heat-labile enterotoxin-producing enterotoxigenic *Escherichia coli* by rice-based vaccine. *Proc. Natl Acad. Sci. USA* 107(19), 8794–8799 (2010).

114 Nochi T, Yuki Y, Katakai Y *et al.* A rice-based oral cholera vaccine induces macaque-specific systemic neutralizing antibodies but does not influence pre-existing intestinal immunity. *J. Immunol.* 183(10), 6538–6544 (2009).

115 Yuki Y, Tokuhara D, Nochi T *et al.* Oral MucoRice expressing double-mutant cholera toxin A and B subunits induces toxin-specific neutralising immunity. *Vaccine* 27(43), 5982–5988 (2009).

116 de Haan A, Geerligs HJ, Huchshorn JP, van Scharrenburg GJ, Palache AM, Wilschut J. Mucosal immunoadjuvant activity of liposomes, induction of systemic IgG and secretory IgA responses in mice by intranasal immunization with an influenza subunit vaccine and coadministered liposomes. *Vaccine* 13(2), 155–162 (1995).

117 de Haan A, Tomee JF, Huchshorn JP, Wilschut J. Liposomes as an immunoadjuvant system for stimulation of mucosal and systemic antibody responses against inactivated measles virus administered intranasally to mice. *Vaccine* 13(14), 1320–1324 (1995).

118 Ernst WA, Kim HJ, Tumpey TM *et al.* Protection against H1, H5, H6 and H9 influenza A infection with liposomal matrix 2 epitope vaccines. *Vaccine* 24(24), 5158–5168 (2006).

119 Hasegawa H, Ichinohe T, Strong P *et al.* Protection against influenza virus infection by intranasal administration of hemagglutinin vaccine with chitin microparticles as an adjuvant. *J. Med. Virol.* 75(1), 130–136 (2005).

120 Marinaro M, Boyaka PN, Finkelman FD *et al.* Oral but not parenteral interleukin (IL)-12 redirects T helper 2 (Th2)-type responses to an oral vaccine without altering mucosal IgA responses. *J. Exp. Med.* 185(3), 415–427 (1997).

121 Reddy ST, Swartz MA, Hubbell JA. Targeting dendritic cells with biomaterials, developing the next generation of vaccines. *Trends Immunol.* 27(12), 573–579 (2006).

- **Describes general features of dendritic cells in the innate and acquired immune systems.**

122 Sharma S, Mukkur TK, Benson HA, Chen Y. Pharmaceutical aspects of intranasal delivery of vaccines using particulate systems. *J. Pharm. Sci.* 98(3), 812–843 (2009).

123 Zurbriggen R, Gluck R. Immunogenicity of IRIV- versus alum-adjuvanted diphtheria and tetanus toxoid vaccines in influenza primed mice. *Vaccine* 17(11–12), 1301–1305 (1999).

124 Alving CR. Immunologic aspects of liposomes: presentation and processing of liposomal protein and phospholipid antigens. *Biochim. Biophys. Acta* 1113(3–4), 307–322 (1992).

125 Brochu H, Polidori A, Pucci B, Vermette P. Drug delivery systems using immobilized intact liposomes: a comparative and critical review. *Curr. Drug Deliv.* 1(3), 299–312 (2004).

126 Gregoriadis, G. Immunological adjuvants: a role for liposomes. *Immunol. Today* 11(3), 89–97 (1990).

127 Amidi M, Romeijn SG, Verhoef JC *et al.* N-trimethyl chitosan (TMC) nanoparticles loaded with influenza subunit antigen for intranasal vaccination: biological properties and immunogenicity in a mouse model. *Vaccine* 25(1), 144–153 (2007).

128 Gilmore JL, Yi X, Quan L, Kabanov AV. Novel nanomaterials for clinical neuroscience. *J. Neuroimmune Pharmacol.* 3(2), 83–94 (2008).

129 Kageyama S, Kitano S, Hirayama M *et al.* Humoral immune responses in patients vaccinated with 1-146 HER2 protein complexed with cholesteryl pullulan nanogel. *Cancer Sci.* 99(3), 601–607 (2008).

