3

Immune Defense at Mucosal Surfaces

Prosper N. Boyaka and Jerry R. McGhee

INTRODUCTION

Mucosal immune responses include a major B-cell component characterized by surface IgA-positive ($SIgA^+$) B-cells that become plasma cells which produce polymeric IgA antibody (Ab). In addition, both T-helper (Th) cells and cytotoxic T-lymphocytes (CTLs) are induced in mucosa-associated lymphoreticular tissues (MALT) *(1)*. These B- and T-cell responses can be induced by pathogens in organized mucosal inductive sites. In fact, the host has evolved a sophisticated network of cells and molecules that maintain the homeostasis of exposed mucosal surfaces *(1,2)*. This system, termed MALT, is anatomically and functionally distinct from the systemic counterpart and is strategically located at the portal of entry of most microorganisms, including specific pathogens. Prior to the development of acquired immune responses, the mucosa are protected by innate defenses including the physical barrier provided by epithelial cells, secreted molecules with antibacterial activity, and the cytolytic activity of natural killer (NK) cells. However, effective protection against virulent mucosal pathogens requires prophylactic immune responses that can be achieved by mucosal vaccines, which, in contrast to systemic vaccines, can trigger both mucosal and systemic immunity. A major challenge for the development of mucosal vaccines will be to overcome the natural tendency of the host to suppress immune responses to orally administered antigens, a state commonly termed oral tolerance. In addition, effective protection against infectious agents will require the development of safe mucosal vaccines capable of promoting targeted immune responses.

THE COMMON MUCOSAL IMMUNE SYSTEM

The mucosal immune system can be divided into organized secondary lymphoid tissue (which allows antigen sampling, uptake, and presentation for initiation of the mucosal immune response) and more diffuse collections of lymphoid cells constituting mucosal effector sites *(2)*. It now well established that Peyer's patches, appendix, and solitary lymphoid nodules in the gastrointestinal (GI) tract constitute the inductive sites of the gut-associated lymphoreticular tissues (GALT). Similarly, the tonsils and adenoids may represent the nasal-associated lymphoreticular tissues (NALT) in the upper airway and aerodigestive tracts. Organized bronchus-associated lymphoreticular tissues

From: *Immunotherapy for Infectious Diseases*
Edited by: J. M. Jacobson © Humana Press Inc., Totowa, NJ

(BALT) *(3)* were also described at airway branches of experimental animals such as rabbits, rats, and guinea pigs, but these structures rarely occur in humans *(4)*. Collectively, GALT and NALT in humans and GALT, BALT, and NALT in experimental species are termed MALT. The mucosal effector tissues include the interstitial tissues of all exocrine glands, e.g., mammary, lacrymal, salivary, and sweat glands, as well as the lamina propria and the epithelium of the GI tract. In addition, lamina propria areas of the upper respiratory and genitourinary tracts are effector sites of this enormously large immune network. MALT is connected with effector sites through migratory patterns of lymphoid cells. Thus, immune effector cells initiated by encounter with antigen at one mucosal inductive site can migrate to distant mucosal effector sites, where they will exert their effector functions. The existence of this interconnected system of inductive and effector sites has been termed the common mucosal immune system (CMIS).

Mucosal Inductive Sites

Peyer's Patches of the GALT

The columnar epithelium that covers the MALT is infiltrated with B- and T-lymphocytes and antigen-presenting cells (APCs), which has led to the term follicle-associated epithelium (FAE). Soluble and particulate lumenal antigens are taken up by a microfold or M cell and delivered to adjacent APCs. M-cells have been described in human Peyer's patches, appendix, and tonsils *(5)*. These cells appear to be ideal for antigen uptake *(6)*. However, M-cells that only contain sparse numbers of lysosomes *(7)* probably do not degrade ingested antigens and thus are not classical APCs *(8)*. M-cells serve as the entry points for uptake; as such they actively ingest soluble proteins as well as particulate antigens, which can include viruses, bacteria, small parasites, and microspheres *(6,9–11)*. In addition to serving as a means of transport for lumenal antigens, the M-cells also provide an entry pathway for pathogens. A recent study suggested that lymphocytes and especially B-cells possess signaling molecules that induce M-cell differentiation of epithelial cells. In this study, mouse Peyer's patch T- and B-cells as well as a human B-cell line (Raji) induced Caco-2 cells to differentiate into M-like cells *(12)*.

Peyer's patches contain a dome region underneath the FAE, as well as underlying follicles that contain five or more germinal centers *(13)*. The dome region is characterized by the presence of T- and B-cells as well as both macrophages and dendritic cells (DCs). The presence of all three major APC types in the dome, e.g., memory B-cells, MØ and DCs make it likely that antigen uptake occurs immediately after release from M cells. Furthermore, Peyer's patch germinal centers differ from those in peripheral lymph nodes and spleen in that relatively high frequencies of $SIgA^+$ B-cells predominate *(14-17)*.

The regulation of Peyer's patch formation in mammals is only partially understood; nevertheless, recent studies suggest that interactions of membrane lymphotoxin (LT)/tumor necrosis factor (TNF) cytokines with LT-β receptor are of central importance in Peyer's patch development *(18,19)*. For example, injection of pregnant mice with lymphotoxin β-receptor Ig (LT-β-R-Ig) fusion protein resulted in loss of Peyer's patches *(19)* and most lymph nodes except the mesenteric lymph nodes. Recent studies with this model showed that Peyer's patches are not strictly required for the induc-

tion of mucosal S-IgA Ab responses and suggest a role for mesenteric lymph nodes as alternative inductive sites in the GI tract. Indeed, S-IgA Abs were induced when mice from LT-β-R-Ig-treated mothers were orally immunized with cholera toxin (CT) and a soluble protein antigen *(20)*. In contrast, neither systemic nor mucosal S-IgA Ab responses were seen after administration of the same oral vaccine regimen to TNF-α and LT-α double knockout mice that lack both Peyer's patches and mesenteric lymph nodes *(20)*. However, Peyer's patches appear to be crucial for the development of oral tolerance to protein antigens since mice from LT-β-R-Ig-treated mothers showed impaired induction of this type of tolerance *(21)*.

Other Mucosal Inductive Sites

The NALT includes the palatine, lingual, and nasopharyngeal tonsils, which collectively create a ring of tissue (Waldeyer's ring) that is strategically positioned at the entry of the digestive and respiratory tracts. These tissues possess structural features resembling both lymph nodes and Peyer's patches, including an FAE with M-cells in tonsillar crypts that are essential for selective antigen uptake. In addition, germinal centers containing B-cells, and professional APCs are also present. Direct unilateral injection of antigens (cholera toxin B subunit [CT-B] and tetanus toxoid [TT]) into the tonsil of human volunteers resulted in the induction of mucosal immune responses manifested by the appearance of antigen-specific IgG- and (to a lesser degree) IgA-producing cells in the noninjected tonsil *(22)*. These studies suggest that the tonsils may serve as an inductive site, analogous to Peyer's patches. Several recent nasal immunization studies have emphasized the importance of the NALT for induction of both mucosal and systemic immune responses that may exceed in magnitude those induced by oral immunization *(22–30)*.

Follicular structures analogous to Peyer's patches are also found in the large intestine, with especially pronounced accumulations in the rectum. In fact, monkeys immunized intrarectally with simian immunodeficiency virus (SIV) developed both T- and B-cell-mediated immune responses, including the induction of anti-SIV Abs in rectal washes and genital secretions *(31,32)*. Similarly, mice immunized intrarectally with CT or recombinant vaccinia virus expressing gp120 of SIV exhibited Abs responses in genital tract secretions as well as in serum; this immunization route was frequently superior to either the intragastric or intravaginal route *(33)*.

Homing of Effector Lymphocytes into Mucosal Compartments

Early studies in rabbits showed that GALT B-cells repopulated the gut with IgA plasma cells, suggesting a direct connection for B-cell migration between Peyer's patches and GI tract lamina propria *(34,35)*. Furthermore, orally immunized experimental animals possessed antigen-specific precursors of IgA plasma cells in GALT-associated mesenteric lymph nodes, which repopulated the lamina propria of the gut and the mammary, lacrymal, and salivary glands *(36–39)*. These studies, when combined with others showing that oral immunization led to S-IgA antibodies in multiple mucosal sites, served as the basis for suggesting a "common" mucosal immune system in humans *(40–42)*. Studies in recent years have unveiled molecular mechanisms involved in the migration of immune cells into the GI tract and, to a lesser extent, homing into other mucosal effector sites.

Lymphocyte Homing in the GI Tract

Naive lymphocytes enter mucosal or systemic lymphoid tissues from the blood through the endothelium via specialized high endothelial venules (HEVs) *(43)*. In GALT, HEV are present in the interfollicular zones rich in T-cells *(44)*. The mucosal addressin cell adhesion molecule-1 (MAdCAM-1) is the major addressin expressed by Peyer's patch HEV *(45)*. The major homing receptors expressed by lymphocytes are the integrins, which represent a large class of molecules characterized by a heterodimeric structure of α and β chains. In general, expression of the α4 chain paired with either β1 or β7 integrins differentiates between homing receptors for the skin or gut, respectively. Thus, the α4β1 pair allows binding to vascular cell adhesion molecule-1 (VCAM-1) and is associated with homing to inflamed sites and skin *(46,47)*. Pairing of α4 with β7 represents the major integrin molecule responsible for lymphocyte binding to MAdCAM-1 expressed on HEVs in Peyer's patches *(48)*. A number of studies have now established that MAdCAM-1 is the major mucosal homing receptor ligand *(48-50)*. In addition to α4β7 integrin, L-selectin, which also binds to carbohydrate-decorated MAdCAM-1, is an important initial receptor for homing into GALT HEVs. Interestingly, L-selectin is expressed on all naive lymphocytes; however, memory T- and B-cells can be separated into $\alpha4\beta7^{hi}$, L-selectin^{+}, and L-selectin^{-} subsets *(51)*.

