TABLE 31.2 Functions/Biological Properties of IgA

Protective Functions in External Secretions
Prevention of Ag absorption from mucosal surfaces due to the formation of Ag-IgA complexes
Mucus trapping (IgA-mucin complexes entrap microorganisms)
Virus neutralization (in some experiments nonneutralizing Abs may also be protective)
Enzyme and toxin neutralization
Enhancement of antimicrobial activities of innate factors (e.g., lysozyme, lactoperoxidase, and lactoferrin)
Biological Activities in Tissues
Inhibition of C activation in some experiments (polymeric IgA or glycan-altered IgA may activate complement by the alternative or lectin pathways)
Enhancement (opsonization) or inhibition of phagocytosis
Inhibition of type I and II hypersensitivity reactions (e.g., anaphylaxis and Arthus reaction)
Degranulation of eosinophils
Intracellular virus neutralization
Elimination of Ag-IgA immune complexes by ECs and hepatocytes expressing IgA receptors
Ab-dependent cell-mediated cytotoxicity
Inhibition of NK cell activity
Inhibition of the release of inflammatory cytokines

or perfusates of mucosal tissues, especially in the GI tract, contain pIgA as the dominant form (Figure 31.10C).

Au: Pls provide callout for Table 31.2.

Structure of Secretory IgA (S-IgA)

When compared to its serum counterpart, IgA in external secretions (called S-IgA) displays unique structural features with respect to its molecular form, chain composition, and IgA subclass distribution (91). Examinations of sera and mucosal secretions, culture supernatants, and cell lysates, and immunohistochemical studies of systemic and mucosal lymphoid tissues indicated that pIgA contains J chain as a typical component and that J chain–containing pIgA is capable of binding to pIgR and its extracellular region, SC (9). Consequently, the presence of intracellular J chain and the ability to bind SC have been taken as markers for pIgA- or IgM-producing cells. In humans, almost all serum IgA is present in a monomeric form (sedimentation constant 7S) and contains two heavy (α) and two light (either κ or λ) chains, and ~85% belongs to the IgA1 and ~15% to the IgA2 subclass. Only a small but variable fraction (1% to 10%) of serum IgA is found in a polymeric form and contains an additional polypeptide-joining (J) chain. In contrast, ~90% of S-IgA occurs in a polymeric form (dimers and tetramers with sedimentation constants 11S and 15.5S, respectively) and is associated with J chain acquired during the transepithelial transport via the pIgR. The structure of a typical dimeric IgA molecule is shown in Figure 31.11. Two mIgA molecules are mutually linked by disulfide bridges through their Fc regions; J chain is bound to the penultimate Cys residues of α chains. Although SC interacts noncovalently with the Fc regions of both monomers, it is attached by disulfide bridges to only one of them, and there are no covalent bonds formed between J chain and SC.

Fig. 31.11

Mammalian α chains with molecular mass ~50 kDa contain one variable and three constant region domains. There are high numbers of Cys residues involved in the formation of intrachain and interchain disulfide bridges with another α chain, L and J chains, and SC (91). In addition, α chains can form complexes with a number of plasma and

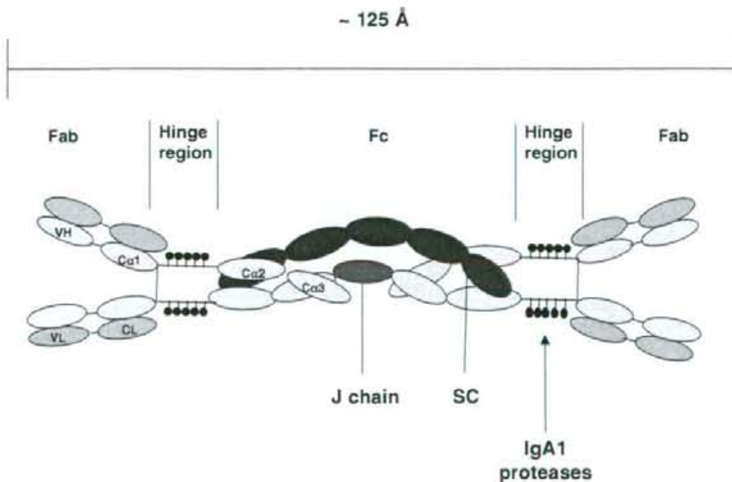

FIGURE 31.11 Molecular dimensions, proteolytic fragments, and domain structure of the human dimeric S-IgA1 molecule. Dimeric or polymeric IgA was formed by joining each IgA by J chain. The dimeric or polymeric forms of IgA are associated with SC, an extracellular domein of pIgR. Hinge region between Fab and Fc is a target site of IgA1 proteases.

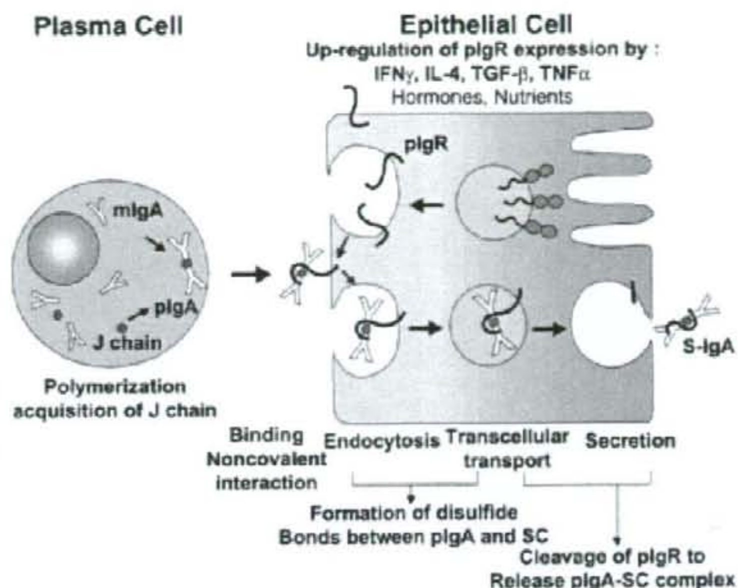

FIGURE 31.12 12 Transcellular transport of pIgA by the pIgR-mediated mechanism and regulation of pIgR expression. Subepithelial PCs produce J chain–associated pIgA that interacts with the epithelial pIgR, and the pIgA-pIgR complex is transcytosed through the epithelial cells and released, after the proteolytic clevage of pIgR, as S-IgA.

secretory proteins including albumin, amylase, lactoferrin, glycosyltransferases, and proteolytic enzymes. An unusual hinge region is present in the middle of the α chain of IgA1, between $C\alpha1$ and $C\alpha2$ domains (46,91). This 13 amino acid–long hinge region is reminiscent of mucin (high content of Pro, Ser, and Thr residues) and carries 3–5 *O*-linked oligosaccharide side chains (Figure 31.11). Although IgA is quite resistant to common proteolytic enzymes, the hinge region contains peptide bonds susceptible to the cleavage by highly substrate-specific IgA1 proteases of bacterial origin (e.g., *Streptococcus pneumoniae, Neisseria meningitidis, N. gonorrhoeae, Haemophilus influenzae,* and several other species of bacteria) (93). Comparative structural and genetic studies of IgA molecules from many species indicate that molecules of the IgA2 subclass represent phylogenetically older forms and that the IgA1 subclass arose in hominoid primates by insertion of a gene segment encoding the hinge region (91). The $Fc\alpha$ region, particularly its C terminus, displays a high degree of sequence homology to the μ chain of IgM, including the characteristic C terminae "tail" (an ~18 amino acid extension over the C terminus of γ, δ, and ε chains of corresponding Ig isotypes) involved in the polymerization and ability of α and μ chains to bind J chain. Both IgA1 and IgA2 contain 6% to 8% of glycans associated in the form of ~2 to 5 *N*-linked side chains within the Fc region; as described earlier, the hinge region of IgA1 also contains *O*-linked glycans (91).

J chain is a chacteristic polypeptide chain present in pIgA and IgM (94). It has a molecular mass of 15 kDa and a single *N*-linked glycan chain and displays an Ig domain folding pattern. Of eight Cys residues, six are involved in three intrachain disulfide bridges, and two participate in linkages to the penultimate Cys residues of α and μ chains. A very high degree of homology exists in the primary structures and antigenic cross-reactivities of mammalian and avian J chains, indicating that the basic properties have remained conserved throughout evolution (91). PCs in mucosal effector tissues and glands assemble pIgA intracellularly from mIgA and J chain as a last step before its externalization (Figure 31.12). Although the incorporation of J chain is not absolutely required for polymerization, the ability of pIgA and IgM to interact with pIgR expressed on ECs or SC depends on the presence of J chain as demonstrated in J chain knockout mice (95).

Fig. 31.12

The pIgR specific for the J chain-containing pIgA and IgM is expressed on the basolateral surfaces of EC of the GI tract and endocervix, acinar, and ductal epithelia of the small and large secretory glands (e.g., lacrimal, mammary, and major and minor salivary glands) in humans (Figure 31.12) (9). In some other species (rats, mice, and rabbits, but not humans), pIgR is also expressed on hepatocytes (9). Structurally, pIgR comprises an extracellular region composed of five Ig domain-like structures, with ~560 amino acids, a 23 amino acid membrane-spanning region, and a cytoplasmic region with ~103 amino acids; the molecular mass of pIgR with attached glycans is ~110 kDa to 120 kDa. The similarity of the general structural features of pIgR from a number of mammalian species indicates

that this receptor and its ability to interact with pIg are conserved in phylogeny.

The N-terminal domain of pIgR interacts with Cα domains (9). The pIgA-pIgR complex is internalized, transcytosed, and finally released at the apical end of the ECs with the entire process taking ~30 minutes (Figure 31.12). Signals for basolateral targeting of pIgR, its endocytosis, and its transcytosis are encoded in the cytoplasmic region of pIgR as revealed by deletion mutants. In the final steps, pIgR is proteolytically cleaved, thus releasing the pIgA-SC complex; the intracellular and transmembrane regions are endocytosed and degraded or released from the apex. Unlike several other receptors, pIgR does not recycle; instead, it remains permanently associated with the ligand as bound SC (9). Therefore, the transport of pIgA and IgM is directly dependent on the availability of pIgR on EC (or hepatocyte) membranes. A number of substances of local and distant origin influence pIgR expression. Cytokines produced locally in mucosal tissues and glands (e.g., IFN-γ, IL-4, TNF-α, and TGF-β) up-regulate in an additive or synergistic pattern the expression of pIgR on established EC lines, usually of intestinal or endometrial origin (9). Similarly, ECs from the female genital tract and mammary gland express pIgR as a consequence of stimulation with hormones, particularly with estrogens, prolactin, and androgens.

Other IgA Receptors

The Fc region of IgA can interact with other receptors expressed on structurally and functionally diverse cell populations including monocytes/MΦ/mesangial cells, PMNs, granulocytes, ECs, hepatocytes, B and T cells, and PCs (96). Some of these receptors have been structurally defined and specific reagents are now available for their detection.