130 Nomura Y, Ikeda M, Yamaguchi N, Aoyama Y, Akiyoshi K. Protein refolding assisted by self-assembled nanogels as novel artificial molecular chaperone. *FEBS Lett.* 553(3), 271–276 (2003).

131 Nochi T, Yuki Y, Takahashi H *et al.* Nanogel antigenic protein-delivery system for adjuvant-free intranasal vaccines. *Nat. Mater.* 9(7), 572–578 (2010).

132 Lehner T. Innate and adaptive mucosal immunity in protection against HIV infection. *Vaccine* 21 (Suppl. 2), S68–S76 (2003).

133 Lehner T, Wang Y, Whittall T, Seidl T. Innate immunity and HIV-1 infection. *Adv. Dent. Res.* 23(1), 19–22 (2011).

134 Tengvall S, Lundqvist A, Eisenberg RJ, Cohen GH, Harandi AM. Mucosal administration of CpG oligodeoxynucleotide elicits strong CC and CXC chemokine responses in the vagina and serves as a potent Th1-tilting adjuvant for recombinant gD2 protein vaccination against genital herpes. *J. Virol.* 80(11), 5283–5291 (2006).

135 Tengvall S, O'Hagan D, Harandi AM. Rectal immunization generates protective immunity in the female genital tract against herpes simplex virus type 2 infection: relative importance of myeloid differentiation factor 88. *Antiviral Res.* 78(3), 202–214 (2008).

136 Mowat AM, Faria AM, Weiner HL. Oral tolerance: physiological basis and clinical applications. In: *Mucosal Immunology.* Mestecky J, Lamm ME, Strober W, Bienenstock J, McGhee JR, Mayer L (Eds.). Elsevier/Academic Press, CA, USA, 487–537 (2004).

137 Frati F, Moingeon P, Marcucci F *et al.* Mucosal immunization application to allergic disease: sublingual immunotherapy. *Allergy Asthma Proc.* 28(1), 35–39 (2007).

138 Moingeon P, Batard T, Fadel R, Frati F, Sieber J, Van Overtvelt L. Immune mechanisms of allergen-specific sublingual immunotherapy. *Allergy* 61(2), 151–165 (2006).

139 Sun JB, Czerkinsky C, Holmgren J. Sublingual 'oral tolerance' induction with antigen conjugated to cholera toxin B subunit generates regulatory T cells that induce apoptosis and depletion of effector T cells. *Scand. J. Immunol.* 66(2–3), 278–286 (2007).

140 Sun JB, Raghavan S, Sjoling A, Lundin S, Holmgren J. Oral tolerance induction with antigen conjugated to cholera toxin B subunit generates both Foxp3$^+$CD25$^+$ and Foxp3$^-$CD25$^-$ CD4$^+$ regulatory T cells. *J. Immunol.* 177(11), 7634–7644 (2006).

141 Hagiwara Y, Kawamura YI, Kataoka K *et al.* A second generation of double mutant cholera toxin adjuvants: enhanced immunity without intracellular trafficking. *J. Immunol.* 177(5), 3045–3054 (2006).

142 van Ginkel FW, Jackson RJ, Yoshino N *et al.* Enterotoxin-based mucosal adjuvants alter antigen trafficking and induce inflammatory responses in the nasal tract. *Infect. Immun.* 73(10), 6892–6902 (2005).

143 van Ginkel FW, Jackson RJ, Yuki Y, McGhee JR. Cutting edge: the mucosal adjuvant cholera toxin redirects vaccine proteins into olfactory tissues. *J. Immunol.* 165(9), 4778–4782 (2000).

144 van Ginkel FW, McGhee JR, Watt JM, Campos-Torres A, Parish LA, Briles DE. Pneumococcal carriage results in ganglioside-mediated olfactory tissue infection. *Proc. Natl Acad. Sci. USA* 100(24), 14363–14367 (2003).

145 Yoshino N, Lu FX, Fujihashi K *et al.* A novel adjuvant for mucosal immunity to HIV-1 gp120 in nonhuman primates. *J. Immunol.* 173(11), 6850–6857 (2004).

146 BenMohamed L, Belkaid Y, Loing E, Brahimi K, Gras-Masse H, Druilhe P. Systemic immune responses induced by mucosal administration of lipopeptides without adjuvant. *Eur. J. Immunol.* 32(8), 2274–2281 (2002).