It is now clear that chemokines are directly involved in lymphocyte homing and that they trigger arrest and cell activation via specific Gsαi receptors *(52)*. For example, loss of secondary lymphoid tissue chemokine (SLC) results in lack of naive T-cell or dendritic cell migration into the spleen or Peyer's patches *(53)*. Furthermore, thymus-expressed chemokine (TECK) mediated human memory T-cell migration into the lamina propria of the GI tract. In fact, the gut homing $\alpha4\beta7^{hi}$ T-cells expressed a TECK receptor, designated G-protein-coupled receptor-9-6, or CCR-9 *(54)*. Interestingly, human $\alpha E\beta7^{+}$ as well as $\alpha4\beta7^{hi}$ CD8 T-cells expressed CCR-9, suggesting that TECK-CCR-9 is also involved in lymphocyte homing and arrest of intraepithelial lymphocytes (IELs) into the GI tract epithelium *(54)*.

Lymphocyte Homing in NALT and Lung-Associated Tissues

Unlike Peyer's patch HEVs which are found in T-cell zones, murine NALT HEVs are found in B-cell zones and express, peripheral node addressin (PNAd) either alone or associated with MAdCAM-1 *(55)*. Furthermore, anti-L-selectin but not anti-MAdCAM-1 Abs blocked the binding of naive lymphocytes to NALT HEV, suggesting predominant roles for L-selectin and PNAd in the binding of naive lymphocytes to these HEVs *(55)*. In a rat model of antigen-induced lung inflammation, the percentage of activated T-cells expressing α4 was increased in the bronchial lumen compared with blood and lymph node T-cells after antigen challenge *(56)*. An interesting approach used to address the homing of human cells in the NALT was the analysis of tissue-specific adhesion molecules after systemic, enteric, or nasal immunization *(57)*. This study showed expression of L-selectin by most effector B-cells induced by systemic immunization, with only a small proportion expressing α4β7; the opposite was seen after enteric (oral or rectal) immunization. Interestingly, effector B-cells induced by intranasal immunization displayed a more promiscuous pattern of adhesion molecules, with a large majority of these cells expressing both L-selectin and α4β7 *(57)*.

IMMUNE RESPONSES IN MUCOSAL SURFACES

Mucosal Innate Immune Responses

In the mucosa, innate defense includes the physical barrier provided by epithelial cells and cilia movement, mucus production, secreted molecules with antibacterial activity, and the cytolytic activity of NK cells. Recent studies have demonstrated that a number of innate molecules produced at mucosal surfaces (including cytokines, chemokines, and defensins) can provide the necessary signals to enhance systemic or both systemic and mucosal immunity to antigens.

Barriere Function of Epithelial Cells

Mucosal surfaces are covered by a layer of epithelial cells that prevent the entry of exogenous antigens into the host. The physical protection of the largest mucosal surface, i.e., the GI tract, involves a monolayer of tightly joined absorptive epithelial cells termed enterocytes, which constitute a highly specialized selective barrier that allows the absorption of nutrients while preventing the entry of pathogens *(2)*. The barrier effect of intestinal epithelial cells is facilitated by the mucus blanket that covers these cells and prevents the penetration of microorganisms and the diffusion of molecules toward the intestinal surface. Mucus resembles glycoprotein and glycolipid receptors that occur on enterocyte membranes, tending to interfere with the attachment of microorganisms. The barrier effect of the epithelial surface is ensured by the continuous renewal of the epithelial cell layer. By this process, which results in complete renewal of the absorptive enterocyte layer every 2–3 days, damaged or infected enterocytes are replaced by crypt epithelial cells, which differentiate into enterocytes as they migrate toward the desquamation zone at the villus tip. The epithelia of other mucosal surfaces (including the oral cavity, pharynx, tonsils, urethra, and vagina) are made of stratified epithelial cells that lack tight junctions. However, the renewal of exposed epithelial cell layers by cells from subjacent layers and mucus secretion contribute to the permeability barrier effect on these surfaces as well.

Mucosal Antimicrobial Peptides

Epithelial cells also secrete antimicrobial peptides such as defensins, inflammatory cytokines, and chemokines, which contribute to mucosal innate immune responses. In this regard, the human intestinal α-defensins (HDs) HD-5 and HD-6 were identified in intestinal Paneth cells and in the human reproductive tract *(58)*. The α-Defensin are also secreted by tracheal epithelial cells, and they are homologous to peptides that function as mediators of nonoxidative microbial cell killing in human neutrophils (termed human neutrophil petide [HNPs]) *(59,60)*. The β-defensins, and in particular human β-defensin-1 (hBD-1), are expressed in the epithelial cells of the oral mucosa, trachea, and bronchi, as well as mammary and salivary glands in humans *(61-63)*. Human intestinal epithelial cells were reported to express hBD-1 constitutively, whereas hBD-2 was only seen in inflamed colon or after bacterial infection of a colonic epithelial cell line *(64)*. Secretory phospholipase A2 (S-PLA2) is an antimicrobial peptide present in granules of small intestinal Paneth cells and human polymorphonuclear neutrophils (PMNs). The S-PLA2 molecule is released by Paneth cells upon exposure to cholinergic agonists, bacteria, or lipopolysaccharide (LPS). High concentrations of

S-PLA2 are also found in human tears. In contrast to other PLA2 molecules produced by mammalian cells, the S-PLA2 preferentially removes bacterial phosphatidyl glycerol and phosphatidyl ethanolamine, a property that can explain the potent antimicrobial activity of S-PLA2 *(65,66)*. Other antimicrobials produced of mucosal surfaces include lysozyme, peroxidases, cathelin-associated peptides, and lactoferrin. In this regard, lactoferrin was recently reported to inhibit HIV-1 replication at the level of viral fusion/entry *(67)*.

Proinflammatory Cytokines and Chemokines

It is now well established that epithelial cells produce proinflammatory cytokines, including interleukin (IL)-1, IL-6, tumor necrosis factor-α (TNF-α), and granulocyte/macrophage colony-stimulating factor (GM-CSF) in response to pathogen invasion *(68,69)*. Interestingly, epithelial cells also express CxC and CC chemokines. For example, bacterial or parasitic (i.e., *Cryptosporidium parvum*) infections of intestinal epithelial cells were shown to upregulate expression and secretion of the CxC chemokines IL-8 and GRO-α *(70)*. Bacterial infection of intestinal epithelial cell lines was also reported to stimulate the expression of the CC chemokines monocyte chemotactic protein-1 (MCP-1), RANTES, and macrophage inflammatory protein-3α (MIP-3α) *(71,72)*, and freshly isolated colon epithelial cells produced an array of chemokines similar to the cell lines, as well as MIP-1α and MIP-1β *(71)*. More recently, inflammatory protein-10 (IP-10) and monokine inducible by interferon-γ (IFN-γ) (MIG), which are CxC chemokines that are known to attract $CD4^+$ T-cells, were detected in normal intestinal epithelial cells, and their expression was upregulated by infection with invasive bacteria or stimulation with proinflammatory cytokines *(73)*. Furthermore, γδ T-cell receptor-positive (TCR^+) (IELs) produce the C-type chemokine lymphotactin, which is chemotactic for T-cells and NK cells but not for monocytes, neutrophils, or dendritic cells *(74,75)*. Taken together, these studies clearly indicate that the mucosal epithelium has the potential to produce a large spectrum of C, CC, and CxC chemokines and that both epithelial cells and intestinal lymphocytes can contribute to these innate responses.

Mucosal Natural Killer Cells

NK cells are major players in the innate immune system, especially in the GI tract. NK cells occur in both the lamina propria and the intraepithelial compartment as large granular lymphocytes *(76,77)*. Studies performed on human IELs have shown that the αEβ7 integrin is the main surface molecule involved in the lysis process *(77)*. Significant increases in intestinal IEL NK cell activity were seen during the early phase of secondary infection of chickens with the *Eimeria* parasite *(78)*. Furthermore, nonspecific recruitment of cytotoxic effector cells into the intestinal mucosa of enteric virus-infected mice has been reported *(79)*. Humans with inherited deficiency of NK cells experience more severe herpesvirus infections *(80);* however, these individuals clear the virus infection in a fashion comparable to that seen in immunocompetent subjects, suggesting that the role of NK cells may be to limit the extent of certain mucosal viral infections. Finally, NK cells are known to secrete interferon-γ (IFN-γ) and IL-4 after infection. Thus, mucosal NK cells could be major players in the cytokine environment that influences the development of effector T-cells.

Mucosal Adaptive Immune Responses

Cytokines In Mucosal Immunity

It is now well accepted that the functional diversity of the immune response is exemplified by an inverse relationship between antibody and cell-mediated immune responses. This dichotomy is due to Th cell subsets, which are classified as either Th1 or Th2 according to the pattern of cytokines produced *(81)*. Thus, Th1 cells produce IL-2, IFN-γ and lymphotoxin-α (LT-α, also known as TNF-β), LT-β and TNF-α, and Th2 cells produce IL-4, IL-5, IL-6, IL-9, IL 10, and IL-13. The cytokine environment plays a key role in the differentiation of both Th cell subsets from precursor Th0 cells. IL-2 is produced by Th0 cells upon antigen exposure and serves as an important growth factor. IL-12 induces NK cells to produce IFN-γ *(82,83)*, which, together with IL-12, triggers Th0 cells to differentiate along the Th1 pathway. Murine Th1-type responses are associated with development of cell-mediated immunity as manifested by delayed-type hypersensitivity (DTH) as well as by B-cell responses with characteristic IgG Ab subclass patterns.