The best-characterized receptor expressed on monocytes, neutrophils, and eosinophils recognizes Fcα regions of both IgA1 and IgA2 with a certain degree of preference for pIgA, probably due to the presence of multiple binding sites on pIgA. This Fcα receptor, designated as FcαRI (CD89) and detectable by monoclonal Abs, occurs in several isoforms and is heavily glycosylated (96). Another study indicates that CD89 is present in minute quantities also in the circulation in complexes with high molecular mass IgA (96). Additionally, eosinophils, but not PMNs, express a receptor for SC, suggesting that S-IgA has the potential to stimulate eosinophils through an interaction with SC. Detailed analysis of the molecular properties of such complexes revealed that FcαRI and IgA were covalently linked, but the high molecular mass IgA complexes lacked J chain; it can be speculated that the soluble FcαRI is linked to the binding site occupied in pIgA by J chain.

Binding studies indicate that the sites of interactions include the first extracellular domain of FcαRI and the boundary between the Cα2 and Cα3 domains of IgA heavy chains (96). Cross-linking of FcαRI on cell surfaces triggers phagocytosis, superoxide generation, and release of inflammatory mediators from PMNs, eosinophils, and MΦ. A receptor specific for the Fc regions of IgA and IgM has been described and designated as Fcα/μR (97). However, the biological function of the receptor remains to be elucidated.

It has been previously reported that the transferrin receptor (CD71) is surprisingly effective in binding IgA1 molecules, especially in their monomeric form (98). Because the binding of IgA1 is inhibitable by transferrin, it appears that this novel receptor binds two structurally highly dissimilar ligands—transferrin as well as IgA1. Although the function of this receptor remains to be determined, its expression on intestinal ECs suggests it may be involved in the appearance of mIgA in GI tract secretions.

T Cell Help for IgA Production

Earlier studies revealed that addition of culture supernatants from DC-T cell clusters, T cell clones, or T cell hybridomas to cultures of PP or splenic B cells resulted in enhanced secretion of IgA (99). One factor responsible for this activity was subsequently shown to be IL-5 (Figure 31.8) (66,72). Removal of sIgA$^+$ B cells from PP B cell cultures abrogated IgA synthesis, demonstrating that this cytokine affected postswitched IgA-committed B cells (100). If splenic B cells were used, these cells required stimulation with LPS before increased IgA secretion occurred. Taken together, these results suggest that IL-5 induces sIgA$^+$ B cells that are in cell cycle (blasts) to differentiate into IgA-producing cells. Human IL-5 is thought to act mainly as an eosinophil differentiation factor and thus may have little effect on B cell isotype switching and differentiation. It has been reported, however, that human B cells, when stimulated with the bacterium *Moraxella (Branhamella) catarrhalis*, could be induced by IL-5 to secrete IgA, and also to possibly undergo isotype switching to IgA (99). This effect could not be demonstrated using other more conventional B cell mitogens, a finding that demonstrates the importance of the primary *in vitro* activation signal for B cell switching.

IL-6, when added to PP B cells in the absence of any *in vitro* stimulus, causes a marked increase in IgA secretion with little effect on either IgM or IgG synthesis (46,66). IL-6–induced two- to three-fold more IgA secretion than IL-5 (101). The removal of sIgA$^+$ B cells abolished the effect of IL-6, demonstrating that like IL-5, this cytokine also acted on postswitched B cells. In IL-6$^{-/-}$ mice, the numbers of IgA$^+$ B cells in the LP were markedly reduced, and Ab responses following mucosal challenge with OVA or vaccinia virus were greatly diminished (102). It was shown that human appendix sIgA$^+$ B cells express IL-6 receptors, while other B cell subsets do not. Further, appendix B cells were induced by IL-6 to secrete both IgA1 and IgA2 in the

absence of any *in vitro* activation (103). This effect was also shown in IgA-committed B cells, again demonstrating the importance of IL-6 for inducing the terminal differentiation of $sIgA^+$ B cells into IgA-producing PCs (Figure 31.8).

An additional Th2 cytokine, IL-10, has also been shown to play an important role in the induction of IgA synthesis in humans (46,66). Stimulation of human B cells with anti-CD40 and *Staphylococcus aureus* Cowan (SAC) resulted in B cell differentiation for IgM and IgG synthesis in patients with IgA deficiency. Further, naïve $sIgD^+$ B cells could be induced to produce IgA after co-culture with IL-10 in the presence of TGF-β and anti-CD40 (74). Taken together, these findings demonstrate that Th2 cytokines such as IL-5, IL-6, and IL-10 all play major roles in the induction of IgA responses by the generation of IgA-producing cells (Figure 31.8).

Since IL-2 has been shown to enhance IgA synthesis in LPS-stimulated B cell cultures, it would be too simplistic to conclude that Th2-type cells and their derived cytokines are the only elements important in the generation of IgA responses. IL-2 also synergistically augmented IgA synthesis in B cell cultures in the presence of LPS and TGF-β (46,66). Although IFN-γ is not directly involved in the enhancement of IgA B cell responses, this cytokine has been shown to enhance the expression of pIgR, an essential molecule for the transport of S-IgA (9). In summary, an optimal relationship between Th1- and Th2-derived cytokines is essential for the induction, regulation and maintenance of appropriate IgA responses in mucosa-associated tissues.

Further, a helper function of IELs has been proposed for support of IgA synthesis (104). Thus, IELs may be actively involved in the induction and regulation of S-IgA Ab responses at mucosal surfaces. It was shown that the numbers of IgA-producing cells in mucosa-associated tissues, such as the intestinal LP of $TCR\gamma\delta^{-/-}$ mice, was significantly lower than that observed in control ($TCR\gamma\delta^{+/+}$) mice of the same genetic background (104). In contrast, identical numbers of IgM- and IgG-producing cells were found in systemic compartments of $TCR\gamma\delta^{-/-}$ and $TCR\gamma\delta^{+/+}$ mice. Further, when $TCR\gamma\delta^{-/-}$ mice were orally immunized with tetanus toxoid (TT) plus cholera toxin (CT) as mucosal adjuvant, significantly lower IgA anti-TT Ab responses were induced in PPs and LP when compared with identically treated $TCR\gamma\delta^{+/+}$ mice. These findings indicate that $\gamma\delta$ T cells are involved in the induction and regulation of Ag-specific IgA Ab responses in both mucosal and systemic compartments.

ALTERNATIVE INDUCTION PATHWAY FOR MUCOSAL IMMUNITY

Although the CMIS-mediated pathway is a major mechanism for the induction of Ag-specific mucosal immune responses, an alternative way (or CMIS-independent pathway) exists for the induction of appropriate productive immunity at mucosa-associated tissues. This is supported by the fact that Ag-specific immune responses have been induced in PP- and/or ILF-null mice following oral immunization (41,42). Indeed, a number of those pathways have been identified, especially in the GI tract at the levels of Ag sampling and S-IgA Ab production.

Other Ag Sampling Systems in the Intestinal Epithelium

In the CMIS-independent acquired immune system, the epithelium is also likely to play an important role as alternative Ag-sampling routes. At least three different scenarios have been offered regarding the alternative Ag-sampling routes in the epithelium. First, M cells were identified on the intestinal villous epithelium (villous M cells), not in proximity to PPs (Figure 31.4) (105). Villous M cells developed in various PP/ILF-null mice and are capable of taking up bacteria, such as *Salmonella, Yersinia,* and invasin-expressing *Escherichia coli* (105). A recent study has suggested that villous M cells locate closer to the upper half of the villus and are preferentially observed in the terminal ileum when compared with other parts of the small intestine, suggesting that the microflora influences villous M cell development (106). In addition, villous M cells are present in mice lacking both B and T cells, indicating that villous M cell differentiation and maintenance does not require lymphocytes (106).

A second route for Ag uptake is the EC itself. As mentioned earlier, ECs are involved in innate immunity by expressing nonclassical MHC molecules that can be sensed by $\gamma\delta$ IEL and NK cells (25,27,37). In addition, there is evidence to suggest that ECs could process and then present Ags to T cells via MHC class I as well as class II molecules in humans and rodents (37). The presentation by MHC class II exhibits polarity with uptake of Ag primarily apical and presentation basal under normal noninflammatory conditions and in inflammatory conditions when MHC class II expression is enhanced through the action of the MHC class II transactivator, CIITA (37). Under normal circumstances, ECs do not express classical costimulatory molecules such as CD80 and CD86 but may do so, at least in the case of CD86, in the context of intestinal inflammation (37). However, intestinal ECs express a number of potential costimulatory molecules, which, in certain circumstances, are functional. For example, LFA-3 or CD58 is constitutively expressed on ECs *in vivo* and *in vitro,* is upregulated in response to inflammation, and may provide crucial costimulatory signals to mucosal T cells through its ligand, CD2, which is constitutively expressed on mucosal T cells (107).

Recent studies identified various DC populations in the unorganized intestine (Figure 31.4). Among them are DCs located between intestinal ECs. These intraepithelial

DCs are frequent in the terminal ileum and express TJ-associated proteins (e.g., occuludin, claudin 1, and *zona occuludens* 1), and thus are capable of extending their dendritic arms into the lumen via the TJ between ECs (108). A previous study had already demonstrated that CD18-expressing phagocytes were involved in an M cell–independent pathway for bacterial invasion (109). A recent study has revealed that these intraepithelial DCs are characterized by the expression of CX_3CR1 and that the interaction between CX_3CR1 and its ligand fractalkine/CX_3CL1 is required for extension of transepithelial dendrites into the epithelium (110). Once intraepithelial DCs take up luminal Ags, it is likely that they leave the epithelium and migrate into the LP or draining LNs for the presentation of Ag to T cells.

A recent study has demonstrated that Ag/IgG complexes from the intestinal lumen can be taken up into the LP across intestinal ECs through FcRn *in vivo* (10,37). These transported Ag/IgG complexes may be captured by LP DCs because they express FcRn (10). In addition to sampling a wide variety of foreign Ags, the mucosal immune system must contend with the high number of apoptotic ECs that result from the frequency with which the epithelium is replaced. Although most of these apoptotic ECs are shed by the epithelium to the lumen, some of these apoptotic ECs have been shown to be potentially immunogenic and transportable to T cell areas of MLNs by mucosal DCs (111).

Contribution of B1 Cells for Mucosal IgA Responses

In addition to conventional B cells (or B2 cells) located in MALTs (e.g., PPs), peritoneal B1 cells have been considered to be a precursor of intestinal IgA PCs (112113). B1- and B2 cells can be distinguished by their cell surface molecules (*e.g.*, B220, IgM, IgD, CD5, and Mac-1), origins, and growth properties (112). Further, B1 cells exhibit different V_H repertoires and Ig specificities, and they are thought to be specialized in responding to T cell–independent Ag conserved on common pathogens like DNA and phosphatidylcholine, while B2 cells recognize most T-dependent protein Ags (112). Consistent with this notion, IgA production from B1 cells was noted in MHC class II–deficient mice as well as TCR β and δ chain–deficient mice (114115). Of note, about 65% of fecal bacteria were reactive with B1 cell–derived IgA, and 30% of bacteria were bound with B2-derived IgA, indicating that S-IgA derived from B1 cells recognized a large population of commensal bacteria as well as pathogenic bacteria (112).