147 McCluskie MJ, Brazolot Millan CL, Gramzinski RA *et al.* Route and method of delivery of DNA vaccine influence immune responses in mice and non-human primates. *Mol. Med.* 5(5), 287–300 (1999).

148 Montgomery PC, Rafferty DE. Induction of secretory and serum antibody responses following oral administration of antigen with bioadhesive degradable starch microparticles. *Oral Microbiol. Immunol.* 13(3), 139–149 (1998).

149 Song, JH, Kim JI, Kwon HJ *et al.* CCR7-CCL19/CCL21-regulated dendritic cells are responsible for effectiveness of sublingual vaccination. *J. Immunol.* 182(11), 6851–6860 (2009).

150 Song JH, Nguyen HH, Cuburu N *et al.* Sublingual vaccination with influenza virus protects mice against lethal viral infection. *Proc. Natl Acad. Sci. USA* 105(5), 1644–1649 (2008).

151 Zhang T, Hashizume T, Kurita-Ochiai T, Yamamoto M. Sublingual vaccination with outer membrane protein of *Porphyromonas gingivalis* and Flt3 ligand elicits protective immunity in the oral cavity. *Biochem. Biophys. Res. Commun.* 390(3), 937–941 (2009).

152 Knop E, Knop N. Lacrimal drainage-associated lymphoid tissue (LDALT): a part of the human mucosal immune system. *Invest. Ophthalmol. Vis. Sci.* 42(3), 566–574 (2001).

153 Knop N, Knop E. Conjunctiva-associated lymphoid tissue in the human eye. *Invest. Ophthalmol. Vis. Sci.* 41(6), 1270–1209 (2000).

154 Cain C, Phillips TE. Developmental changes in conjunctiva-associated lymphoid tissue of the rabbit. *Invest. Ophthalmol. Vis. Sci.* 49(2), 644–649 (2008).

155 Chodosh J, Nordquist RE, Kennedy RC. Comparative anatomy of mammalian conjunctival lymphoid tissue: a putative mucosal immune site. *Dev. Comp. Immunol.* 22(5–6), 621–630 (1998).

156 Giuliano EA, Moore CP, Phillips TE. Morphological evidence of M cells in healthy canine conjunctiva-associated lymphoid tissue. *Graefes Arch. Clin. Exp. Ophthalmol.* 240(3), 220–226 (2002).

157 Gomes JA, Jindal VK, Gormley PD, Dua HS. Phenotypic analysis of resident lymphoid cells in the conjunctiva and adnexal tissues of rat. *Exp. Eye Res.* 64(6), 991–997 (1997).

158 Knop N, Knop E. Ultrastructural anatomy of CALT follicles in the rabbit reveals characteristics of M-cells, germinal centres and high endothelial venules. *J. Anat.* 207(4), 409–426 (2005).

Immunological Reviews

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Epithelial barrier: an interface for the cross-communication between gut flora and immune system

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Summary: Large numbers of environmental antigens, including commensal bacteria and food-derived antigens, constitutively interact with the epithelial layer of the gastrointestinal (GI) tract. Commensal bacteria peacefully cohabit with the host GI tract and exert multiple beneficial or destructive effects on their host. Intestinal epithelial cells (IECs) constitute the first physical and immunological protective wall against invasive pathogens and a cohabitation niche for commensal bacteria. As the physiological homeostasis of IECs is maintained by multiple biological processes such as apoptosis, autophagy, and the handling of endoplasmic reticulum stress, the aberrant kinetics of these biological events, which have genetic and environmental causes, leads to the development of host intestinal pathogenesis such as inflammatory bowel disease. In addition, IECs recognize and interact with commensal bacteria and give instructions to mucosal immune cells to initiate an immunological balance between active and quiescent conditions, eventually establishing intestinal homeostasis. The mucosal immune system regulates the homeostasis of gut microbiota by producing immunological molecules such as secretory immunoglobulin A, the production of which is mediated by IECs. IECs therefore play a central role in the creation and maintenance of a physiologically and immunologically stable intestinal environment.