For example, IFN-γ induces murine μ → γ2a switches *(84)* and production of complement-fixing IgG2a antibodies. On the other hand, IL-4 production induces Th0 → Th2-type development. The production of IL-4 by Th2 cells is supportive of B-cell switches from sIgM expression to $SIgG1^+$ and to $sIgE^+$ B-cells *(85–87)*. Furthermore, the Th2 cell subset is an effective helper phenotype for supporting the IgA isotype in addition to IgG1, IgG2b, and IgE responses in the mouse system. Both Th1 and Th2 cells are also quite sensitive to cross-regulation. IFN-γ produced by Th1 cells inhibits both Th2 cell proliferation and B-cell isotype switching stimulated by IL-4 *(88,89)*. Likewise, Th2 cells regulate Th1 cell effects by secreting IL-10, which inhibits IFN-γ secretion by Th1 cells. This decreased IFN-γ production allows development of Th2-type cells. It is also clear that Th1- and Th2-type cells express distinct patterns of chemokine receptors *(90,91)*. Thus, CCR5 and the CxC chemokine receptors CxCR3 and CxCR5 are preferentially expressed by human Th1 cell clones, whereas Th2 cells express CCR4 and to a lesser extent CCR3 *(91,92)*.

Studies in the last decade have shown that two Th2 cytokines, IL-5 and IL-6, are of particular importance for inducing $SIgA^+$ B-cells to differentiate into IgA-producing plasma cells *(93-95)*. In this regard, IL-6 induced strikingly high IgA responses in vitro in both mouse *(93–95)* and human *(96)* systems. However, the role of IL-6 in IgA responses in vivo remains to be demonstrated since both reduced *(97)* and normal IgA responses were reported in $IL\text{-}6^{-/-}$ mice *(98)*. IL-10 has also been shown to play an important role in the induction of IgA synthesis, especially in humans *(99-101)*. Finally, high frequencies of Th2 cells producing IL-5, IL-6, and IL-10 were shown in mucosal effector sites (e.g., the intestinal lamina propria and the salivary glands) where IgA responses predominate *(102,103)*.

Secretory IgA Antibodies

The S-IgA Abs constitute the predominant isotype present at mucosal surfaces, and they are the first Abs to come into contact with the microorganisms that have entered the host through the mucosae. Inhibition of microbial adherence is a critical initial step for the protection of the host and is mediated by both specific and nonspecific

mechanisms. For instance, the agglutinating ability of S-IgA specific to capsular polysaccharide of *Hemophilus influenzae* seems to be crucial for avoiding colonization by *H. influenzae (104)*. Finally, another nonspecific mechanism that inhibits microbial adherence is owing to the presence of carbohydrate chains on the S-IgA molecule that bind to bacteria or other antigens *(105–107)*. The S-IgA Abs have been shown to be effective at neutralizing viruses at different steps in the infectious process. In particular, S-IgA specific for influenza hemagglutinin can interfere with the initial binding of influenza virus to target cells or with the internalization and the intracellular replication of the virus *(108)*. The S-IgA can neutralize the catalytic activity of many enzymes of microbial origin (such as neuraminidase, hyaluronidase, glycosyltransferase and IgA-specific protease), as well as the toxic activity of bacterial enterotoxins (cholera toxin and the related heat-labile enterotoxin of *E. coli*). In vitro experiments employing murine polarized epithelial cells have demonstrated that antibodies specific to rotavirus and hepatitis virus can neutralize the respective viruses inside the epithelial cells *(109,110)*, and evidence has been provided that similar mechanisms occur in vivo *(111)*. Similarly, it has been shown that transcytosis of primary HIV isolates is blocked by polymeric IgA specific to HIV envelope proteins *(112)*. These authors have shown that neutralization of HIV transcytosis occurs within the apical recycling endosome and that immune complexes are specifically recycled to the mucosal surface *(112)*.

It should be mentioned that S-IgA appears to be important in limiting inflammation at mucosal surfaces. In fact, IgA Abs are unable to activate complement and interfere with IgM- and IgG-mediated complement activation *(113,114)*. Furthermore, S-IgA inhibits phagocytosis, bactericidal activity, and chemotaxis by neutrophils, monocytes, and macrophages. In addition, IgA can downregulate the synthesis of TNF- α and IL-6 as well as enhance the production of IL-1R antagonists by LPS-activated human monocytes *(115,116)*.

Mucosal Cytotoxic T-Lymphocytes

There is a clear demarcation between inductive sites, which harbor precursor (p)CTLs, and effector sites, which include the lamina propria and the epithelial cells where activated $CD8^+$ CTLs function. it is now established that administration of virus into the GI tract results in a higher frequency of pCTL in Peyer's patches *(117,118)*. For example, reovirus localizes to T-cell regions and is clearly associated with increased $CD8^+$ pCTLs and memory B-cell responses *(119)*. Oral administration of *Vaccinia* to rats resulted in the induction of virus-specific CTLs in Peyer's patches and mesenteric lymph nodes *(120)*. These findings suggest that after enteric infection or immunization, antigen-stimulated CTLs are disseminated from Peyer's patches into mesenteric lymph nodes via the lymphatic drainage *(120)*. Furthermore, virus-specific CTLs are also generated in mucosa-associated tissues by oral immunization with reovirus and rotavirus *(117,118)* and a high frequency of virus-specific CTLs is present in the Peyer's patches as early as 6 days after oral immunization. These studies suggest that oral immunization with live virus can induce antigen-specific CTLs in both mucosal inductive and effector tissues for mucosal responses and in systemic lymphoid tissues as well.

The vaginal infection model of rhesus macaques with SIV has been useful in studies of immunity to SIV in the female reproductive tract *(121,122)*. Recent studies in this model have provided direct evidence that pCTLs occur in female macaque repro-

ductive tissues and that infection with SIV induces CTL responses *(123)*. This important finding has now been extended to vaginal infection with an SIV/HIV-1 chimeric virus (SHIV) containing HIV-1 89.6 env gene *(124)*. Interestingly, all macaques resisted two challenges with virulent SIV, and functional, gag-specific CTLs were present in the peripheral blood *(124)*. Again, it should be emphasized that vaginal Abs were also induced; however, these results clearly indicate that mucosal CTL responses may be of importance in immunity to SIV infection. Recent work has shown that intranasal immunization with SIV/HIV components induces antibody responses in vaginal secretions (reviewed in ref. *125*). It should be noted that intranasal immunization of mice with HIV-1 T-cell epitopes and the mucosal adjuvant CT induced functional CTLs *(126)*. This evidence suggests that mucosal delivery of SIV/HIV components can induce mucosal CTLs that will contribute to immunity.

MUCOSAL ADJUVANTS AND DELIVERY SYSTEMS

Since immune effector cells initiated by triggering mucosal inductive sites can migrate to the systemic compartment and to distant mucosal sites, mucosal administration of vaccines represents an attractive strategy for provision of immunity in both the mucosal and systemic compartments. Unfortunately, probably because the mucosal surfaces are continuously exposed to a myriad of exogenous antigens, most protein antigens are poorly immunogenic when given mucosally. Furthermore, oral delivery of antigen can instead result in immunologic unresponsiveness (oral tolerance). Therefore, adjuvants or antigen delivery systems are needed to ensure the development of effective immune responses to mucosally delivered antigens. For reasons still to be elucidated, classic systemic adjuvants such as alum are unable to stimulate mucosal S-IgA Ab responses. Unlike many protein antigens, the bacterial enterotoxin CT is highly immunogenic when administered by mucosal routes *(127)*. Furthermore, CT and the related heat-labile toxin (LT)-I from *E. coli* are effective adjuvants that promote mucosal and systemic immune responses to coadministered antigens *(128–130)*. However, the toxicity of these molecules precludes their use in humans. Recombinant attenuated bacterial and viral vectors were found to be effective mucosal delivery systems for induction of mucosal immunity *(131,132)*. Again, however, toxicity issues will need to be addressed before their use in humans. Some of the strategies to develop safe mucosal vaccines are discussed below.

Nontoxic Enterotoxin Derivatives

Although CT and LT were identified as effective mucosal adjuvants, the enterotoxicity of these molecules has precluded their use in human vaccines. The main strategy undertaken to make these molecules more suitable for use in humans consisted of developing mutants that lack the adenosine diphosphate (ADP) ribosyl transferase activity of the native toxins. Other approaches include substitution of the B subunit by a B-cell targeting moiety and the covalent binding of protein antigens to CT-B or LT-B.

ADP-Ribosylation-Defective Mutants of CT and LT

Mutants defective in ADP-ribosyl transferase activity were generated by single amino acid substitutions in the ADP-ribosylation activity site of the A subunit of CT or LT or in the protease-sensitive loop of LT. In this regard, cholera toxin is a heterologous macromolecule consisting of two structurally and functionally separate A and B

subunits *(133,134)*. The B subunit of CT consists of five identical 11.6-kD peptides that bind to GM1 gangliosides *(135)*. The binding of CT-B to GM1 ganglioside on epithelia allows the A subunit to reach the cytosol of target cells, where it binds to nicotinamide (N) ADP and catalyzes the ADP ribosylation of Gsα protein. The later guanosine triphosphate (GTP) binding protein activates adenyl cyclase with subsequent elevation of cyclic adenosine monophosphate (cAMP) in epithelial cells followed by secretion of water and chloride ions into the intestinal lumen *(136)*. The labile toxin from *E. coli* is closely related to CT, and the two enterotoxins share 80% amino acid sequence homology *(137)*. Although both CT and LT bind GM1 gangliosides, LT also exhibits an affinity for GM2 and asialo-GM1 *(134)*.