AU: Is this supposed to indicate "B one negative"? If not, cut the hyphen.

B1- and B2 cell responses have distinct IgA-associated cytokine requirements. It was shown that IL-5, a well-known IgA-enhancing cytokine, and IL-15 are also involved in the proliferation and differentiation of B1 but not of B2 cells into IgA-producing cells (113). Thus, mucosal EC-derived IL-15 promoted the proliferation and differentiation of B1 cells into IgA-producing cells. Based upon these findings, it appears that intestinal B1 cells migrate presumably from the peritoneal cavity into mucosal effector sites, where they further differentiate into IgA-producing PCs under the influence of IL-5 and IL-15 for IgA production against T-independent Ags and commensal microflora-associated Ags.

AU: "B one negative"? If no, cut the hyphen.

Although precisely where CSR might occur for B1 cells is an unresolved issue, several lines of evidence have demonstrated B1 cell migration into intestinal LP. Using *aly/aly* mice that carried a point mutation in the NIK, there was a complete absence of B cell populations in the intestinal LP, but elevated B cell levels were seen in the peritoneal cavity (54). In this context, another report proposed that the migratory pathway of B1 cells to the peritoneal cavity depended upon the BCL/CXCL13 produced by peritoneal MΦ (85). These results imply that the NIK-mediated pathway is involved in the B1 cell mucosal migration, which may be dependent on specific but not yet identified chemokine receptor(s). Additionally, a recent study showed that peritoneal B cells express comparable levels of the receptor for S1P, a lipid mediator, and that S1P plays an important role in the regulation of peritoneal B cell trafficking into the intestine (116). It was also previously reported that B1 cells existed in nasal passages (113), but the actual molecular machinery for B1 cell migration into nasal passages remains an open question. These findings suggested the presence of a unique migration mechanism for the continuous supply of B1 cells as a part of a CMIS-independent mucosal immunity.

MICROBIAL MUCOSAL IMMUNE SYSTEM

Protection

Mucosal Igs

Large amounts of Ig are delivered onto mucosal surfaces as a result of receptor-mediated transepithelial transport and passive transudation of plasma-derived Igs. Depending upon the species as well as type of external secretion, IgA, IgM, and IgG are present in variable proportions (Table 31.1). Igs of all of these isotypes provide, by different mechanisms, protection against pathogenic microorganisms, interact with commensal microbiota, and interfere with the absorption of undigested food Ags from the large surface area of the digestive tract.

The dominant presence of S-IgA has several important functional advantages that render Abs of this isotype and molecular form particularly suitable for functioning in the mucosal environment (117). Dimeric and tetrameric S-IgA and pentameric S-IgM display 4 to 10 Ag binding sites. Although of lower affinity than, for example, IgG Abs of the same specificity, this multivalency of pIg enhances their effectiveness over mIg by at least an order of magnitude. The

presence of such low-affinity IgA Abs that are also "polyreactive" and thus are capable of binding to a variety of bacterial Ags, and autoAgs have been shown in human external secretions. Further, the intrinsic resistance of pIgA to proteolysis, enhanced by association with SC, is of functional advantage in secretions, particularly those of the GI tract rich in proteolytic enzymes. Finally, due to the inability to activate complement (C) and thus generate C3 and C5 fragments, IgA displays strong anti-inflammatory properties. This fact is of special importance in the GI tract in which the external milieu rich in microbial and food Ags, and the internal milieu are separated by only a single layer of ECs.

As demonstrated in a number of studies, mucosal Ig inhibits the absorption of soluble and particulate Ags from mucosal surfaces by forming large immune complexes. Further, endogenous commensal microorganisms are coated *in vivo* with corresponding Abs that, in turn, prevent their adherence to epithelial receptors. In this respect, mucosal Abs and especially IgA may function by two independent mechanisms. Specific Abs interact with corresponding Ags through the Ag binding site. In addition, glycans that are abundant on the Fc region of IgA can aggregate bacteria based upon the interaction of bacterial glycan-binding lectins, with glycan side chains present on IgA molecules (117). Consequently, such IgA-coated bacteria are prevented from adhering to ECs expressing analogous mannose-rich glycans on their luminal surfaces without the need for Ag-specific Abs.

Biologically active Ags such as viruses, enzymes, and toxins can be effectively neutralized by mucosal Abs (117). The neutralization activity that is operational in a fluid phase may also extend to the intracellular compartment. In addition, it was demonstrated that virus-specific pIgA also exhibit their neutralization activity intracellularly (117). Apparently, the transcytosis route of pIgA intercepts the pathways involved in virus assembly, resulting in intracellular neutralization. Further, elimination of immune complexes composed of noninfectious Ags, absorbed by ECs and corresponding internalized pIgA Abs, has been demonstrated *in vitro*. Small circulating immune complexes containing soluble Ags and pIgA can be eliminated from the circulation into the bile by binding to hepatocytes that in some species (e.g., rats, mice, and rabbits) express pIgR (117). It appears that this mechanism of disposal of immune complexes is primarily restricted to species whose plasma IgA is dominated by pIgA, which in humans represent normally only a minor component (117). However, it is possible that immune complexes containing locally produced pIgA and absorbed Ags that may be formed within mucosal tissues are eliminated by this mechanism.

The noninflammatory nature of IgA is probably of considerable importance for the maintenance of the structural and functional integrity of mucosal tissues (117). The concept that IgA Abs are anti-inflammatory is exemplified by studies in which intact, native, and fully glycosylated human IgA Abs failed to activate C when complexed with Ags; actually in competition experiments, IgA effectively inhibited C activation by IgM and IgG Abs (117). Close examination of the frequently cited ability to activate C reveals that this may be largely due to artificial aggregation and conformational alterations caused by purification procedures and binding to hydrophobic surfaces in C activation assays and aberrancies in glycosylation frequently seen in IgA proteins. Indeed, specific IgA Abs with modified glycan moieties have been shown to activate the alternative and perhaps the lectin pathways of C activation.

Although phagocytic cells including monocytes/MΦ, PMNs, and eosinophils express receptors for the Fc region of IgA (96), the ability of IgA alone to effectively promote phagocytosis of bacteria remains controversial and depends upon the experimental system used in such studies. However, the binding of IgA and IgA-containing immune complexes to such receptors may provide transducing signals for cell activation, proliferation, and oxidative metabolism and prompt degranulation of eosinophils with local inflammatory consequences and extensive tissue damage (96).

The function of mucosal S-IgA also depends on the subclass distribution of specific Abs (46,91,117). Naturally occurring and immunization-induced Abs to protein and glycoprotein Ags are predominantly present in the IgA1 subclass, while Abs to polysaccharide Ags, LPS, and lipoteichoic acid are mainly of the IgA2 subclass. Because of its unique hinge region, IgA1 is susceptible to the cleavage by bacterial IgA1 proteases that are considered as one of the virulence factors produced by *S. pneumoniae, H. influenzae, N. gonorrhoeae, N. meningitides,* and other microorganisms (93). It was also shown that bacteria coated with Fabα are refractory to IgM- and IgG-mediated and C-dependent killing action due to blocking. The antibacterial activity of IgA may be further potentiated by cooperation with innate factors of immunity including the peroxidase system, mucin, lactoferrin, and lysozyme (117).

Although S-IgA is the dominant isotype in most external secretions, the protective effects of Abs of IgM and IgG isotypes are evident from many studies. In external secretions of some IgA-deficient individuals, S-IgM and IgG may functionally compensate for the absence of S-IgA (118). Further, systemic immunization, particularly with conjugated polysaccharide-protein vaccines induces, vigorous and long-lasting IgG immune responses that protect children from infections with upper respiratory tract pathogens causing otitis media and meningitis (*H. influenzae, N. meningitides*) (119). In animal species (e.g., horses, cows, and pigs) in which prenatal transplacental active transport of IgG is not operational, consumption of milk rich in IgG is of life-saving importance. Abs of this isotype are absorbed during the first 7 to 14 days of life from the gut into the circulation presumably by the action of FcRn (10).

Mucosal CTLs

In the mucosal setting, natural infection of the epithelium by enteric (rotavirus or reovirus) or by respiratory viral pathogens (influenza or RSV) leads to endogenous viral peptide processing that induces pCTLs to become effector (activated) and memory CTLs. Most virus-specific CTLs are $CD8^+$ $TCR\alpha\beta^+$, and recognition of viral peptides is associated with MHC class I presentation by infected cells. In this regard, high numbers of $CD8^+$ T cells reside in the mucosal epithelium as a subpopulation of IELs (25). These $CD8^+$ IELs are thought to represent an important cytotoxic effector population that can eliminate virus-infected ECs. When freshly isolated IELs were examined using a redirected cytotoxicity assay, these T lymphocytes were found to constitutively possess lytic activity (28).

Significant progress is being made in areas related to the roles of APCs for induction of pCTLs and for mechanisms of perforin-mediated or Fas-Fas ligand-associated killing of target cells (28). It should be noted that the same processes occur during host responses to intracellular bacteria, to tumor-associated antigens, and in certain mucosal parasite infections. Although this focus is on $CD8^+$ CTLs, cell- and Ab-mediated cytotoxicity and NK cell activity are major responses associated with IELs (28).

An obvious question is how a CTL immune response is initiated given that mucosal inductive sites, which harbor pCTLs, are separate from effector sites, such as infected ECs where activated $CD8^+$ CTLs function. A partial answer is that the M cell has specific receptors for mucosal viruses, best exemplified by reovirus. As described earlier, the sigma protein of the reovirus enters the M cell in both NALT and GALT (59). It is likely, though less well documented, that other enteric viruses, such as rotavirus and respiratory pathogens, such as influenza and RSV, also enter the mucosal inductive pathway via M cells (59). Further, it now established that administration of virus into the GI tract results in the induction of increased pCTL frequencies in PPs (28). These findings suggest that, after enteric infection or immunization, Ag-stimulated CTLs are disseminated from PPs into MLNs via the lymphatic drainage. Further, virus-specific CTLs were also found among LP lymphocytes, IELs, and spleen cells of mice mucosally immunized with reovirus or rotavirus (28). Although mucosal effector tissues such as intestinal epithelium contain high numbers of $\gamma\delta$ T cells in addition to $\alpha\beta$ T cells, virus-specific CTLs in IELs were associated with the latter T cell subset (25,28). These studies suggest that oral immunization with live virus can induce Ag-specific CTLs in both mucosal inductive and effector tissues and in systemic lymphoid tissues.