Keywords: *commensal bacteria, IECs, physiological inflammation*

Introduction

In our gastrointestinal (GI) tracts, 10^{13} to 10^{14} microorganisms peacefully cohabit with the mucosal immune system to establish an 'inside world' together (1). These microorganisms perform multiple functions for their host, such as preventing infections caused by pathogens, digesting and providing nutrients such as vitamin B and folate, and shaping the mucosal immune systems (2–4). Commensal bacteria diffuse in the GI tract, constantly encountering the host epithelial monolayer. The epithelial monolayer consists of several subsets of intestinal epithelial cells (IECs) including M cells, goblet cells, Paneth cells, enteroendocrine cells, and columnar epithelial cells. The epithelial monolayer generally separates the host from the external environment to avoid the hazards associated with the intrusion of undesired antigens, such as pathogenic bacteria and viruses, as well as some of commensal

but opportunistic bacteria. Although IECs create a physiological barrier, there is also a mucus layer that acts as a barrier against both harmless commensal and hazardous pathogenic bacteria. Disruption of this barrier system allows invasion of bacteria, which can lead to detrimental inflammation, destruction of the digestive tract, and conditions such as the establishment of infection and inflammatory bowel disease (IBD). In addition, commensal bacteria spatiotemporally direct the organization of the mucosal immune system, which is mediated by effector molecules produced by IECs. Although pathogens induce the expression of pro-inflammatory cytokines and chemokines in IECs, leading to the recruitment of mucosal innate immune cells that initiate acute and chronic inflammatory responses (5–7), in this review article we mainly describe the cellular and molecular crosstalk between commensal bacteria and IECs. This crosstalk is closely associated with the maintenance of the physiological and immunological homeostasis of the GI tract. The homeostatic condition of the mucosal immune system contributes to the maintenance of normal gut microbiota. From recent genome-wide association studies (GWASs), a set of genes involved in the homeostasis of IECs and the mucosal immune system has been reported as being closely associated with the prevention of IBD (8, 9).

The intestinal barrier system as a cohabitation and disease initiation site for commensal bacteria

Although commensal bacteria reside peacefully in the gut mucosa, they share common microbe-associated molecular patterns (MAMPs) with the pathogenic bacteria that invade though the intestinal epithelium (10). Commensal bacteria, therefore, have the potential to activate immune responses through pattern recognition receptors such as Toll-like receptors (TLRs) and nucleotide-oligomerization domain (NOD)-like receptors (NLRs) (10, 11). Mild or physiologically acceptable levels of stimulatory signals provided by commensal flora are essential for the development and maintenance of an appropriate mucosal immune system, which together with the epithelial barrier system provides the first line of defense against foreign undesired antigens (3). To avoid unnecessary and excessive active immune responses at the intestinal epithelium, a dual safety control system is employed: physical separation of commensal bacteria from the host, and negative regulation of extreme and active immune responses. Indeed, under normal conditions, immune responses to commensal bacteria are downregulated to avoid any pathological events but maintain a tremor condition for immediate triggering of necessary active immune responses against unwanted antigens, which is described as physiological inflammation (12, 13). For the creation and maintenance of physiological inflammation, two physically, chemically, and immunologically critical walls, the mucus layer and the intestinal epithelial layer, play vital roles. Commensal bacteria are thus separated from the host by the two layers of intestinal wall. At same time, these two segregation systems provide an environment suitable for cohabitation by commensal microorganisms.