Two CT mutants were contructed by substitution of serine by phenylalanine at position 61 (CT-S61F) and glutamate by lysine at position 112 (CT-E112K) in the ADP-ribosyl transferase activity center of the CT gene from *Vibrio cholerae* 01 strain GP14. Similar substitutions in LT have been shown to inactivate ADP-ribosyl-transferase activity and enterotoxicity completely *(138,139)*. The levels of antigen-specific serum IgG and secretory IgA Abs induced by the mutants are comparable to those induced by wild-type CT and are significantly higher than those induced by recombinant CT-B *(140, 141)*. Furthermore, the mutant CT-E112K, like nCT, induces Th2-type responses through a preferential inhibition of Th1-type $CD4^+$ T-cells, and both nCT and mCTs enhanced the expression of costimulatory molecules of the B7 family and their corresponding receptors *(142,143)*. Mutations in other sites of the CT molecule were reported to induce nontoxic derivatives, but the adjuvant activity was also affected. For example, the CT-106S mCT, with a partial knockout of the ADP-ribosylating activity, exhibited an adjuvant activity lower that that of wild-type CT *(144)*.

Mutant LT molecules with either a residual ADP-ribosyltransferase activity (e.g., LT-72R) or totally devoid of such enzymatic activity (e.g., LT-7K and LT-63K) can function as mucosal adjuvants when intranasally administered to mice together with unrelated antigens *(26,145,146)*. When mLTs were tested as mucosal adjuvants, they generally induced mucosal and systemic Ab responses comparable to those of nLTs, although higher doses of mLT were often needed *(147)*. Since LT induces a mixed $CD4^+$ Th1- (i.e., IFN-γ) and Th2-type (i.e., IL-4, IL-5, IL-6, and IL-10) response *(148)*, one might envision the use of mutants of LT where both Th1- and Th2-type responses are desired.

Other CT and LT Derivatives

It has also been hypothesized that the strong toxic effect of CT and LT could be largely owing to their promiscuous binding to cells via their B subunits. This assumption led to the construction of a fusion protein consisting of CTA1 and two Ig binding domains (DD) of staphylococcal protein A, which binds IgG, IgE, IgA, and IgM *(149)*. The CTA1-DD fusion protein displayed adjuvant activity when given by the nasal route and promoted both mucosal and systemic immune responses *(149)*. More detailed analyses of CTA1-DD adjuvanticity have shown that this CT derivative promotes both T-cell-dependent and -independent responses and that both the ADP-ribosylation and Ig binding activities were required *(150,151)*.

Another approach used to develop nontoxic derivatives of CT consisted of genetically substituting the entire CT-A subunit, or the toxic CT-A1 portion, with a protein antigen. Thus, nasal or oral immunization with the chimeric fusion protein made of

CT-B/A2 and a *Streptococcus mutans* protein adhesin elicited antigen-specific mucosal and systemic immunity *(152)*. Similarly, nasal immunization with CT-B conjugated to a *Schistosoma mansoni* antigen protected infected animals from schistosomiasis *(153)*. It is important to note that mucosal administration of low doses of antigen coupled to CT-B was shown to induce tolerance *(154,155)*. Thus, caution is recommended when using antigen coupled to CT-B for induction of mucosal immunity or tolerance.

Cytokines and Chemokines as Mucosal Adjuvants

The use of cytokines and chemokines to enhance the immune responses to mucosal vaccines is an attractive strategy for several reasons. First, cytokines and chemokines act by often known mechanisms through specific interactions with corresponding receptors. Furthermore, whereas important adverse effects are often associated with large and repeated parenteral cytokine doses generally required for the effective targeting of tissues/organs, only low serum cytokine levels are achieved after mucosal delivery of these regulatory molecules *(156)*. Finally, cytokines/chemokines that influence the development of Th cell subsets can help promote targeted Th1-type responses for protection against intracellular pathogens or Th2-type responses required for protection against soluble antigens and toxins.

The cytokines IL-1, IL-6, and IL-12 were recently tested for their ability to enhance mucosal and systemic immune responses to nasal vaccines. A nasal vaccine of TT given with either IL-6 or IL-12 induced serum TT-specific IgG Ab responses that protected mice against lethal challenge with tetanus toxin, suggesting that both IL-6 and IL-12 can enhance protective systemic immunity to mucosal vaccines *(157)*. Furthermore, IL-12 but not IL-6 as an adjuvant induced high titers of S-IgA Ab responses in the GI tract, vaginal washes, and saliva *(157)*. In another system, mice nasally immunized with soluble influenza H1 and N1 proteins and IL-12 developed anti-influenza systemic and mucosal immunity, further demonstrating that nasal IL-12 does not require additional stimuli for induction of S-IgA Ab responses *(158)*. Nasal administration of protein antigens with IL-1 also enhanced systemic and mucosal immune responses to coadministered antigens *(159)*. As an illustration of the potential of regulatory cytokines to promote targeted immunity, IL-12 was shown to redirect CT-induced antigen-specific Th2-type responses toward the Th1-type when given by oral *(160)* or intranasal routes *(156)*. In addition, IL-12 could also promote both Th1- and Th2-type responses when administered by a separate mucosal route than a vaccine regimen containing CT as an adjuvant *(156)*.

As mentioned above, a number of innate molecules are secreted in mucosal epithelia. To test whether these molecules could provide signals to bridge the innate and adaptive mucosal immune systems, protein antigens were given nasally with α-defensins, (i.e., HNPs) *(161)*, lymphotactin *(162)*, or RANTES *(163)*. All of these vaccine regimens were found to promote systemic immune responses to the coadministered antigen *(161–163)*. Furthermore, whereas defensins failed to promote mucosal S-IgA Ab responses, significant S-IgA Abs were induced by the CC chemokine RANTES and the C chemokine lymphotactin *(161–163)*. The adjuvant activity of lymphotactin resulted in Th1- and Th2-type responses, whereas only Th1- and selected Th2-type cytokines were produced by RANTES-induced $CD4^+$ Th cells *(162,163)*.

Immunostimulating DNA Sequences and Saponin Derivatives

Immunostimulatory DNA Sequences

Bacterial but not eukaryotic DNA contain immunostimulatory sequences consisting of short palindromic nucleotides centered around a CpG dinucleotide core, e.g., 5′-purine-purine-CG-pyrimidine-pyrimidine-3′ or CpG motifs *(164)*. It is now clear that CpG motifs can induce B-cell proliferation and Ig synthesis as well as cytokine secretion (i.e., IL-6, IFN-α, IFN-β, IFN-γ, IL-12, and IL-18) by a variety of immune cells *(165)*. Since CpG motifs create a cytokine microenvironment favoring Th1-type responses, they can be used as adjuvants to stimulate antigen-specific Th1-type responses or to redirect harmful allergic or Th2-dominated autoimmune responses. Indeed, coinjection of bacterial DNA or CpG motifs with a DNA vaccine or with a protein antigen promotes Th1-type responses even in mice with a preexisting Th2-type of immunity *(166,167)*. In addition, vaccination of mice with hen egg lysozyme (HEL) and a CpG oligonucleotide in incomplete Freund's adjuvant induced a Th1-type response comparable to that achieved by injecting HEL in complete Freund's adjuvant *(168)*. It has also been reported that CpG motifs can enhance systemic as well as mucosal immune responses when given intranasally to mice *(169)*. The observation that these CpG motifs can also function as mucosal adjuvants was confirmed by the finding that delivery to lungs of hepatitis B surface antigen (HBsAg) with CpG DNA resulted in high HBsAg-specific mucosal and systemic immune responses *(170)*.

Saponin Derivatives

Immunostimulating complexes (ISCOMs) are cage-like particules generated after addition of cholesterol to the Quil A from the bark of the *Quillaja saponaria* Molina tree *(171)*. Since antigens can be incorporated into ISCOMs, these particules represent good delivery systems for mucosal vaccines. In fact, ISCOMs are effective oral delivery systems that promote mucosal and systemic immunity *(172)*. It is believed that the cage-like structure of ISCOMs protects both the antigen and Quil A from degradation in the GI tract. However, ISCOMs appeared to be toxic after parenteral immunization of experimental animals. It is possible that ISCOMs are less toxic after oral delivery. This point will need to be carefully addressed before considering a broader use of ISCOMs.

QS-21 is a highly purified complex triterpene glycoside isolated from the bark of the *Quillaja saponaria* Molina tree *(173,174)*. This molecule promotes both humoral and cell-mediated immunity when added to systemic vaccine formulations *(175–177)* and is now being tested in several parenteral vaccine formulations *(173)*. QS-21 was reported to act as an adjuvant for both systemic and mucosal immunity to a nasally administered DNA vaccine *(178)*. More recently, it has been shown that QS-21 also acts as adjuvant when administered by the oral route *(179)*. Interestingly, low oral QS-21 doses promoted mucosal S-IgA Abs responses, whereas no S-IgA responses were induced by high oral QS-21 *(179)*. On the other hand, stronger Th1-type responses were seen after immunization with high oral QS-21 doses *(179)*.

CONCLUSIONS

The increasing numbers of bacteria that are resistant to antibiotic therapy and the inefficiency of antiviral drugs to resolve virus infections leave vaccines as the most promising immunoprophylactic approach against infectious diseases. Mucosal sur-

faces, which are the main portal of entry for exogenous pathogens, are protected by a first line of innate defenses provided by epithelial cells, NK cells, and IELs. Although regulation of these innate defenses is only partially understood, a growing body of evidence shows that mucosal innate factors can provide the necessary signals for the development of adaptive immunity. It is also clear that effective protection of mucosal surfaces can only be achieved by vaccines promoting both systemic and mucosal immunity. A number of mucosal adjuvants and delivery systems capable of inducing mucosal S-IgA Abs as well as systemic immunity have been identified. However, toxicity issues preclude their use in humans (i.e., native enterotoxin, as well as the complex saponin derivatives such as Quil A and recombinant bacterial and viral vectors). Safe mucosal adjuvants and vaccination strategies are being developed to induce targeted Th1- or Th2-type immunity for optimal protection against different pathogens.