Detailed studies of immune responses after nasal infection with influenza virus have also revealed that both humoral and cellular pathways are involved in virus clearance (120). However, it was shown that using mice lacking $CD8^+$ T cells (β2-microglobulin knockout mice) or treating with anti-CD8 mAbs did not alter clearance of influenza (121). These results support the presence of multiple pathways for immunity and suggest that $CD4^+$ Th cell pathways are important for mucosal Ab responses and $CD8^+$ CTLs for respiratory tract immunity. Several studies have also established that effector CTLs protect mice from RSV infection. The murine RSV model was used to determine the relative importance of $CD4^+$ T cells, including Th1 and Th2 subsets, which resulted in inflammation versus immunity. These studies clearly suggest that $CD4^+$ IFN-γ-producing Th1 cells as well as $CD8^+$ T cells are associated with recovery, while $CD4^+$ Th2-type pathways are not (122). Interestingly, priming with inactivated RSV or F glycoprotein induced $CD4^+$ Th2 cells while live RSV elicited the Th1-type pathway. When one considers mucosal vaccine development for virus infections, these findings suggest that the outcome of Th1- (including induction of CTLs) and Th2-type immune responses could be regulated by the nature and form of viral Ag used for immunization.

CTLs also play an important role in the inhibition of HIV infection. It was shown that CTLs recognized Ags derived from gp120, p27, nef, gag, tat, and pol proteins (123,124). Thus, infection of rhesus macaques with simian immunodeficient virus (SIV) resulted in the induction of CTL responses in the vaginal mucosa, which played a crucial role in the control of viral replication in the acute phase of viral infection. CTLs killed HIV-infected cells in a granzyme, perforin, or FasL-dependent manner. It is interesting to note that chemokines (e.g., CCL3, 4, and 5, and CXCL12) were produced by CTLs, which prevented HIV infection by blocking CCR5 and CXCR4, which are specific receptors for HIV infection.

Symbiotic Interactions with the Mucosal Microbiota

Mucosal surfaces of the oral cavity; the GI, genital, and respiratory tracts; and conjunctiva are populated by a large number of bacteria of more than 200 species with a characteristic distribution (Figure 31.13) (125). The mucosal microbiota comprises some 10^{14} bacteria present mostly in the large intestine. Considering the relative numbers of host's eukaryotic cells and prokaryotic bacteria, it is estimated that the mucosal microbiota outnumbers mammalian host cells by a factor of at least 10. Mutually beneficial coexistence of the mucosal microbiota with the effective mucosal immune system is one of the most interesting problems in mucosal immunology. Although the innate and specific immune factors present in the mucosal secretions and tissues may limit the adherence of bacteria to mucosal ECs and prevent penetration of such bacteria into the mucosal tissues with subsequent systemic dissemination, the mucosal microbiota continues to survive with remarkable tenacity in the presence of an immune response

Fig. 31.13

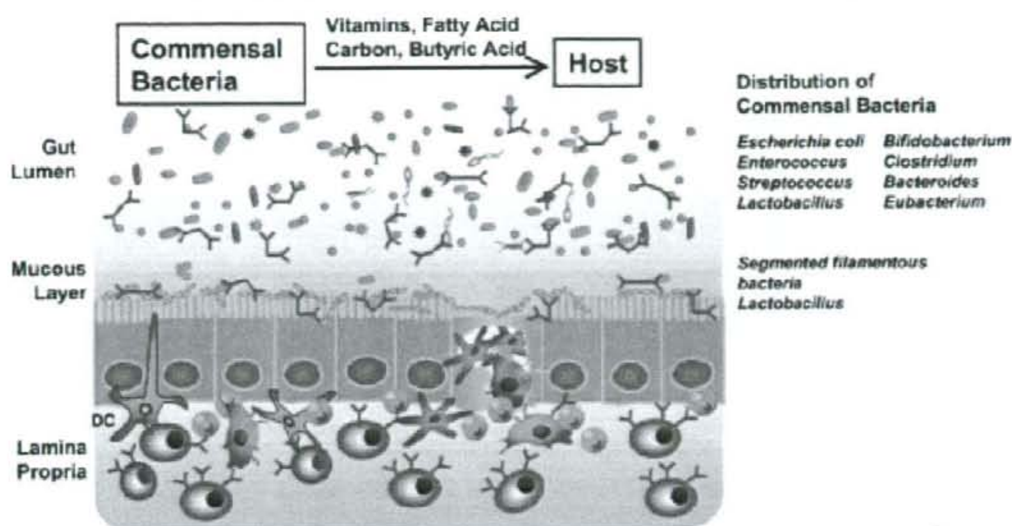

FIGURE 31.13 Symbiotic interactions between the mucosal immune system and commensal microbiota. Most microorganisms are present in the lumen and some preferably reside in the mucous layer. The host mucosal immune system regulates the diversity and quantity of intestinal microflora by secreting S-IgA Abs into the lumen. These Abs have been suggested to originate from B1 cells. These Abs may limit the adherence of commensal bacteria to ECs for an appropriate cohabitation environment and thus their ability to continuously produce beneficial bioactive molecules such as vitamins, fatty acids, carbon, and butyric acid for the host.

manifested by corresponding Abs (47). As a matter of fact, oral, intestinal, and probably other mucosal bacteria are coated *in vivo* with Abs, particularly of the IgA isotype, that may prevent their adherence to the epithelial receptors but do not significantly interfere with their elimination and metabolism (112). Therefore, products generated as a result of fermentation by mucosal bacteria such as butyric acid, fatty acids and vitamins can be important sources of energy and carbon for the human host, thus further stressing the immunological and physiological interdependence of the host on the mucosal microbiota (Figure 31.13) (125).

Quantitative and Qualitative Aspects of the Mucosal Microbiota

Quantitative data concerning the distribution of the indigenous microbiota on mucosal surfaces of the oral cavity, conjunctiva, and genital, GI, and respiratory tracts indicate that of approximately 10^{14} of bacteria, 99.9% are present in the large intestine (125). In this locale, bacteria are found free in the lumen and in feces, bound to the desquamated ECs, entrapped in the mucus layer, and deep in intestinal crypts (Figure 31.13). Despite the inherent difficulties with representative sampling, culture conditions, identification of cultured bacteria, as well as obvious host variables (e.g., hormonal status, diet, use of antibiotics, etc.), hundreds of species in 40 to 50 bacterial genera have been identified and described. These studies revealed that gram-negative and gram-positive, spore- and non–spore-forming, and aerobic as well as strictly anaerobic bacteria are present and are characteristically distributed in specific mucosal compartments. Although it is beyond the scope of this chapter to provide detailed information concerning the specific species distribution of indigenous microbiota in individual mucosal compartments, a brief summary of the colonic microbiota may illustrate the most important points. The intestinal microbiota is acquired shortly after birth and its composition is greatly influenced by the route of delivery (vaginal versus Cesarean section), the environment, and most profoundly by the diet (breastfeeding versus bottled formula and addition of solid food). Colonic microbiota changes from the dominant bifidobacteria at the initial stage to other species, particularly *Bacteroides* and anaerobic cocci with a significant presence of coliforms, streptococci and clostridia. Quantitative representation of bacteria in feces from adults indicates the dominance of bacteria of the genera *Bacteroides, Clostridium, Eubacterium, Lactobacillus, Streptococcus,* and *Bifidobacterium*;

E. coli constitutes only a minor contribution (~1%) of the colonic microbiota.

Regulation of Mucosal Immune Development and Immunological Homeostasis by Commensal Microbiota

The presence of the mucosal microbiota has a profound influence on the evolution and functionality of the immune system (47,126,127). As evidenced by a large number of studies performed on gnotobiotic (germ-free) animals, the development of both mucosal and systemic lymphoid tissues and the hosts' ensuing ability to respond to environmental Ags is, to a large extent, dependent on the previous exposure to a mucosal microbiota. Specifically, when compared to conventionally reared animals, lymphoid tissues of germ-free animals are hypotrophic, lack well-developed GCs, display minute numbers of mucosal PCs, and respond poorly to mitogens and polyclonal stimulants. Upon colonization with even a few representative species of mucosal microbiota, a prompt development of lymphoid tissues and restoration of responsiveness to a plethora of Ags and other stimuli ensues. Importantly, the development and responsiveness of both humoral and cell-mediated compartments of the immune system are profoundly affected by the mucosal microbiota as documented by the presence and numbers of B cells and ultimately Ab-forming cells (AFC) in mucosal and nonmucosal tissues, levels of mucosal and plasma Abs, and T cells of various phenotypes in the IEL and LP compartments of mucosal tissues, as well as in the systemic secondary lymphoid tissues (47,126,127).

The presence of the mucosal microbiota in the intestinal lumen induces S-IgA Ab synthesis. It was shown that induction of polyreactive S-IgA Ab responses to commensal bacteria is mainly derived from T cell–independent B1 cells (112). The T cell–independent IgA Abs originating from B1 cells possessed reactivity to conserved bacterial products (e.g., phosphorylcholine), which resulted in the nondiscriminating blockade of commensal bacterial attachment to mucosal surfaces. In this B1 cell Ab production pathway, IL-15 may be involved in IgA production facilitated by the intestinal microbiota, since it was previously reported that B1 cells proliferated in the LP when reacted with IL-15, an event induced by TLR-mediated signaling (113).

Another study revealed a unique pathway for specific Ab production against commensal microbiota in the intestine. It was shown that intestinal MΦ rapidly kill commensal bacteria, while intestinal DCs retain small numbers of live commensal bacteria and migrate only into MLNs but do not penetrate beyond MLNs (128). This function ensures a commensal bacteria-specific IgA Ab response that is specifically produced at the gut mucosa, but not in systemic immune compartments.

Mucosal microbiota-mediated innate immunity (e.g., TLR-mediated signaling) also plays an important role in the maintenance of mucosal homeostasis (47,126,127). For instance, the TLR-mediated cross-talk between the mucosal microbiota and IECs is biologically significant in the maintenance of epithelial homeostasis. EC cycles such as renewal, differentiation, and mitosis are significantly changed in germ-free or MyD88-deficient mice (22). In this regard, it was shown that TLR-mediated signals from the intestinal microbiota regulated the production of tissue protective factors such as IL-6, KC-1, and heat shock proteins (22). In addition to ECs, it was shown that TLRs were selectively expressed on $CD25^+$ $CD4^+$ Treg cells, which have been considered to be involved in the induction of oral tolerance, as discussed laer in this chapter (129). Thus, bacterial products such as LPS directly enhance their survival and proliferation. These products and host responses may explain why C3H/HeJ mice lacking TLR4-mediated signaling and germ-free mice show less sensitivity to oral tolerance induction (130).

Additional examples of molecules in the microbiota-dependent intestinal homeostasis are bacterial DNA. Bacterial DNA contains unmethylated CpG motifs within consensus sequences and is the ligand for TLR9 (11). Several lines of evidence have demonstrated that CpG targets DCs to induce the inhibitory environment (131), and it has been considered that some types of DCs are involved in these inhibitory immune responses (51,52); it appears that the intestinal microbiota stimulates intestinal DCs through the interactions between CpG and TLR9 for the maintenance of intestinal homeostasis. These inhibitory effects of bacterial DNA have led to the development of probiotics-mediated anti-inflammatory therapy, as discussed later in this chapter (131).