Mucus layer

The GI epithelial layer is entirely covered by a thick and sticky mucus gel layer. The mucus layer consists of many bioactive molecules, including mucins, glycoproteins, trefoil factors, anti-microbial peptides, and secretory IgA (SIgA) antibodies (14). In general, the mucus layer can be separated into two layers, an inner and an outer layer, which are the hallmark of the GI tract (14–16). The thickness of these two layers varies in each part of the GI tract. The inner layer is approximately 50 μm thick, and the outer layer is approximately 100 μm thick in the murine colon (15, 17). The thickness of the mucus layer is dependent on commensal bacteria, as suggested by data showing that the outer mucus layer of germfree mice is thicker than that of bacteria-colonized mice (17). Protein content generally does not differ between the inner and outer layer; however, the concentration of mucin 2(Muc2) is higher in the inner layer than in the outer layer (17). This provides the inner mucus layer with the ability to firmly attach to the epithelial layer. Commensal bacteria usually colonize the outer mucus layer (17), suggesting that the inner layer has a potent defensive function. Indeed, unusual colonization by commensal bacteria is observed in *Muc2*-deficient mice, which lack an inner mucus layer (17). This abnormal localization, especially when the bacteria penetrate the crypt region, leads to the spontaneous development of acute followed by chronic inflammation that resembles human ulcerative colitis and adenomas in the small intestine (17–19), indicating the critical role of mucus and mucins in the exclusion of undesired intrusion of commensal bacteria. However, pathogenic bacteria have evolved to produce mucus-degrading enzymes such as glycosydases, which break down mucin oligosaccharides and digest mucus barriers to reach the host epithelial layer (14). Although specific commensal bacteria such as *Candidatus arthromitus*, which are commonly referred to as segmented filamentous bacteria (SFB), closely associate with murine ileal IECs (20), how these bacteria pass through the mucus layer to colonize the epithelial layer is not yet fully understood.

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Intestinal epithelial layer

The intestine is covered by a monolayer of epithelial cells. As well as constituting a physiological barrier, IECs produce digestive enzymes, absorb nutrients, and undertake the immunological functions of detecting and sampling antigens, secreting anti-microbial molecules, and shaping and guiding mucosal immune responses (21). To exert these functions, several specialized epithelial cell subsets, including M cells, goblet cells, Paneth cells, endocrine cells, and columnar epithelial cells, cooperatively form a physical and immunological network for the creation and maintenance of homeostasis between the environments inside and outside the intestine (*Fig.* 1).

IECs are constantly supplied from pluripotent stem cells residing in the crypt region of villi and secondary mucosa-associated lymphoid tissues (MALT) such as Peyer's Patches (PPs). Some of these crypt stem cells, now identified as leucine-rich repeat-containing G-protein-coupled receptor 5 (Lgr5)$^{+}$ cells (22), are capable of differentiating into goblet cells, enteroendocrine cells, or enterocytes during migration upwards from the crypt. Crypt stem cells differentiate into Paneth cells as well and migrate downwards to the bottom of the villi (23). Terminally differentiated goblet cells, enteroendocrine cells, and enterocytes reach the tip of the villi within a few days, undergo apoptosis, and are extruded into the intestinal lumen (24). Crypt stem cells residing in the base of PPs differentiate into columnar epithelial cells and M cells in regions of follicle-associated epithelium (FAE) that cover the PPs (25). It remains to be identified whether crypt stem cells residing in villi possess the same characteristics and differentiation kinetics as secondary MALT crypt stem cells.

M cells

M cells are mainly located in the regions of FAE that cover the secondary lymphoid structures associated with the mucosal immune system, such as PPs, colonic patches, isolated lymphoid follicles (ILFs), and nasopharynx-associated lymphoid tissues (NALTs) (26–29). M cells have unique morphological