REFERENCES

1. McGhee JR, Lamm ME, Strober W. Mucosal immune responses: an overview. In: Ogra PL, et al. (eds). Mucosal Immunology. San Diego: Academic, 1999, pp. 485–506.
2. McGhee JR, Kiyono H. The mucosal immune system. In: Paul WE (ed). Fundamental Immunology. Philadelphia: Lippincott-Raven, 1999, pp. 909–945.
3. Bienenstock J, McDermot MR, Clancy RL. Respiratory tract defenses: role of mucosal lymphoid tissues. In: Ogra PL, et al. (eds). Mucosal Immunology. San Diego: Academic, 1999, pp. 283–292.
4. Pabst R. Is BALT a major component of the human lung immune system? Immunol Today 1992; 13:119–122.
5. Owen RL, Jones AL. Epithelial cell specialization within human Peyer's patches: an ultrastructural study of intestinal lymphoid follicles. Gastroenterology 1974; 66:189–203.
6. Neutra MR, Frey A, Kraehenbuhl JP. Epithelial M cells: gateways for mucosal infection and immunization. Cell 1996; 86:345–348.
7. Owen RL, Apple RT, Bhalla DK. Morphometric and cytochemical analysis of lysosomes in rat Peyer's patch follicle epithelium: their reduction in volume fraction and acid phosphatase content in M cells compared to adjacent enterocytes. Anat Rec 1986; 216:521–527.
8. Neutra MR, Kreahenbuhl JP. Cellular and molecular basis for antigen transport across epithelial barriers. In: Ogra PL, et al. (eds). Mucosal Immunology. San Diego: Academic, 1999, pp. 110–114.
9. Wolf JL, Bye WA. The membranous epithelial (M) cell and the mucosal immune system. Ann Rev Medi 1984; 35:95–112.
10. Gebert A, Rothkotter HJ, Pabst R. M cells in Peyer's patches of the intestine. Int Rev Cytol 1996; 167:91–159.
11. 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 1995; 279:433–436.
12. Kerneis S, Bogdanova A, Kraehenbuhl JP, Pringault E. Conversion by Peyer's patch lymphocytes of human enterocytes into M cells that transport bacteria. Science 1997; 277:949–952.
13. Brandtzaeg P, Baklien K, Bjerke K, Rognum TO, Scott H, Valnes K. Nature and properties of the human gastrointestinal immune system. In: Miller K, Nicklin S (eds). Immunology of the Gastrointestinal Tract. Boca Raton, FL: CRC, 1987, pp. 1–85.
14. George A, Cebra JJ. Responses of single germinal-center B cells in T-cell-dependent microculture. Proc Natl Acad Sci USA 1991; 88:11–15.
15. Butcher EC, Rouse RV, Coffman RL, Nottenburg CN, Hardy RR, Weissman IL. Surface phenotype of Peyer's patch germinal center cells: implications for the role of germinal centers in B cell differentiation. J Immunol 1982; 129:2698–2707.

16. Lebman DA, Griffin PM, Cebra JJ. Relationship between expression of IgA by Peyer's patch cells and functional IgA memory cells. J Exp Med 1987; 166:1405–1418.
17. Weinstein PD, Cebra JJ. The preference for switching to IgA expression by Peyer's patch germinal center B cells is likely due to the intrinsic influence of their microenvironment. J Immunol 1991; 147:4126–4135.
18. Erickson SL, de Sauvage FJ, Kikly K, et al. Decreased sensitivity to tumour-necrosis factor but normal T-cell development in TNF receptor-2-deficient mice. Nature 1994; 372:560–563.
19. Rennert PD, Browning JL, Mebius R, Mackay F, Hochman PS. Surface lymphotoxin alpha/beta complex is required for the development of peripheral lymphoid organs. J Exp Med 1996; 184:1999–2006.
20. 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 2000; 164:5184 5191.
21. Fujihashi K, Dohi T, Rennert PD, et al. Peyer's patches are required for oral tolerance to proteins. Proc Natl Acad Sci USA 2001; 98:3310–3315.
22. Quiding-Jarbrink M, Granstrom G, Nordstrom I, Holmgren J, Czerkinsky C. Induction of compartmentalized B-cell responses in human tonsils. Infect Immun 1995; 63:853–857.
23. Lubeck MD, Natuk RJ, Chengalvala M, et al. Immunogenicity of recombinant adenovirus-human immunodeficiency virus vaccines in chimpanzees following intranasal administration. AIDS Res Hum Retroviruses 1994;10:1443–1449.
24. Gallichan WS, Rosenthal KL. Specific secretory immune responses in the female genital tract following intranasal immunization with a recombinant adenovirus expressing glycoprotein B of herpes simplex virus. Vaccine 1995; 13:1589–1595.
25. Pal S, Peterson EM, de la Maza LM. Intranasal immunization induces long-term protection in mice against a *Chlamydia trachomatis* genital challenge. Infect Immun 1996; 64:5341–5348.
26. Di Tommaso A, Saletti G, Pizza M, et al. Induction of antigen-specific antibodies in vaginal secretions by using a nontoxic mutant of heat-labile enterotoxin as a mucosal adjuvant. Infect Immun 1996; 64:974–979.
27. Staats HF, Jackson RJ, Marinaro M, Takahashi I, Kiyono H, McGhee JR. Mucosal immunity to infection with implications for vaccine development. Curr Opin Immunol 1994; 6:572–583.
28. Russell MW, Moldoveanu Z, White PL, Sibert GJ, Mestecky J, Michalek SM. Salivary, nasal, genital, and systemic antibody responses in monkeys immunized intranasally with a bacterial protein antigen and the cholera toxin B subunit. Infect Immun 1996; 64:1272–1283.
29. Johansson EL, Rask C, Fredriksson M, Eriksson K, Czerkinsky C, Holmgren J. Antibodies and antibody-secreting cells in the female genital tract after vaginal or intranasal immunization with cholera toxin B subunit or conjugates. Infect Immun 1998; 66:514–520.
30. Bergquist C, Johansson EL, Lagergard T, Holmgren J, Rudin A. Intranasal vaccination of humans with recombinant cholera toxin B subunit induces systemic and local antibody responses in the upper respiratory tract and the vagina. Infect Immun 1997; 65:2676–2684.
31. Lehner T, Bergmeier LA, Panagiotidi C, et al. Induction of mucosal and systemic immunity to a recombinant simian immunodeficiency viral protein. Science 1992; 258:1365–1369.
32. Lehner T, Brookes R, Panagiotidi C, et al. T- and B-cell functions and epitope expression in nonhuman primates immunized with simian immunodeficiency virus antigen by the rectal route. Proc Natl Acad Sci USA 1993; 90:8638–8642.
33. Moldoveanu Z, Russell MW, Wu HY, Huang WQ, Compans RW, Mestecky J. Compartmentalization within the common mucosal immune system. Adv Exp Med Biol 1995; 371A:97–101.

34. Craig SW, Cebra JJ. Peyer's patches: an enriched source of precursors for IgA-producing immunocytes in the rabbit. J Exp Med 1971; 134:188–200.
35. Craig SW, Cebra JJ. Rabbit Peyer's patches, appendix, and popliteal lymph node B lymphocytes: a comparative analysis of their membrane immunoglobulin components and plasma cell precursor potential. J Immunol 1975; 114:492–502.
36. McDermott MR, Bienenstock J. Evidence for a common mucosal immunologic system. I. Migration of B immunoblasts into intestinal, respiratory, and genital tissues. J Immunol 1979; 122:1892–1898.
37. McWilliams M, Phillips-Quagliata JM, Lamm ME. Characteristics of mesenteric lymph node cells homing to gut-associated lymphoid tissue in syngeneic mice. J Immunol 1975; 115:54–58.
38. McWilliams M, Phillips-Quagliata JM, Lamm ME. Mesenteric lymph node B lymphoblasts which home to the small intestine are precommitted to IgA synthesis. J Exp Med 1977; 145:866–875.
39. Roux ME, McWilliams M, Phillips-Quagliata JM, Weisz-Carrington P, Lamm ME. Origin of IgA-secreting plasma cells in the mammary gland. J Exp Med 1977; 146:1311–1322.
40. Czerkinsky C, Prince SJ, Michalek SM, et al. IgA antibody-producing cells in peripheral blood after antigen ingestion: evidence for a common mucosal immune system in humans. Proc Natl Acad Sci USA 1987; 84:2449–2453.
41. Kantele A, Arvilommi H, Jokinen I. Specific immunoglobulin-secreting human blood cells after peroral vaccination against *Salmonella* typhi. J Infect Dis 1986; 153:1126–1131.
42. Quiding M, Nordstrom I, Kilander A, et al. Intestinal immune responses in humans. Oral cholera vaccination induces strong intestinal antibody responses and interferon-gamma production and evokes local immunological memory. J Clin Invest 1991; 88:143–148.
43. Kraal G, Mebius RE. High endothelial venules: lymphocyte traffic control and controlled traffic. Adv Immunol 1997; 65:347–395.
44. Butcher EC. Lymphocyte homing and intestinal immunity. In: Ogra PL, et al. (eds). Mucosal Immunology. San Diego: Academic, 1999, pp. 507–522.
45. Berlin C, Berg EL, Briskin MJ, et al. Alpha 4 beta 7 integrin mediates lymphocyte binding to the mucosal vascular addressin MAdCAM-1. Cell 1993; 74:185–195.
46. Bevilacqua MP. Endothelial-leukocyte adhesion molecules. Annu Rev Immunol 1993; 11:767–804.
47. Osborn L. Leukocyte adhesion to endothelium in inflammation. Cell 1990; 62:3–6.
48. Holzmann B, McIntyre BW, Weissman IL. Identification of a murine Peyer's patch—specific lymphocyte homing receptor as an integrin molecule with an alpha chain homologous to human VLA-4 alpha. Cell 1989; 56:37–46.
49. Bell RG, Issekutz T. Expression of a protective intestinal immune response can be inhibited at three distinct sites by treatment with anti-alpha 4 integrin. J Immunol 1993; 151: 4790–4802.
50. Hamann A, Andrew DP, Jablonski-Westrich D, Holzmann B, Butcher EC. Role of alpha 4-integrins in lymphocyte homing to mucosal tissues in vivo. J Immunol 1994; 152: 3282–3293.
51. Rott LS, Briskin MJ, Andrew DP, Berg EL, Butcher EC. A fundamental subdivision of circulating lymphocytes defined by adhesion to mucosal addressin cell adhesion molecule-1. Comparison with vascular cell adhesion molecule-1 and correlation with beta 7 integrins and memory differentiation. J Immunol 1996; 156:3727–3736.
52. Campbell JJ, Hedrick J, Zlotnik A, Siani MA, Thompson DA, Butcher EC. Chemokines and the arrest of lymphocytes rolling under flow conditions. Science 1998; 279:381–384.
53. Gunn MD, Kyuwa S, Tam C, et al. Mice lacking expression of secondary lymphoid organ chemokine have defects in lymphocyte homing and dendritic cell localization [see comments]. J Exp Med 1999; 189:451–460.