MUCOSAL TOLERANCE

Basic Concepts

In addition to the protective function of Ag-specific S-IgA and serum IgG Ab responses after mucosal immunization, the mucosal route of Ag delivery can also induce systemic unresponsiveness (132). Oral administration of a single high dose or repeated oral delivery of low doses of proteins has been shown to induce systemic unresponsiveness. Additional studies have shown that the nasal administration of proteins also induces systemic unresponsiveness and has led to the more general term mucosal tolerance to include nasal or oral Ag induction of unresponsiveness (132). The inhibition of Ag-specific immune responses in systemic compartments by mucosal Ag delivery is important for the prevention of overstimulation of responses and frequently encountered and hypersensitivity responses to food proteins and allergens. Further, this system could potentially be applied to the prevention and possibly treatment of autoimmune diseases by feeding relevant Ags.

Role of PPs in Oral Tolerance

The precise site of Ag uptake in the GI tract during oral tolerance induction has not been firmly established. One possible route is that Ags may enter the GALT via M cells and lead to APC–T cell interactions that down-regulate T and B cell responses. Some investigators have suggested that organized lymphoid tissue in the GI tract was not required for oral tolerance to OVA, since B cell–defective mice, which contain poorly developed PPs, were fully tolerized at the level of T cells (133). The availability of mice without PPs has allowed reinvestigation of the notion that GALT may be involved in oral tolerance. In one study, it was shown that mice that lack GALT but retain MLNs could be orally tolerized to OVA (134). However, others found that mice that lack PPs but retain MLNs were resistant to oral tolerance to protein (135); however, these mice showed normal mucosal S-IgA Ab responses to oral protein given with CT as adjuvant (41,42). Although one cannot yet conclude whether GALT is a strict requirement for oral tolerance to proteins, it is plausible to suggest that the nature of the Ag itself may influence the site of entry into the host.

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CD4⁺ T Cells in Oral Tolerance

The $\alpha\beta$ T cells appear to be the major players in down-regulation of systemic immune responses to orally administered Ags. It is generally agreed that the status of oral tolerance can be explained by: 1) clonal anergy or deletion of T cells, or 2) by active suppression by regulatory-type T cells through the secretion of inhibitory cytokines (132). Low doses of oral Ag tend to favor the latter form of inhibition, while higher doses of feeding induce clonal anergy of immunocompetent T cells. These two forms of oral tolerance are not mutually exclusive and may occur simultaneously following oral administration of Ags.

Anergy is defined as a state of T cell unresponsiveness characterized by the lack of proliferation and IL-2 synthesis and diminished IL-2R expression, a condition reversed by pre-culturing T cells with IL-2 (132). It was shown that Th1-type cells appear to be more sensitive to the induction of tolerance *in vitro* than Th2-type cells; *in vivo* evidence has demonstrated that Th1 cells are likely to be anergized in oral tolerance. This may be an oversimplification since it has been shown that oral tolerance can be induced in mice defective in Th1 ($STAT4^{-/-}$) or Th2 ($STAT6^{-/-}$) cells (136). Further, to identify which lymphocyte compartment (e.g., $CD4^+$ versus $CD8^+$ T cells) preferentially mediates the induction of oral tolerance, cell transfer experiments were performed using SCID and *nu/nu* mice, demonstrating that oral tolerance was induced by anergized $CD4^+$ but not $CD8^+$ T cells (137).

Clonal deletion has been considered as another pathway for the induction of oral tolerance. Clonal deletion of Ag-specific $CD4^+$ T cells was detected after oral feeding of high doses of Ag (132). Accumulating evidence has revealed that the clonal deletion induced by feeding high doses of Ag was due to an increase in the susceptibility of lymphocyte apoptosis via increased expression of caspase.

Mucosal Regulatory T Cell Networks

Regulatory- and suppressor-type T cells are crucial players in the induction of peripheral tolerance to self and foreign Ags. It is now accepted that several populations of T cells expressing CD4 or CD8 show regulatory or suppressive functions to other T cell-mediated mucosal immune responses. They can be classified as: 1) naïve, or those which have not yet encountered Ag; 2) activated (effector); and 3) memory, where both effector and memory T cells have engaged in the regulation of immune responses (48,132). $CD4^+$ T cells including $CD4^+CD25^+Foxp3^+$ Treg cells, Th3 cells (secreting TGF-β), and Tr1 cells (secreting IL-10) and $CD8^+$ T cells were shown to be key players for the creation of a mucosal regulatory T cell network in the establishment of quiescent immunity (or mucosally induced tolerance) (Figures 31.7 and 31.14).

Fig 31.14

CD25⁺ CD4⁺ Treg Cells

Recent evidence has revealed that naturally arising $CD25^+$ $CD4^+$ Treg cells play a pivotal role in the negative control of a variety of physiological and pathological immune responses (138). Naturally arising Treg cells specifically express a forkhead winged-helix transcription factor family member (Foxp3) for the process of Treg cell lineage commitment. Thus, Foxp3 deficiency results in early onset, fatal, systemic autoimmune disease. The $Foxp3^+$ $CD25^+$ $CD4^+$ Treg cells are present in the PPs, suggesting that $Foxp3^+$ $CD25^+$ $CD4^+$ Treg cells are involved in the maintenance of intestinal homeostasis.

In addition to inhibitory cytokines, such as IL-10 and TGF-β, several inhibitory molecules have been shown to be expressed on $CD25^+$ $CD4^+$ Treg cells (138). Although naïve T cells express CTLA-4 (or CD152) after activation, which competes with the cellular interaction between CD28 on effector T cells and CD80/CD86 on APCs, $CD25^+$ $CD4^+$ Treg cells constitutively express CTLA-4 (Figures 31.7 and 31.14). The CTLA-4 on Treg cells plays a pivotal role in the maintenance of mucosal homeostasis (139). Thus, anti-CTLA-4 mAb treatment inhibited the regulatory function of $CD25^+$ $CD4^+$ Treg cells and subsequently led to the development of IBD. Several mechanisms have been proposed for the inhibitory function of CTLA-4 on Treg cells, including the enhancement of Treg cell activity through the interaction with CD80/CD86 on APCs (138). Since ECs also express CD80 as mentioned earlier (37), it is possible that Treg cells recognize CD80 on the ECs exhibit their immunosuppressive function. Another possible mechanism is the induction of the inhibitory molecule, IDO (140). As

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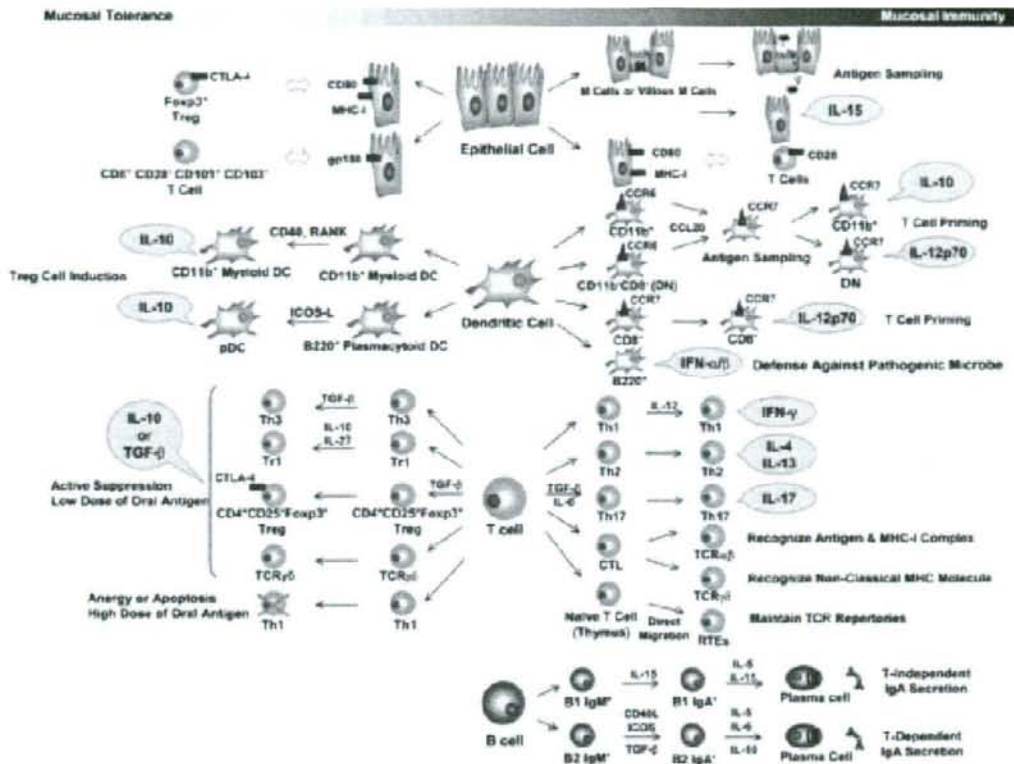

FIGURE 31.14 Cellular and molecular mechanisms for the induction and regulation of mucosal immunity and tolerance. A unique aspect of the mucosal immune system is the simultaneous presence of active (e.g., effector T cells and S-IgA Abs) and quiescent (e.g., Treg cells and mucosal tolerance) immunity. To smoothly operate two opposite types of immunity, ECs, DCs, and T cells play central roles by providing appropriate inflammatory and anti-inflammatory cytokine networks together with costimulatory and chemokine molecular families.

mentioned earlier, IDO exhibits immunosuppressive effects by catalyzing the catabolism of tryptophan, an essential amino acid for T cell proliferation (20). In addition to CTLA-4, Treg cells also express high levels of glucocorticoid-induced TNF-like receptor (GITR), another important suppressive function-associated molecule, that contributes to the maintenance of a quiescent condition in mucosal immune compartments (138). Thus, transfer of $GITR^{high}$ Treg cell–depleted spleen cells to athymic nude mice resulted in the development of autoimmune diseases including gastritis (141). GITR-mediated signaling triggers Treg cell proliferation, which has been considered to be one molecular mechanism for GITR-mediated suppressive function of Treg cells in the presence of IL-2, but detailed mechanisms remain unclear.

Although naturally arising $CD25^+$ $CD4^+$ Treg cells develop in the thymus and their survival in the periphery is dependent on IL-2 (138), accumulating evidence has demonstrated that $Foxp3^+$ Treg cells can be induced from $Foxp3^-$ precursors and, like naturally arising $CD25^+$ $CD4^+$ Treg cells, the induced Treg cells express CD25 and CTLA-4, and also produce IL-10 and TGF-β (142). It is interesting to note that cytokines such as TGF-β and IL-10 contribute to the differentiation of Treg cells (142). As mentioned earlier, TGF-β and IL-10 are predominantly produced in the intestinal compartments; thus, the GI tract seems to be an optimal environment for naturally inducing the differentiation of Treg cells for the creation of immunologic homeostasis in the otherwise harsh environment of the gut.