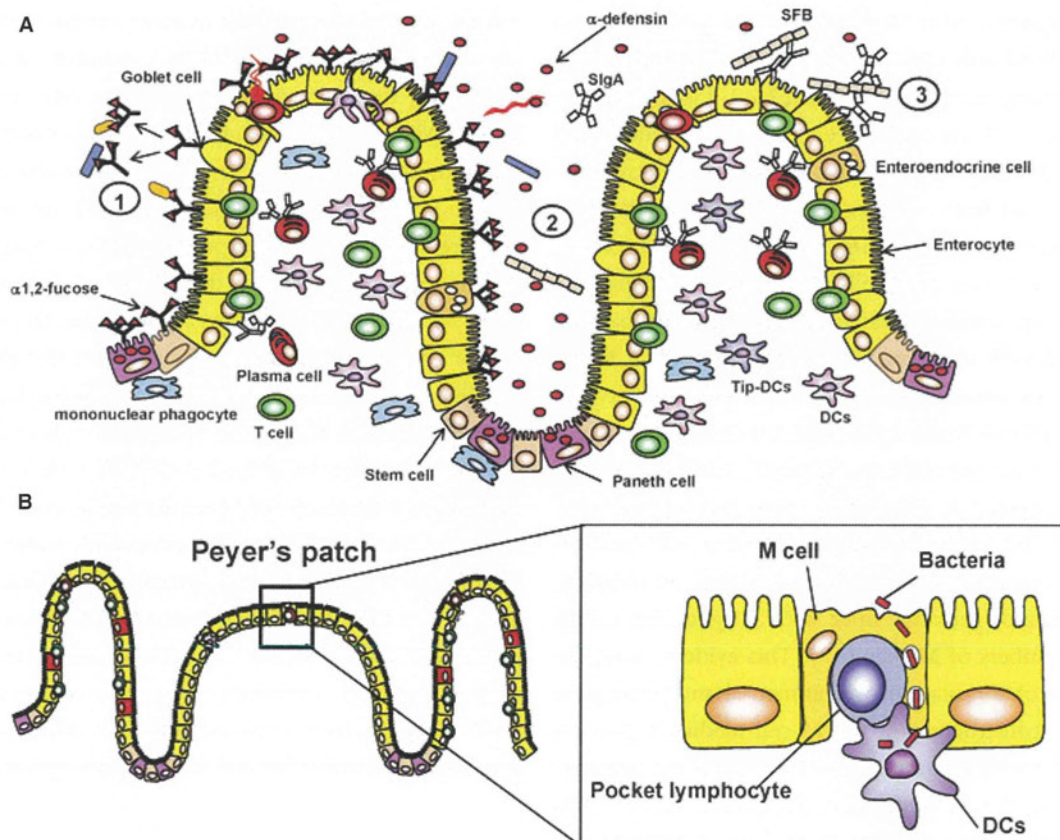

Fig. 1. Homeostasis of commensal bacteria is maintained by mucosal immune factors. (A) At the villous epithelial layer, ileal enterocytes express the terminally fucosylated carbohydrate moiety on their apical membrane and goblet cells produce fucosylated mucins that affect the homeostais of *Bacteroides* ①. Paneth cells produce anti-microbial molecules (e.g. α-defensin) ② and enterocytes transport and release secretary IgA (SIgA) into the luminal cavity ③. These factors inhibit the abnormal location and expansion of segmented filamentous bacteria (SFB). (B) Peyer's Patch M cells are specialized as antigen-sampling cells. Pathogenic and non-pathogenic commensal bacteria are taken up by M cells and initiate mucosal immune responses. DC, dendritic cell; Tip-DC, TNF-α/iNOS-producing dendritic cell.

features. Their apical cell membrane is covered by short, irregular microvilli that are totally different to those on columnar epithelial cells (*Fig. 1B*). These unique microvilli might enable microorganisms to access the apical surface of M cells. Indeed, many pathological agents are reported to invade through PP M cells (30). The other unique morphological feature of M cells is the pocket-like microfold structure located on the basal side of the plasma membrane that contains lymphocytes such as B cells, T cells, macrophages, and/or dendritic cells (DCs) (31) (*Fig. 1B*). These pocket lymphocytes, especially CCR6^{high} CD11c^{int} B cells, produce survival signals for M cells (32). Indeed, B-cell-deficient mice have been shown to possess few M cells in their PPs (31).

M cells also have unique glycosylation patterns on the epithelial surface. PP M cells express α(1,2)-fucose but not N-acetyl-D-glucosamine, which are detected by *Ulex europaeus* agglutinin-1 (UEA-1) and wheat germ agglutinin (WGA), respectively (33). Thus, M cells are histologically identified as UEA-1^{+}WGA^{-} cells. Monoclonal antibody NKM 16-2-4, which recognizes UEA-1 related α (1,2) fucose associated with M cells, was shown to recognize murine PP M cells but not goblet cells and columnar epithelial cells (34). It was recently shown that the α (1,2) fucose moiety recognized by NKM 16-2-4 is also used by villous M cells and fucosylated epithelial cells (described below) (35). Recently, glycoprotein 2 (GP2), a membrane-bound protein, has been reported as a new specific protein-associated marker of M cells (36). GP2 is expressed on the apical plasma membrane of M cells and interacts with FimH, a component of type I pilli expressed on Gram-negative bacteria such as *Escherichia coli* and *Salmonella typhimurium*, leading to the binding and transcytosis of bacteria (37). Interaction between GP2 and bacterial pilli has an important role in the initiation of antigen-specific immune responses against *S. typhimurium* (37).