54. Zabel BA, Agace W, Campbell JJ, et al. Human G protein-coupled receptor GPR-9-6/CC chemokine receptor 9 is selectively expressed on intestinal homing T lymphocytes, mucosal lymphocytes, and thymocytes and is required for thymus-expressed chemokine-mediated chemotaxis. J Exp Med 1999; 190:1241–1256.
55. Csencsits KL, Jutila MA, Pascual DW. Nasal-associated lymphoid tissue: phenotypic and functional evidence for the primary role of peripheral node addressin in naive lymphocyte adhesion to high endothelial venules in a mucosal site. J Immunol 1999; 163:1382–1389.
56. Richards IM, Kolbasa KP, Hatfield CA, et al. Role of very late activation antigen-4 in the antigen-induced accumulation of eosinophils and lymphocytes in the lungs and airway lumen of sensitized brown Norway rats. Am J Respir Cell Mol Biol 1996; 15:172–183.
57. Quiding-Jabrink M, Nordstrom I, Granstrom G, et al. Differential expression of tissue-specific adhesion molecules on human circulating antibody-forming cells after systemic, enteric, and nasal immunizations. A molecular basis for the compartmentalization of effector B cell responses. J Clin Invest 1997; 99:1281–1286.
58. Quayle AJ, Porter EM, Nussbaum AA, et al. Gene expression, immunolocalization, and secretion of human defensin-5 in human female reproductive tract. Am J Pathol 1998; 152:1247–1258.
59. Porter EM, Liu L, Oren A, Anton PA, Ganz T. Localization of human intestinal defensin 5 in Paneth cell granules. Infect Immun 1997; 65:2389–2395.
60. Porter EM, van Dam E, Valore EV, Ganz T. Broad-spectrum antimicrobial activity of human intestinal defensin 5. Infect Immun 1997; 65:2396–3401.
61. Zhao C, Wang I, Lehrer RI. Widespread expression of beta-defensin hBD-1 in human secretory glands and epithelial cells. FEBS Lett 1996; 396:319–322.
62. Singh PK, Jia HP, Wiles K, et al. Production of beta-defensins by human airway epithelia. Proc Natl Acad Sci USA 1998; 95:14961–14966.
63. Mathews M, Jia HP, Guthmiller JM, et al. Production of beta-defensin antimicrobial peptides by the oral mucosa and salivary glands. Infect Immun 1999; 67:2740–2745.
64. O'Neil DA, Porter EM, Elewaut D, et al. Expression and regulation of the human beta-defensins hBD-1 and hBD-2 in intestinal epithelium. J Immunol 1999; 163:6718–6724.
65. Weinrauch Y, Elsbach P, Madsen LM, Foreman A, Weiss J. The potent anti-*Staphylococcus aureus* activity of a sterile rabbit inflammatory fluid is due to a 14-kD phospholipase A2. J Clin Invest 1996; 97:250–257.
66. Harwig SS, Tan L, Qu XD, Cho Y, Eisenhauer PB, Lehrer RI. Bactericidal properties of murine intestinal phospholipase A2. J Clin Invest 1995; 95:603–610.
67. Moriuchi M, Moriuchi H. A milk protein lactoferrin enhances human T cell leukemia virus type i and suppresses HIV-1 infection. J Immunol 2001; 166:4231–4236.
68. Jung HC, Eckmann L, Yang SK, et al. A distinct array of proinflammatory cytokines is expressed in human colon epithelial cells in response to bacterial invasion. J Clin Invest 1995; 95:55–65.
69. Mayer L, Blumberg RS. Antigen presentating cells: epithelial cells. In: Ogra PL, et al. (eds). Mucosal Immunology. San Diego: Academic, 1999, pp. 365–379.
70. Laurent F, Eckmann L, Savidge TC, et al. *Cryptosporidium parvum* infection of human intestinal epithelial cells induces the polarized secretion of C-X-C chemokines. Infect Immun 1997; 65:5067–5073.
71. Yang SK, Eckmann L, Panja A, Kagnoff MF. Differential and regulated expression of C-X-C, C-C, and C-chemokines by human colon epithelial cells. Gastroenterology 1997; 113:1214–1223.
72. Izadpanah A, Dwinell MB, Eckmann L, Varki NM, Kagnoff MF. Regulated MIP-3alpha/CCL20 production by human intestinal epithelium: mechanism for modulating mucosal immunity. Am J Physiol (Gastrointest Liver Physiol) 2001; 280:G710–G719.
73. Dwinell MB, Lugering N, Eckmann L, Kagnoff MF. Regulated production of interferon-inducible T-cell chemoattractants by human intestinal epithelial cells. Gastroenterology 2001; 120:49–59.

74. Boismenu R, Feng L, Xia YY, Chang JC, Havran WL. Chemokine expression by intraepithelial gamma delta T cells. Implications for the recruitment of inflammatory cells to damaged epithelia. J Immunol 1996; 157:985–992.
75. Hedrick JA, Saylor V, Figueroa D, et al. Lymphotactin is produced by NK cells and attracts both NK cells and T cells in vivo. J Immunol 1997; 158:1533–1540.
76. Tagliabue A, Befus AD, Clark DA, Bienenstock J. Characteristics of natural killer cells in the murine intestinal epithelium and lamina propria. J Exp Med 1982; 155:1785–1796.
77. Roberts AI, O'Connell SM, Biancone L, Brolin RE, Ebert EC. Spontaneous cytotoxicity of intestinal intraepithelial lymphocytes: clues to the mechanism. Clin Exp Immunol 1993; 94:527–532.
78. Lillehoj HS. Intestinal intraepithelial and splenic natural killer cell responses to Eimerian infections in inbred chickens. Infect Immun 1989; 57:1879–1884.
79. Carman PS, Ernst PB, Rosenthal KL, Clark DA, Befus AD, Bienenstock J. Intraepithelial leukocytes contain a unique subpopulation of NK-like cytotoxic cells active in the defense of gut epithelium to enteric murine coronavirus. J Immunol 1986; 136:1548–1553.
80. Biron CA, Byron KS, Sullivan JL. Severe herpesvirus infections in an adolescent without natural killer cells. N Engl J Med 1989; 320:1731–1735.
81. Mosmann TR, Coffman RL. TH1 and TH2 cells: different patterns of lymphokine secretion lead to different functional properties. Ann Rev Immunol 1989; 7:145–173.
82. Kobayashi M, Fitz L, Ryan M, et al. Identification and purification of natural killer cell stimulatory factor (NKSF), a cytokine with multiple biologic effects on human lymphocytes. J Exp Med 1989; 170:827–845.
83. Chan SH, Perussia B, Gupta JW, et al. Induction of interferon gamma production by natural killer cell stimulatory factor: characterization of the responder cells and synergy with other inducers. J Exp Med 1991; 173:869–879.
84. Snapper CM, Paul WE. Interferon-gamma and B cell stimulatory factor-1 reciprocally regulate Ig isotype production. Science 1987; 236:944–947.
85. Finkelman FD, Holmes J, Katona IM, et al. Lymphokine control of in vivo immunoglobulin isotype selection. Annu Rev Immunol 1990; 8:303–333.
86. Esser C, Radbruch A. Immunoglobulin class switching: molecular and cellular analysis. Annu Rev Immunol 1990; 8:717–735.
87. Coffman RL, Seymour BW, Lebman DA, et al. The role of helper T cell products in mouse B cell differentiation and isotype regulation. Immunol Rev 1998; 102:5–28.
88. Gajewski TF, Fitch FW. Anti-proliferative effect of IFN-gamma in immune regulation. I. IFN-gamma inhibits the proliferation of Th2 but not Th1 murine helper T lymphocyte clones. J Immunol 1988; 140:4245–4252.
89. Golding B. Cytokine regulation of humoral immune responses. Topics Vaccine Adjuvant Res 1991, pp. 37–45.
90. Bonecchi R, Bianchi G, Bordignon PP, et al. Differential expression of chemokine receptors and chemotactic responsiveness of type 1 T helper cells (Th1s) and Th2s. J Exp Med 1998; 187:129–134.
91. Sallusto F, Lenig D, Mackay CR, Lanzavecchia A. Flexible programs of chemokine receptor expression on human polarized T helper 1 and 2 lymphocytes. J Exp Med 1998; 187:875–883.
92. Imai T, Nagira M, Takagi S, et al. Selective recruitment of CCR4-bearing Th2 cells toward antigen-presenting cells by the CC chemokines thymus and activation-regulated chemokine and macrophage-derived chemokine. Int Immunol 1999; 11:81–88.
93. Beagley KW, Eldridge JH, Kiyono H, et al. Recombinant murine IL-5 induces high rate IgA synthesis in cycling IgA-positive Peyer's patch B cells. J Immunol 1988; 141:2035–2042.
94. Beagley KW, Eldridge JH, Lee F, et al. Interleukins and IgA synthesis. Human and murine interleukin 6 induce high rate IgA secretion in IgA-committed B cells. J Exp Med 1989; 169:2133–2148.