Th3 Cells

Th3 cells were initially discovered as a form of suppressor T cell subset that accounted for oral tolerance (Figures 31.7 and 31.14) (48,132). The finding of CD4$^+$ T cell clones generated after induction of oral tolerance to myelin basic protein (MBP), led to the description of a new phenotype of regulatory or suppressor T cell. Clones of CD4$^+$ T cells were MBP-specific, and of 48 clones assessed, 42 produced the active form of TGF-β, which is also an essential cytokine to induce Th3 cells (143). Th3 cells have different cytokine requirements for their growth from CD25$^+$ CD4$^+$ Treg cells. As mentioned earlier, the survival of CD25$^+$ CD4$^+$ Treg cells is dependent upon IL-2 (138), while *in vitro* differentiation of Th3 cells is enhanced by TGF-β, IL-4, and IL-10 (48). These findings suggest that Th3 cells are a different linage from naturally arising CD25$^+$ CD4$^+$ Treg cells, but it is still unclear whether Th3 cells are the same as induced Treg cells because of the lack of a specific marker for Th3 cells. It was previously shown that TGF-β was produced by intestinal DCs (51,52), which has been considered to be the source of cytokines for the induction of Th3 cells in the intestine. Additionally, since TGF-β production was induced by CTLA-4, which is constitutively expressed on naturally arising Treg cells (142), it is possible that TGF-β production from Treg cells through CTLA-4–mediated signaling may stimulate the differentiation of both induced Treg cells and Th3 cells.

Tr1 Cells

Another regulatory type of T cell is one that secretes IL-10 and TGF-β and has been termed a *Tr1 cell* (Figures 31.7 and 31.14) (144). Similar to IL-10–secreting induced-type Treg cells, Tr1 cells are induced by Ag stimulation in the presence of IL-10, which is abundantly produced by DCs in GALT and in pulmonary tissues, as mentioned earlier (51,52). The function of Tr1 cells is to suppress Ag-specific effector T cell responses in a cytokine-dependent manner. Ag-specific activation of TCR is required for the Tr1 suppressive function, but Tr1 cells can also mediate bystander suppressive activity against other Ags once they are activated. Although the migratory capacity of Tr1 cells has not been elucidated yet, it is interesting to note that Tr1 cells in the blood circulation express the GI tract migration chemokine receptor, CCR9, suggesting that these cells intrinsically home to the intestine (145). Consistent with this idea, previous studies reported an important role for Tr1 cells in IBD and celiac diseases (144,146). Although Tr1 cells do not express Foxp3 the lack of Tr1 cells resulted in the development of intestinal inflammation. Hence, Tr1 cells have been considered to be a unique subset of regulatory T cells, which is distinct from the CD4$^+$ CD25$^+$ Treg cell subset, important in the control of undesired hyperimmune responses in the intestine.

CD8$^+$ Suppressor T Cells

The first identified population of regulatory T cells thought to be involved in oral tolerance was a CD8$^+$ suppressor T cell subset (Figure 31.14) (147). However, their functions and characteristics have not been clearly defined. It was reported that CD8$^+$ CD28$^-$ suppressor T cells induced the up-regulation of Ig-like transcript 3 (ILT3) and ILT4 expressed on human monocytes and DCs, rendering these APCs tolerogenic by inducing Ag-specific unresponsiveness of CD4$^+$ T cells through reduced expression of costimulatory molecules (148). Subsequent study has revealed that ILT3/4 expression in human vascular endothelial cells was up-regulated by IL-10 (149). Since IL-10 is abundantly produced in the intestinal compartments, it is plausible that IL-10 produced in the intestinal compartments may regulate ILT3/4-mediated suppressive function as suppressor T cell–mediated maintenance of intestinal homeostasis. In addition, it was suggested that CD8$^+$ CD122$^+$ (IL-2/IL-15 receptor β chain) T cells behave like naturally occurring regulatory T cells, where the depletion of the CD8$^+$ T cells resulted in the high incidence of pulmonary inflammation (150). Although CD8$^+$ CD122$^+$ T cells are also involved in the intestinal immune system, the finding further suggested the existence of multiple layers of a mucosal regulatory network for the creation and maintenance of the quiescent status of the immune environment in the mucosal compartments of both the GI and respiratory tracts.

Factors in Determining the Type of Regulatory T Cells

Recent studies have identified several factors determining whether T cells differentiate into pathogenic or regulatory T cells. For instance, as mentioned earlier, TGF-β is known to be an essential molecule for the induction of Treg cells and Th17 cells; however, Th17 cells additionally require IL-6 for their development. Therefore, in the presence of IL-6 plus TGF-β, only Th17 cells developed (62,63). A recent separate study shows that stimulation with TGF-β and IL-6 triggers initial lineage commitment of Th17, but IL-23 is required for the full differentiation of Th17 cells (151). In contrast to the effects of IL-23 for the full differentiation of IL-6– and TGF-β-treated T cells into Th17 cells, IL-27 plus IL-6 and TGF-β–induced T cells producing IL-10, which resembled Tr1 cells (152,153). Thus, it seems that IL-23 and IL-27 both play important roles in the fate decision of IL-6– and TGF-β–exposed T cells either become pathogenic Th17 cells or regulatory Tr1 cells. Several separate studies have revealed that mucosal DC–derived retinoic acid, a key molecule for the induction of gut-homing $\alpha 4\beta 7$ integrin and CCR9, as mentioned earlier (88,89), also enhanced conversion of TGF-β–treated T cells to Treg cells and simultaneously suppressed the

differentiation to Th17 cells (154–157). These data suggest the presence of versatile pathways for regulating T cell fate.

Role of IELs in Mucosal Tolerance

Since the intestinal epithelium is directly and continuously exposed to gut environmental Ags, it was logical to consider that IELs and IECs contribute to the mucosal regulatory network for the induction of mucosal tolerance (25,132). Interestingly, it has been shown that depletion of TCR$\gamma\delta$ cells resulted in the failure to induce the systemic unresponsiveness after oral administration of Ags (158). Another study demonstrated that when either TCR$\delta^{-/-}$ or TCR$\delta^{+/+}$ mice were immunized orally with a high dose of OVA prior to parenteral challenge, systemic IgG and IgE Ab responses were markedly reduced in both types of mice (159). Reduced T cell proliferative responses and delayed-type hypersensitivity were seen in both TCR$\delta^{-/-}$ and TCR$\delta^{+/+}$ mice given high-doses of OVA. In contrast, while oral tolerance associated with increased levels of IL-10 synthesis was induced by low-dose OVA in TCR$\delta^{+/+}$ mice, TCR$\delta^{-/-}$ mice were not tolerized and failed to produce IL-10 (159). These findings indicate that $\gamma\delta$ T cells play an important role in IL-10–mediated, low-dose oral tolerance induction, but are not essential participants in the induction of systemic tolerance induced by oral administration of large doses of Ag. It has been suggested that oral tolerance induced by repeated administration of small doses of Ag is mediated by T cells involved in the generation of active suppression, while systemic unresponsiveness induced by large doses of Ag is caused by clonal anergy or clonal deletion (132). Thus, it is likely that $\gamma\delta$ T cells play regulatory roles for the induction of active suppression, although they are not involved in the induction of clonal anergy or deletion.

DCs and IECs in Oral Tolerance

It has been demonstrated that intestinal DCs contribute to the induction of tolerance. The initial evidence of DC involvement in the induction of oral tolerance was provided by the demonstration that Flt3 ligand-mediated expansion of DCs led to enhanced oral tolerance (160). Among several kinds of DCs in the intestinal compartments, two subsets of DCs have gained attention. As mentioned earlier, CD11b^{+} myeloid DCs in the PPs have the unique feature of producing predominantly IL-10 in response to CD40 ligation or receptor activator of NF-κB (RANK)/RANK ligand interactions (51,52). A second DC population involved in the induction of oral tolerance is pDCs. One study described that CD11c^{low} DCs displayed a plasmacytoid morphology and a stable immature phenotype and secreted IL-10 for the induction of IL-10–secreting Tr1 cells, and a recent study has revealed that inducible costimulator ligand (ICOS-L) plays an important role in pDC-mediated Tr1 induction (161). High levels of ICOS-L expression on pDCs allow them to induce the differentiation of naïve CD4 T cells to IL-10 but not the other Th2 cytokines (161). In addition to Tr1 cells, pDCs induced IL-10–producing CD8^{+} regulatory T cells (162). Taken together, IL-10 produced by DCs is a key factor in the differentiation of regulatory-type T cells.

ECs are also thought to contribute to the induction of oral tolerance by capturing and presenting luminal Ag by MHC molecules with low expression of costimulatory molecules (37). As an additional pathway of IEC-mediated oral tolerance, it was demonstrated that gp180-mediated interaction between IECs and CD8^{+} CD28^{-} CD101^{+} CD103^{+} cells caused the CD8^{+} CD28^{-} CD101^{+} CD103^{+} cells to develop into regulatory cells (163). IECs also mediated suppression of CD4^{+} T cell activation in a cell contact-dependent and TGF-β–independent manner (164). A recent study has demonstrated that IEC-primed T cells secreted lower amounts of IFN-γ and IL-2 and exhibited an increased expression of IL-10 and Foxp3, providing direct evidence that IECs induced IL-10–producing Foxp3^{+} T cells (165). Thus, IECs can be involved in the creation of a mucosal regulatory network in two phases including Ag-presentation and priming of regulatory-type T cells.

Nasal Tolerance

The initial dogma that mucosal tolerance requires intestinal processing of the Ag was challenged by the observation that systemic unresponsiveness could be achieved by administration of the Ag via the nasal or aerosol routes (132). These routes were found to require lower doses of Ags than did oral administration, a discrepancy that can be explained by the dilution effect, as well as the potential degradation of the Ag in the GI tract. Although the precise mechanism behind nasally induced tolerance is not yet known, several studies have demonstrated a similar pathway for the induction of both nasal tolerance and oral tolerance. For example, the membrane-bound form of TGF-β–expressing CD4^{+} Foxp3^{+} T cells are involved in tolerance induction to inhaled Ag (166). It was also shown that airway pDCs suppressed the generation of effector T cells primed by myeloid DCs via the induction of IL-10–producing Treg cells (167).

Mucosal Tolerance in Humans

Increasing attention is being paid to oral tolerance and the role it could play in the prevention or treatment of autoimmune diseases, including multiple sclerosis, rheumatoid arthritis, uveitis, as well as type I diabetes and contact hypersensitivity (132). Indeed, humans immunized with the neoantigen KLH either by the oral or nasal route developed systemic unresponsiveness evaluated by DTH and T cell proliferative responses. However, B cell responses

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were primed in both systemic and mucosal sites. In other studies, humans naturally ingesting the dietary Ags bovine gamma globulin, OVA, and soybean protein developed a T cell tolerance characterized by anergy (132). Antigen-specific Th3 cells secreting TGF-β have been observed in the blood of multiple sclerosis patients orally treated with a bovine myelin preparation, demonstrating that oral administration of autoantigen can induce antigen-specific TGF-β–secreting cells in a human autoimmune disease.