Bacterial stimulation is involved in the induction and function of M cells. The number of M cells increases after conventionalization of specific-pathogen-free mice (38). In addition, mono-association of germfree mice with *S. typhimurium* results in increased numbers of M cells (39). This evidence suggests the importance of stimulation by commensal and pathogenic bacteria in the construction of the M cell-mediated gateway system for the mucosal immune systems. However, the specific bacterial signaling that initiates the differentiation of M cells from PP crypt stem cells has not yet been identified (31). TLR2- and TLR4-mediated bacterial signaling molecules such as the peptidoglycans and lipopolysaccharides (LPSs) promote the transcytosis of microparticles by M cells, although these MAMP–TLR signaling cascades do not influence on the development of M cells (31, 40).

In addition to the typical M cells located in the FAE covering MALT (e.g. PPs), cells known as villous M cells that share M cell traits have been found in the villous epithelium of the small intestine (41). Villous M cells share the morphological (short, irregular microvilli; microfold formation) and biological (UEA-1^{+}WGA^{-}) characteristics of classical PP M cells (41). The other major arm of the mucosal immune system, in addition to the digestive tract, is the respiratory tract. Analogous to the intestinal antigen-sampling system consisting of villous M cells, the epithelium of the nasal passage contains respiratory M cells distantly located from NALTs (e.g. in the turbinate portion of the nasal cavity) (42). Antigen sampling in the upper respiratory system is thus handled by classical M cells located in the FAE of NALTs (NALT M cells) and respiratory M cells. Identification of villous M cells and respiratory M cells further emphasizes the dynamism of the gateway and the antigen-sampling system of the aero-digestive mucosal immune system.

Administration of stress-inducing molecules such as dextran sodium sulfate (DSS), cholera toxin, or non-steroidal anti-inflammatory drugs has been shown to induce UEA-1^{+} cells in the intestine (35). Although UEA-1 positivity is one hallmark of M cells, these stress-induced cells are also co-stained with WGA (WGA^{+}), morphologically indistinguishable from typical columnar epithelial cells, and they possess less ability to sample bacteria than classical M cells (35). Importantly, these cells are negative for GP2 expression (35), a marker of M cells. We therefore propose that these cells are different from the other M cell family members, despite the fact that the cells harbor similar glycosylation characteristics that are recognized by UEA-1, and thus, should be named 'fucosylated columnar epithelial cells (F-ECs)' (*Table 1*). Goblet cells and Paneth cells have also been shown to react with UEA-1 (43). It is therefore difficult to distinguish between classical M cells, F-ECs, goblet cells, and Paneth cells by only using UEA-1 reactivity. GP2 is now considered an additional criterion for defining M cells, at least in the intestine. F-ECs interact with viruses and pathogenic and non-pathogenic bacteria through terminal fucose residues (44–48). Therefore, these terminal fucoses expressed on F-ECs might have a role as entry sites and might produce a suitable environment for symbiotic microorganisms.

Goblet cells

Goblet cells are secretory epithelial cells that produce mucin, trefoil peptides, Fcγ binding protein, and resistin-like molecule β (RELMβ), which are all major components of mucus (14). The percentage of goblet cells relative to the total

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Table 1. **Properties of M cells, villous M cells, and F-ECs of murine small intestine and nasal tissues**

	UEA-1	WGA	Microvilli	Pocket lymphocytes	Glycoprotein 2	NKM 16-2-4	Bacterial antigen sampling	Fucosultransferase
PP M cell	+	−	Irregular	+	+	+	+	Fut1
Villous M cell	+	−	Irregular	+	−	+	+	Fut2
F-ECs	+	+	Dense	−	−	+	−	Fut2
NALT M cell	+	−	Irregular	+	?	+	+	?
Respiratory M cell	+	−	Irregular	−	?	+	+	?