95. Beagley KW, Eldridge JH, Aicher WK, et al. Peyer's patch B cells with memory cell characteristics undergo terminal differentiation within 24 hours in response to interleukin-6. Cytokine 1991; 3:107–116.
96. Fujihashi K, McGhee JR, Lue C, et al. Human appendix B cells naturally express receptors for and respond to interleukin 6 with selective IgA1 and IgA2 synthesis. J Clin Invest 1991; 88:248–252.
97. Ramsay AJ, Husband AJ, Ramshaw IA, et al. The role of interleukin-6 in mucosal IgA antibody responses in vivo. Science 1994; 264:561–563.
98. Bromander AK, Ekman L, Kopf M, Nedrud JG, Lycke NY. IL-6-deficient mice exhibit normal mucosal IgA responses to local immunizations and *Helicobacter felis* infection. J Immunol 1996; 156:4290–4207.
99. Briere F, Bridon JM, Chevet D, et al. Interleukin 10 induces B lymphocytes from IgA-deficient patients to secrete IgA. J Clin Invest 1994; 94:97–104.
100. Defrance T, Vanbervliet B, Briere F, Durand I, Rousset F, Banchereau J. Interleukin 10 and transforming growth factor beta cooperate to induce anti-CD40-activated naive human B cells to secrete immunoglobulin A. J Exp Med 1992; 175:671–682.
101. Nonoyama S, Farrington M, Ishida H, Howard M, Ochs HD. Activated B cells from patients with common variable immunodeficiency proliferate and synthesize immunoglobulin. J Clin Invest 1993; 92:1282–1287.
102. Mega J, McGhee JR, Kiyono H. Cytokine- and Ig-producing T cells in mucosal effector tissues: analysis of IL-5- and IFN-gamma-producing T cells, T cell receptor expression, and IgA plasma cells from mouse salivary gland-associated tissues. J Immunol 1992; 148:2030-2039.
103. Taguchi T, McGhee JR, Coffman RL, et al. Analysis of Th1 and Th2 cells in murine gut-associated tissues. Frequencies of CD4+ and CD8+ T cells that secrete IFN-gamma and IL-5. J Immunol 1990; 145:68–77.
104. Kauppi-Korkeila M, van Alphen L, Madore D, Saarinen L, Kayhty H. Mechanism of antibody-mediated reduction of nasopharyngeal colonization by *Haemophilus influenzae* type b studied in an infant rat model. J Infect Dis 1996; 174:1337–1340.
105. Davin JC, Senterre J, Mahieu PR. The high lectin-binding capacity of human secretory IgA protects nonspecifically mucosae against environmental antigens. Biol Neonate 1991; 59:121–125.
106. Wold AE, Mestecky J, Tomana M, et al. Secretory immunoglobulin A carries oligosaccharide receptors for *Escherichia coli* type 1 fimbrial. Infect Immun 1990; 58:3073–3077.
107. Wold AE, Motas C, Svanborg C, Mestecky J. Lectin receptors on IgA isotypes. Scand J Immunol 1994; 39:195–201.
108. Armstrong SJ, Dimmock NJ. Neutralization of influenza virus by low concentrations of hemagglutinin-specific polymeric immunoglobulin A inhibits viral fusion activity, but activation of the ribonucleoprotein is also inhibited. J Virol 1992; 66:3823–3832.
109. Mazanec MB, Coudret CL, Fletcher DR. Intracellular neutralization of influenza virus by immunoglobulin A anti-hemagglutinin monoclonal antibodies. J Virol 1995; 69:1339–1343.
110. Mazanec MB, Kaetzel CS, Lamm ME, Fletcher D, Nedrud JG. Intracellular neutralization of virus by immunoglobulin A antibodies. Pro Natl Acad Sci USA 1992; 89:6901–6905.
111. Burns JW, Siadat-Pajouh M, Krishnaney AA, Greenberg HB. Protective effect of rotavirus VP6-specific IgA monoclonal antibodies that lack neutralizing activity. Science 1996; 272:104–107.
112. Bomsel M, Heyman M, Hocini H, et al. Intracellular neutralization of HIV transcytosis across tight epithelial barriers by anti-HIV envelope protein dIgA or IgM. Immunity 1998; 9:277–287.
113. Griffiss JM, Goroff DK. IgA blocks IgM and IgG-initiated immune lysis by separate molecular mechanisms. J Immunol 1983; 130:2882–2885.

114. Russell MW, Mansa B. Complement-fixing properties of human IgA antibodies. Alternative pathway complement activation by plastic-bound, but not specific antigen-bound, IgA. Scand J Immunol 1989; 30:175–183.
115. Wolf HM, Fischer MB, Puhringer H, Samstag A, Vogel E, Eibl MM. Human serum IgA downregulates the release of inflammatory cytokines (tumor necrosis factor-alpha, interleukin-6) in human monocytes. Blood 1994; 83:1278–1288.
116. Wolf HM, Hauber I, Gulle H, et al. Anti-inflammatory properties of human serum IgA: induction of IL-1 receptor antagonist and Fc alpha R (CD89)-mediated down-regulation of tumour necrosis factor-alpha (TNF-alpha) and IL-6 in human monocytes. Clin Exp Immunol 1996; 105:537–543.
117. Offit PA, Cunningham SL, Dudzik KI. Memory and distribution of virus-specific cytotoxic T lymphocytes (CTLs) and CTL precursors after rotavirus infection. J Virol 1991; 65:1318–1324.
118. London SD, Rubin DH, Cebra JJ. Gut mucosal immunization with reovirus serotype 1/L stimulates virus-specific cytotoxic T cell precursors as well as IgA memory cells in Peyer's patches. J Exp Med 1987; 165:830–847.
119. London SD, Cebra-Thomas JA, Rubin DH, Cebra JJ. CD8 lymphocyte subpopulations in Peyer's patches induced by reovirus serotype 1 infection. J Immunol 1990; 144:3187–3194.
120. Issekutz TB. The response of gut-associated T lymphocytes to intestinal viral immunization. J Immunol 1984; 133:2955–2960.
121. Marx PA, Compans RW, Gettie A, et al. Protection against vaginal SIV transmission with microencapsulated vaccine. Science 1993; 260:1323–1327.
122. Miller CJ, Alexander NJ, Sutjipto S, et al. Genital mucosal transmission of simian immunodeficiency virus: animal model for heterosexual transmission of human immunodeficiency virus. J Virol 1989; 63:4277–4284.
123. Lohman BL, Miller CJ, McChesney MB. Antiviral cytotoxic T lymphocytes in vaginal mucosa of simian immunodeficiency virus-infected rhesus macaques. J Immunol 1995; 155:5855–5860.
124. Miller CJ, McChesney MB, Lu X, et al. Rhesus macaques previously infected with simian/human immunodeficiency virus are protected from vaginal challenge with pathogenic SIVmac239. J Virol 1997; 71:1911–1921.
125. Miller CJ. Mucosal transmission of simian immunodeficiency virus. Curr Top Microbiol Immunol 1994; 188:107–122.
126. Porgador A, Staats HF, Faiola B, Gilboa E, Palker TJ. Intranasal immunization with CTL epitope peptides from HIV-1 or ovalbumin and the mucosal adjuvant cholera toxin induces peptide-specific CTLs and protection against tumor development in vivo. J Immunol 1997; 158:834–841.
127. Elson CO, Ealding W. Generalized systemic and mucosal immunity in mice after mucosal stimulation with cholera toxin. J Immunol 1984; 132:2736–2741.
128. Elson CO, Ealding W. Cholera toxin feeding did not induce oral tolerance in mice and abrogated oral tolerance to an unrelated protein antigen. J Immunol 1984; 133:2892–2897.
129. Clements JD, Hartzog NM, Lyon FL. Adjuvant activity of *Escherichia coli* heat-labile enterotoxin and effect on the induction of oral tolerance in mice to unrelated protein antigens. Vaccine 1988; 6:269–277.
130. Lycke N, Holmgren J. Strong adjuvant properties of cholera toxin on gut mucosal immune responses to orally presented antigens. Immunology 1986; 59:301–308.
131. VanCott JL, Staats HF, Pascual DW, et al. Regulation of mucosal and systemic antibody responses by T helper cell subsets, macrophages, and derived cytokines following oral immunization with live recombinant *Salmonella.* J Immunol 1996; 156:1504-1514.
132. Van Ginkel FW, Liu C, Simecka JW, et al. Intratracheal gene delivery with adenoviral vector induces elevated systemic IgG and mucosal IgA antibodies to adenovirus and beta-galactosidase. 1995; Hum Gene Ther 1995; 6:895–903.