Pilot clinical trials of oral tolerance have been conducted in patients with autoimmune diseases, and promising clinical benefits have been reported (132). Despite encouraging initial results regarding oral delivery of autoantigens for the treatment of human autoimmune diseases, a followup study did not demonstrate statistically significant beneficial effects. Further, oral feeding of autoantigen in mice resulted in the generation of antigen-specific $CD8^+$ CTL responses that could lead to the aggravation of autoimmune disease (132). Thus, one must also keep in mind that oral administration of autoantigen may induce undesirable $CD8^+$ CTLs that may worsen the disease instead of preventing the development of autoimmunity.

A description of extensive experiments and clinical studies based on the exploitation of principles of mucosal tolerance in the prevention and treatmenet of T and B cell–mediated hypersensitivity diseases (e.g., contact dermatitis and inhalation allergies), other autoimmune diseases (e.g., uveoretinitis, glomerulonephritis, and diabetes), and prolonged survival of allografts are beyond the scope of this review. However, these efforts have not yet reached fruition. Thus, the experience of most investigators is that once antigen-specific systemic immune response has been induced, it is difficult to achieve a reversal through mucosal tolerance.

MUCOSAL IMMUNE SYSTEM FOR HOST DEFENSE

Mucosal Vaccines

Mucosal surfaces are also the most frequent portals of entry of common viral, bacterial, fungal, and parasitic agents causing both local and systemic infectious diseases. The fascinating characteristics of the mucosal immune system in the prevention of infections by pathogens has led to much attention for the development of mucosal (e.g., oral and nasal) vaccines (6). Mucosal vaccines offer numerous advantages over traditional injection-type parenteral vaccines, including needleless and easy administration. Most important, mucosal vaccines can induce both mucosal and systemic immune responses, while parenteral immunization yields only systemic immune responses. Hence, traditional parenteral immunization does not induce mucosal immunity, which would inhibit the initial attachment of pathogens, while mucosal vaccines can establish a first line of immunological defense at mucosal sites as well as provide a systemic immune surveillance to detect and destroy invading pathogens. Therefore, numerous studies have been conducted to harness the enormous potential of the mucosal immune system to induce protective immune responses at the site of entry of infectious agents.

However, due to the difficulties with dosing of relevant Ags, their limited absorption, proteolytic degradation, low pH, and detergent activity by bile salts, unique Ag delivery systems and mucosal adjuvants have been explored to avoid such problems (6). At a minimum, these systems should protect the Ag from physical and biological elimination. In addition, a major research focus has been aimed at molecular and cellular elucidation of key immunological mechanisms for the simultaneous induction and regulation of active (e.g., S-IgA) and silent (e.g., mucosal tolerance) immune responses.

Administration Route of Mucosal Vaccines

Stimulation of local and generalized mucosal immune responses can be achieved by ingestion of Ags or their introduction by the rectal route. The former route exploits the inductive potential of lymphoepithelial tissues distributed in the small intestine, while the latter route primarily stimulates cells accumulated in structures termed *rectal tonsils* (6). Immune responses induced by infections or immunization through the nasal mucosa and oropharyngeal lymphoid tissues (Waldeyer's ring) have been evaluated with particular emphasis on local respiratory tract pathogens such as influenza, parainfluenza, and respiratory syncytial viruses (120). Individuals naturally infected or locally immunized with attenuated viruses responded by formation of S-IgA and IgG Abs in nasal secretions and when examined also in saliva (120). In general, nasal immunization in contrast to intestinal administration, induces prominent systemic immune responses manifested by the presence of AFCs in peripheral blood with mucosal as well as systemic homing receptors, and plasma Ab responses. Examination of other external secretions of nasally immunized humans and animals revealed another significant feature: the female genital tract secretions contained high levels of antimicrobial Abs of IgA and IgG isotypes, which, in some experiments, were higher than those induced by local, oral, rectal, or systemic immunizations (168). Thus, it appears that nasal exposure with Ags is the route of choice for the induction of female genital tract responses.

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The mucosal immune system of the female and male genital tracts displays several features distinct from other mucosal sites, such as the absence of lymphoepithelial structures analogous to intestinal PPs, and a dominance of IgG-AFCs in tissues and IgG in cervical mucus, vaginal

washes, and serum (168,169). Although repeated administration of Ags with adjuvants, or infection with live viruses generated local immune responses, Abs were absent or present in low levels in secretions of remote glands, probably due to the lack of organized inductive sites, equivalent to PPs, in the genital tract. However, systemic immunization followed by local mucosal booster or targeted immunization in the vicinity of local LNs enhanced genital tract responses (168,169). Further, sequential combination of several immunization routes (systemic, oral, rectal, vaginal, tracheal, or nasal) generates better results than repeated immunization at a single site.

Enterotoxin-Based Mucosal Adjuvants

Two bacterial enterotoxins, CT and the closely related heat LT, are the most well-studied mucosal adjuvants, which are derived from *Vibrio cholerae* and *Escherichia coli*, respectively (6,170,171). They are not only potent immunogens, but also adjuvants that enhance both mucosal and systemic immune responses against mucosally coadministered Ags. CT and LT are structurally similar (83% homology at the amino acid level) hexameric toxins consisting of two structurally and functionally separate A (CT-A or LT-A) and B (CT-B_5 or LT-B_5) subunits (6,170,171). The A subunit possesses ADP-ribosyltransferase activity, and the B subunit participates in the binding to host cells. Different binding activities between CT-B and LT-B have been reported. The CT-B binds to GM1-ganglioside, whereas the LT-B binds to GM1-ganglioside as well as asialo GM1 and GM2. The binding of B subunits to their receptors on ECs allows the A subunit to reach the cytosol of target cells, where it binds to nicotinamide adenosyl diphosphate (NADP) and catalyzes the ADP ribosylation of Gsα. The latter GTP-binding protein activates adenyl cyclase with subsequent elevation of cAMP in ECs, followed by secretion of water and chloride ions into the intestinal lumen. Although both CT and LT have strong adjuvant activities, the clinical use of CT and LT has been hampered by the fact that both enterotoxins induce severe diarrhea after oral administration or from natural infection. Both enterotoxins also have undesirable side effects involving their entry into the central nervous system when given by the nasal route (6,170,171).

To circumvent toxicity linked to these enterotoxins, several groups have attempted to generate mutants of CT (mCT) and LT (mLT) devoid of their toxic activity or replacement of the toxic A subunit (6,170,171). The first approach involves the introduction of single amino acid substitutions in the active site (i.e., the site responsible for the ADP-ribosylation activity) of the A subunit of CT or LT or in the protease sensitive loop of LT. mCTs constructed by substitution of serine by phenylalanine at position 61 (CT-S61F) and glutamate by lysine at position 112 (CT-E112K) in the ADP-ribosyltransferase activity center of the CT gene from *V. cholerae* 01 strain GP14 display no ADP-ribosyltransferase activity or enterotoxicity (6,170,171). The levels of Ag-specific serum IgG and S-IgA Abs induced by the mutants are comparable to those induced by wild-type CT. Further, the mutant CT-E112K, like native CT, induces Th2-type responses through a preferential inhibition of Th1-type CD4$^+$ T cells. Subsequent studies demonstrated that the mutant forms of CT were effective for the induction of immune responses against tetanus toxin, *Streptococcus pneumoniae*, influenza virus, diphtheria toxin, HIV, and botulinum neurotoxin (6,170,171). Mutations in other sites of the CT molecule were reported to induce nontoxic derivatives, but the adjuvant activity was also affected. Similarly, mLTs have also been successfully developed as safe mucosal adjuvants and used with vaccines against measles virus, tetanus toxin, *Helicobacter pylori*, and influenza among many others, and some of them have been examined clinically (6,170,171).

Another strategy that exploits the binding potential of CT and CT-B to gangliosides on mucosal cells involves the genetic construction of recombinant chimeric proteins. The toxic subunit A of CT consists of two segments, A1 (carrier of toxicity) and A2, that interact with the B subunit. Genetic replacement of the A1 segment with DNA encoding a desired Ag (e.g., Ag I/II of *Streptococcus mutans*) results in the assembly of a molecule composed of CT-B/A2-Ag (6,170,171). When given intragastrically and especially nasally, potent Ag-specific humoral immune responses were generated in mice.

In addition to CT and LT, several toxins have been shown to exhibit mucosal adjuvant activity. For instance, Shiga toxin 1 (STX1) and the mutant form of STX1 have been shown to exhibit mucosal adjuvanticity. A genetically engineered pertussis toxin (PTX) developed by removal of its ADP-ribosylating activity and was an effective adjuvant for enhancing mucosal immune responses (6). The PTX recognizes glycoprotein with a branched mannose core and an N-acetyl glucosamine expressed on various types of mammalian cells. Nasal immunization with tetanus toxin and PTX augmented parenteral and mucosal Ab responses. *Zonula occludens* toxin (Zot) is a single polypeptide encoded by the filamentous bacteriophage infecting toxigenic strain of *V. cholerae*. Nasal or rectal immunization with Zot resulted in the induction of plasma IgG and mucosal S-IgA Ab responses against coadministered Ag mediated by both Th1- and Th2-type cells (6). CTA1-DD is composed of an enzymatically active CT-A and a dimer of an Ig-binding element of *Staphylococcus aureus* protein A. Thus, it targets to B cells (172). When CTA1-DD was applied nasally, it enhanced Ag-specific immune responses in both mucosal and systemic sites without causing inflammation. A subsequent study indicated that the adjuvanticity of CTA1-DD was at least mediated by promoting GC formation (172). Surprisingly, CTA1-DD is nontoxic although it contains the intact form of holotoxin.

It should be noted that, except for a few results, almost all mutant forms of adjuvants derived from the bacterial toxin retained full adjuvant activity at least after nasal and parenteral immunization, but possess less adjuvant activity when they were given orally. The reasons for different adjuvant activities after nasal versus oral delivery remains an open question, and further experiments are necessary for effective oral delivery use of toxin-based mutant adjuvants.

Mucosal Cytokines, Chemokines, and Innate Factors as Adjuvants

Mucosal delivery of cytokines allowed the use of these molecules that primarily interact with their corresponding receptors without the important adverse effects that are often associated with the large and repeated parenteral cytokine doses generally required for the effective targeting of tissues/organs. Considerable numbers of cytokines such as type I IFNs, IL-1, IL-2, IL-12, IL-15, and IL-18 have been shown to have mucosal adjuvant activity (6). Although these cytokines showed adjuvant activities after a single use, previous reports have provided evidence for synergistic effects of cytokines. For instance, the adjuvant activity for induction of mucosal S-IgA and systemic IgG Abs after simultaneous administration of IL-1, IL-12, and IL-18 was much stronger than those induced by the treatment with each cytokine alone or a combination of IL-12 plus IL-18. In contrast to the synergistic effects of IL-1, IL-12, and IL-18, coexpression of IL-15 with IL-12 did not enhance adjuvanticity. These findings suggest that the adjuvant mechanism mediated by cytokines is complex and should be carefully examined for the suitability of a particular cytokine for use in the development of effective mucosal vaccines.