Well known morphological and physiological features of M cells are the short and irregular microvilli with pocket formation and antigen sampling/transporting capacity. Peyer's Patch (PP) M cells react with NKM16-2-4 and express Glycoprotein 2 (GP2), thereby typical murine M cells are identified as UEA-1$^+$, WGA$^-$, NKM16-2-4$^+$, and GP2$^+$ cells. Subsets of M cells including villous M cells, nasopharynx-associated lymphoid tissues (NALT) M cells, and respiratory M cells all have the same short and irregular microvilli and antigen sampling capacity as PP M cells. F-EC, fucoslated columnar epithelial cells; EC, epithelial cells; UEA-1, *Ulex europaeus* agglutinin-1; WGA, wheat germ agglutinin; Fut, fucosyltransferase.

number of epithelial cells increases from the duodenum (approximately 4%) to the descending colon (approximately 16%) (23). Fewer numbers and smaller sizes of goblet cells are detected in the cecum of germfree mice compared with conventional mice (49), suggesting that microflora might regulate the differentiation and maturation of goblet cells. Goblet cells have a pivotal role in regulating the localization of commensal bacteria in the intestinal lumen and in maintaining the homeostasis of the gut microenvironment by producing mucins.

Paneth cells

Paneth cells are located at the basal sites of crypts of Lieberkuhn in the small intestine, but they are not observed in the colon (23), although Paneth cell-related cells are recently observed in colon crypt region (50). Paneth cells contain a massive endoplasmic reticulum (ER) and Golgi network system that contains many cytoplasmic granules. These secretory Paneth cells discharge various antimicrobial factors (e.g. α-defensins, RELMβ, RegIIIγ) that might contribute to the prevention of bacterial invasion of the crypt regions (12, 51–54). In addition, it has been reported that Paneth cells create a niche for crypt stem cells by producing EGF, TGF-α, Wnt3, and Dll4, which are essential trophic molecules for the maintenance of Lgr5$^+$ stem cells (50).

As briefly indicated above, Paneth cells secrete a wide variety of bactericidal molecules such as defensins, lysozymes, cathelicidins, $sPLA_2$, Ang4, and c-type lectins such as RegIIIγ (55), which contribute to the protection of the gut epithelium from infection by enteric pathogens (51–54, 56). The expression of these bactericidal proteins is induced by the cellular components of pathogenic and non-pathogenic bacteria such as LPS and lipoteichoic acid, which are recognized by TLRs associated with Paneth cells (51, 57). α-Defensin is one of the major antimicrobial peptides produced by Paneth cells, and it protects against the invasion of pathogenic bacteria such as *S. typhimurium* (53, 54). In addition, ectopic expression of human α-defensin 5 restricts the residence of SFB, a well-known pathobiont in the mouse ileum. Genetic removal of α-defensin results in the aberrant expansion of SFB, leading to the induction of pro-inflammatory T-helper 17 (Th17) cells (58). Importantly, impaired production of α-defensin is observed in patients with IBD (59), suggesting that α-defensin and Paneth cells have bactericidal roles in the exclusion of pathogens and pathobionts and the prevention of undesired inflammation. Paneth cell-intrinsic myeloid differentiation factor 88 (MyD88) activity also mediates the induction of RELMβ, RegIIIβ, RegIIIγ, and CRP-ductin in response to commensal bacteria (60). Of these antimicrobial molecules, RegIIIγ, which is mediated by TLR4–MyD88 signaling cascades controlled by commensal bacteria-derived LPS, is critical in the prevention of infection by vancomycin-resistant *Enterococcus* (51).

Enteroendocrine cells

Enteroendocrine cells, like goblet cells, are a group of secretory epithelial cells scattered in the intestinal epithelium. The total number of enteroendocrine cells is estimated to be approximately 1% of the total number of IECs (61). These cells specialize in producing hormones and neuropeptides such as serotonin and secretin, which are important for mucosa-protecting functions such as luminal secretion (62). Although the interaction between microorganisms and enteroendocrine cells has not yet been extensively investigated, it is hypothesized that bacteria and enteroendocrine cells are involved in the communication between the gut and the nervous system. Post-infectious irritable bowel syndrome (PI-IBS), a GI disorder, is occasionally provoked after acute infection by pathogenic bacteria such as *Campylobacter*, *Shigella*, and *Salmonella* (63). One of the major clinical features of PI-IBS