133. Gill DM. The arrangement of subunits in cholera toxin. Biochemistry 1976; 15:1242–1248.
134. Spangler BD. Structure and function of cholera toxin and the related *Escherichia coli* heat-labile enterotoxin. Microbiol Rev 1992; 56:622–647.
135. Heyningen SV. Cholera toxin: interaction of subunits with ganglioside GM1. Science 1974; 183:656–657.
136. Field M, Rao MC, Chang EB. Intestinal electrolyte transport and diarrheal disease (1). N Engl J Med 1989; 321:800–806.
137. Dallas WS, Falkow S. Amino acid sequence homology between cholera toxin and *Escherichia coli* heat-labile toxin. Nature 1980; 288:499–501.
138. Tsuji T, Inoue T, Miyama A, Okamoto K, Honda T, Miwatani T. A single amino acid substitution in the A subunit of *Escherichia coli* enterotoxin results in a loss of its toxic activity. J Biol Chem 1990; 265:22520–22525.
139. Harford S, Dykes CW, Hobden AN, Read MJ, Halliday IJ. Inactivation of the *Escherichia coli* heat-labile enterotoxin by in vitro mutagenesis of the A-subunit gene. Eur J Biochem 1989; 183:311–316.
140. Yamamoto S, Takeda Y, Yamamoto M, et al. Mutants in the ADP-ribosyltransferase cleft of cholera toxin lack diarrheagenicity but retain adjuvanticity. J Exp Med 1997; 185:1203–1210.
141. Yamamoto S, Kiyono H, Yamamoto M, et al. A nontoxic mutant of cholera toxin elicits Th2-type responses for enhanced mucosal immunity. Pro Natl Acad Sci USA 1997; 94:5267–5272.
142. Yamamoto M, Kiyono H, Yamamoto S, et al. Direct effects on antigen-presenting cells and T lymphocytes explain the adjuvanticity of a nontoxic cholera toxin mutant. J Immunol 1999; 162:7015–7021.
143. Cong Y, Weaver CT, Elson CO. The mucosal adjuvanticity of cholera toxin involves enhancement of costimulatory activity by selective upregulation of B7.2 expression. J Immunol 1997; 159:5301–5308.
144. Douce G, Fontana M, Pizza M, Rappuoli R, Dougan G. Intranasal immunogenicity and adjuvanticity of site-directed mutant derivatives of cholera toxin. Infect Immun 1997; 65:2821–2828.
145. Douce G, Turcotte C, Cropley I, et al. Mutants of *Escherichia coli* heat-labile toxin lacking ADP-ribosyltransferase activity act as nontoxic, mucosal adjuvants. Proc Natl Acad Sci USA 1995; 92:1644–1648.
146. Giuliani MM, Del Giudice G, Giannelli V, et al. Mucosal adjuvanticity and immunogenicity of LTR72, a novel mutant of *Escherichia coli* heat-labile enterotoxin with partial knockout of ADP-ribosyltransferase activity. J Exp Med 1998; 187:1123–1132.
147. Rappuoli R, Pizza M, Douce G, Dougan G. Structure and mucosal adjuvanticity of cholera and *Escherichia coli* heat-labile enterotoxins. Immunol Today 1999; 20:493–500.
148. Takahashi I, Marinaro M, Kiyono H, et al. Mechanisms for mucosal immunogenicity and adjuvancy of *Escherichia coli* labile enterotoxin. J Infect Dis 1996; 173, no. 3: 627–635.
149. Agren LC, Ekman L, Lowenadler B, Lycke NY. Genetically engineered nontoxic vaccine adjuvant that combines B cell targeting with immunomodulation by cholera toxin A1 subunit. J Immunol 1997; 158:3936–3946.
150. Agren L, Sverremark E, Ekman L, et al. The ADP-ribosylating CTA1-DD adjuvant enhances T cell-dependent and independent responses by direct action on B cells involving anti-apoptotic Bcl-2- and germinal center-promoting effects. J Immunol 2000; 164:6276–6286.
151. Agren LC, Ekman L, Lowenadler B, Nedrud JG, Lycke NY. Adjuvanticity of the cholera toxin A1-based gene fusion protein, CTA1-DD, is critically dependent on the ADP-ribosyltransferase and Ig-binding activity. J Immunol 1999; 162:2432–2440.

152. Hajishengallis G, Hollingshead SK, Koga T, Russell MW. Mucosal immunization with a bacterial protein antigen genetically coupled to cholera toxin A2/B subunits. J Immunol 1995; 154:4322–4332.
153. Sun JB, Mielcarek N, Lakew M, et al. Intranasal administration of a *Schistosoma mansoni* glutathione S-transferase-cholera toxoid conjugate vaccine evokes antiparasitic and antipathological immunity in mice. J Immunol 1999; 163:1045–1052.
154. Sun JB, Rask C, Olsson T, Holmgren J, Czerkinsky C. Treatment of experimental autoimmune encephalomyelitis by feeding myelin basic protein conjugated to cholera toxin B subunit. Proc Natl Acad Sci USA 1996; 93:7196–7201.
155. Bergerot I, Ploix C, Petersen J, et al. A cholera toxoid-insulin conjugate as an oral vaccine against spontaneous autoimmune diabetes. Proc Natl Acad Sci USA 1997; 94:4610–4614.
156. Marinaro M, Boyaka PN, Jackson RJ, et al. Use of intranasal IL-12 to target predominantly Th1 responses to nasal and Th2 responses to oral vaccines given with cholera toxin. J Immunol 1999; 162:114–121.
157. Boyaka PN, Marinaro M, Jackson RJ, et al. IL-12 is an effective adjuvant for induction of mucosal immunity. J Immunol 1999; 162: 122–128.
158. Arulanandam BP, O'Toole M, Metzger DW. Intranasal interleukin-12 is a powerful adjuvant for protective mucosal immunity. J Infect Dis 1999; 180:940–949.
159. Staats HF, Ennis, Jr. FA. IL-1 is an effective adjuvant for mucosal and systemic immune responses when coadministered with protein immunogens. J Immunol 1999; 162:6141–6147.
160. 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 1997; 185:415–427.
161. Lillard JW, Jr, Boyaka PN, Chertov O, Oppenheim JJ, McGhee JR. Mechanisms for induction of acquired host immunity by neutrophil peptide defensins. Proc Natl Acad Sci USA 1999; 96:651–656.
162. Lillard JW, Jr, Boyaka PN, Hedrick JA, Zlotnik A, McGhee JR. Lymphotactin acts as an innate mucosal adjuvant. J Immunol 1999; 162:1959–1965.
163. Lillard JW, Boyaka PN, Taub DD, McGhee JR. RANTES potentiates antigen-specific mucosal immune responses. J Immunol 2001; 166:162–169.
164. Krieg AM. CpG DNA: a pathogenic factor in systemic lupus erythematosus? J Clin Immunol 1995; 15:284–292.
165. Tighe H, Corr M, Roman M, Raz E. Gene vaccination: plasmid DNA is more than just a blueprint. Immunol Today 1998; 19:89–97.
166. Roman M, Martin-Orozco E, Goodman JS, et al. Immunostimulatory DNA sequences function as T helper-1-promoting adjuvants. Nat Med 1997; 3:849–854.
167. 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 1996; 93:2879–2883.
168. Chu RS, Targoni OS, Krieg AM, Lehmann PV, Harding CV. CpG oligodeoxynucleotides act as adjuvants that switch on T helper 1 (Th1) immunity. J Exp Med 1997; 186:1623–1631.
169. Moldoveanu Z, Love-Homan L, Huang WQ, Krieg AM. CpG DNA, a novel immune enhancer for systemic and mucosal immunization with influenza virus. Vaccine 1998; 16:1216–1224.
170. 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 1998; 161:4463–4466.
171. Elson CO, Dertzbaugh MT. Mucosal adjuvants. In: Ogra PL, et al. (eds). Mucosal Immunology. San Diego: Academic, 1999, pp. 817–838.

172. Mowat AM, Donachie AM, Reid G, Jarrett O. Immune-stimulating complexes containing Quil A and protein antigen prime class I MHC-restricted T lymphocytes in vivo and are immunogenic by the oral route. Immunology 1991; 72:317–322.
173. Kensil CR, Kammer R. QS-21: A water-soluble triterpene glycoside adjuvant. Expert Opin Invest Drugs 1998; 7:1475–1482.
174. Kensil CR, Patel U, Lennick M, and Marciani D. Separation and characterization of saponins with adjuvant activity from *Quillaja saponaria* Molina cortex. J Immunol 1991; 146:431–437.
175. Livingston P, Zhang S, Adluri S, et al. Tumor cell reactivity mediated by IgM antibodies in sera from melanoma patients vaccinated with GM2 ganglioside covalently linked to KLH is increased by IgG antibodies. Cancer Immunol Immunother 1997; 43:324–330.
176. Coughlin RT, Fattom A, Chu C, White AC, Winston S. Adjuvant activity of QS-21 for experimental *E. coli* 018 polysaccharide vaccines. Vaccine 1995. 13:17–21.
177. Newman MJ, Wu JY, Gardner BH, et al. Saponin adjuvant induction of ovalbumin-specific CD8+ cytotoxic T lymphocyte responses. J Immunol 1992; 148:2357–2362.
178. Sasaki S, Sumino K, Hamajima K, et al. Induction of systemic and mucosal immune responses to human immunodeficiency virus type 1 by a DNA vaccine formulated with QS-21 saponin adjuvant via intramuscular and intranasal routes. J Virol 1998; 72:4931–4939.
179. Boyaka PN, Marinaro M, Jackson RJ, et al. Oral QS-21 requires early IL-4 help for induction of mucosal and systemic immunity. J Immunol 2001; 166:2283–2290.