In addition to cytokines, chemokines have been shown to act as innate-type mucosal adjuvants (6). For instance, nasal administration of XCL1/lymphotactin with Ag resulted in the marked enhancement of Ag-specific S-IgA Abs in various mucosal secretions (e.g., feces, saliva, vaginal, and nasal washes) and plasma IgG Ab responses. Similarly, nasal coadministration of RANTES with Ag induced high levels of S-IgA, plasma IgG, and preferential Th1-type responses. MIP-1 is another CC chemokine that was analyzed for its ability to act as a mucosal adjuvant. MIP-1 contains two homologous subtypes, MIP-1α and MIP-1β. It was demonstrated that nasal administration of MIP-1α enhanced Ag-specific Ab responses to coadministered Ag in systemic but not in mucosal sites. In contrast, MIP-1β promoted mucosal S-IgA Ab responses with less efficient induction of systemic immune responses, although they both share the same ligand (CCR5).

Defensins belong to a family of antimicrobial peptides produced by Paneth cells, as mentioned earlier (4,5). Defensins also possess chemotactic activity against T cells and exert adjuvant activity (6). It is interesting to note that no mucosal S-IgA Ab responses were induced after nasal immunization with defensins, although they promoted systemic IgG Ab responses associated with IFN-γ, IL-5, IL-6, and IL-10 production. Thus, defensins are unique adjuvants that enhance systemic immune responses without induction of mucosal S-IgA Ab production.

PRR-Targeted Mucosal Adjuvants

Innate immunity plays a pivotal role in host defense against invading microbial pathogens at early stages of infection through recognition by PRRs (11). The PRR-mediated signals induce cytokine production like type I IFN, IL-1 and IL-12, as well as antimicrobial peptides like defensins that are all known to have adjuvant activity, as discussed earlier. As one may expect from the fact that cytokines that function as mucosal adjuvants, PAMPs, also act as mucosal adjuvants. For example, monophosphoryl lipid A (MLA) that has already been shown to be a systemic adjuvant pre-clinically and clinically effectively works as a mucosal adjuvant (6). Until now, the target cells of TLR agonists remained obscure but were presumably DCs, since TLR4 expression is very low or absent on ECs, as mentioned earlier. TLR2 is also a target of a specific mucosal adjuvant. Muramyl dipeptide (MDP) is derived from the cell wall of mycobacteria and has been shown to be one of the ligands for TLR2. Before identification of TLR2, MDP was shown to stimulate PP cells for the enhancement of IgA Ab responses (6). Recent studies have demonstrated that mycoplasma-derived MΦ-activating 2 kDa lipopeptide (MALP-2) promoted Th2-type responses, plasma IgG, and mucosal S-IgA Ab responses against coadministered Ags such as β-galactosidase and HIV-1 Tat protein through TLR2 (173).

The discovery that gene-associated molecules (e.g., DNA and RNA) had immune-stimulating activities allowed us to extend this system for development of mucosal adjuvants. Bacterial, but not eukaryotic, DNA generally contains nonmethylated "CpG motifs" and acts as a ligand for TLR9, thus, initiating innate and adaptive immunity (11). Thus, plasmid DNA for gene vaccination can be functionally divided into two distinct units: a transcription unit and an adjuvant/mitogen unit (131). The latter unit contains immunostimulatory sequences consisting of short palindromic nucleotides centered on a CpG dinucleotide core. 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 (131). Numerous studies have shown that mucosal administration of Ag with CpG promoted mucosal S-IgA, plasma IgG, and T cell responses, including $CD8^+$ CTLs and $CD4^+$ Th1 cells accompanied with type I IFN production by DCs, which induced protective immunity against various types of infections, such as

Streptococcus pneumoniae, HIV, HSV-2, and *Helicobacter pylori* (131).

Mucosal Ag Delivery Systems

The mucosal delivery of Ag is another important subject in the area of mucosal vaccine development. Various approaches toward the development of an ideal mucosal Ag delivery system have been developed using inert particles, including biodegradable polymer-based particles (microspheres and nanospheres) as well as lipid-based particles such as liposomes and ISCOMs (6,174). Incorporation of Ags into these particles usually protects them from proteolytic degradation by mucosal enzymes and acids; however particles by themselves are nonimmunogenic. Further, variation in microsphere chemical composition allows generation of particles with fast or slow degradation to stimulate long-lasting responses. Several different Ags can be incorporated into a single preparation, and other substances such as cytokines can be co-incorporated with Ags to show ensuing immune responses (6). These obvious attractive features are, however, counterbalanced by serious disadvantages. Specifically, the disappointingly low uptake from mucosal surfaces (<1%), low rate of incorporation, and the use of organic solvents that may denature Ags are negative features.

To overcome this limitation, several modifications have been attempted to deliver the Ag selectively to M cells, the major targets for delivery of encapsulated Ag. Lectins have been widely exploited to gain or to enhance access to M cells. It has been considered that *Ulex europaeus agglutinin* 1 (UEA1), a lectin specific for α-L-fucose residues, binds almost exclusively to the apical surface of M cells of murine PPs and NALT (44). The unique reactivity of UEA1 to M cells allowed the selective and effective delivery of microspheres or liposomes to M cells after oral administration, which led to the significant enhancement of Ag-specific Ab responses (175). Recent advances in biomedical technology have been utilized to identify organic molecules or peptides that mimic the functional activity of UEA-1 using mixture-based positional scanning of synthetic combinatorial libraries or of phage peptide libraries. The former study revealed that a digalloyl D-Lysine amide construct and a tetragalloyl D-Lysine amide construct bound effectively to M cells, and the coating of particles with these compounds resulted in the selective delivery of the particles to M cells with high efficacy (176). The latter study demonstrated that specific peptide (YQCSYTMPHPPV) selectively bound to the M cell–rich subepithelial dome region of the PPs and enhanced the delivery of microspheres to M cells (177). In addition to these molecules, an M cell–specific monoclonal antibody (mAb) was recently developed, which can be used for the M cell–targetted delivery of vaccine antigen (178). This mAb recognized carbohydrate-modified molecules selectively expressed on M cells and thus, effectively delivered the conjugated vaccine Ag to M cells for the induction of mucosal and systemic Ag-specific immune responses (178).

Microbial adhesins have been applied to the targeted delivery of synthetic particles to M cells. As expected given the selectivity of ligands, enhanced Ag uptake was achieved by coating polystyrene nanospheres with *Yersinia*-derived invasin, a ligand for $\beta 1$ integrins on the apical side of M cells (44,59). Similarly, reoviruses are known to invade through M cells using a 45-kDa viral haemagglutinin sigma one ($\sigma 1$) protein (44,59). Subsequent studies demonstrated that mucosal immune responses were significantly increased by mucosal immunization by coupling a reovirus-derived $\sigma 1$ protein (179). As another approach, hybrid Ag delivery vehicles have been developed (180). These vehicles are composed of a synthetic liposome and virus, such as influenza and Sendai viruses (6). Using fusion activity that originates from the virus, the virus-mimicked liposomes could effectively deliver the encapsulated Ag to MALTs and induce high levels of Ag-specific immune responses.

Attenuated live microorganisms have been developed as vaccines, and some of them have already been used as mucosal vaccines (e.g., poliovirus, *S. typhi* Ty21a, and *V. cholerae*) (6,181,182) since the attenuation may not affect the natural abilities of bacteria to survive in the hostile environment of the intestinal and respiratory tracts and to bind to M cells to promote vaccine uptake for the effective induction of mucosal and systemic immune responses. Recent progress in genetic technology has allowed the creation of a new application of attenuated vaccines for Ag delivery, namely recombinant attenuated vaccines carrying DNA encoding heterologous Ag (6,181,182). In the attenuated recombinant vaccines, several genes determining pathogenicity have been mutated or disrupted, and a gene encoding a heterologous Ag has been inserted, which ensures both safety and effectiveness. Vectors that have been tested include various species of attenuated bacteria such as *S. typhi*, *Shigella flexneri*, and *Listeria monocytogenes*, *V. cholerae*, *Lactobacillus*, and *Y. enterocolitica* (181). Similarly, recombinant viruses have been established as vehicles for mucosal vaccine delivery (182). Since CTL responses appear to be pivotal in chronic viral infections, these efforts have been aimed at developing mucosal vaccines that induce both mucosal S-IgA and CTL responses to prevent initial contact of pathogens with host cells in mucosal sites and for surveillance of virus-infected cells, respectively. Several types of viruses have the advantage of their natural transmission via mucosal sites. These include poxvirus, adenovirus, HSV, adeno-associated virus, alphavirus (e.g., Semliki Forest virus and Sindbis virus), vesicular stomatitis virus, and poliovirus (182).

Novel molecular methods have allowed the production of subunit vaccines in transgenic plants (183). Plant-based vaccines offer some advantages over other systems,

including: (1) the ability to carry out posttranslational modifications similar to eukaryotes; (2) ease of production of large quantities at reduced costs; (3) no requirement to use a human pathogen. Assemblies of one or more Ags that retain both T and B cell epitopes have been expressed in genetically modified plants (GM plants) and are now being tested for their potential use as human or animal vaccines. To date, many plant species have been employed for vaccine usage. Early studies used tobacco and potato plants, but now tomato, banana, corn, lupine, lettuce, wheat, rice, and other plants are being used for this purpose (183). To circumvent potential denaturation of Ag during cooking, recombinant plants such as tomatoes, lettuce, and bananas have been developed. To overcome the "cold chain" problem, grain (e.g., corn, wheat, and rice) are suitable because they can be stored at ambient temperature for a long time. Along these lines, a rice-based vaccine technology was introduced. In mice orally immunized with rice expressing CT-B kept over 18 monthes under normal temperature condition (or without any refergiration), CT-B–specific intestinal S-IgA Ab responses were elicited, which enabled them to protect against CT-induced diarrhea (178). Like this one example, considerable progress has been achieved to show that protective immune responses are induced in animal model studies and, more recently, in completing application trials for target animals for veterinary vaccines (183). Thus, although many problems need to be overcome for use of this technology clinically, including low yields and inconsistent product quality for GM plant standards, there is no doubt that plant-based vaccines are a most promising mucosal vaccine system.

MUCOSAL DISEASES AND IMMUNOTHERAPY

IgA Deficiency

Deficiency of IgA is the most common primary immunodeficiency disease in humans (184). Serological data indicate that in Western Europe and the United States, one out of 400 to 700 individuals are affected; in Japan, the disease is less frequent (~1:18,000). Deficiency of IgA frequently escapes detection, because a large percentage of afflicted individuals have no clinical symptoms. In an absolute majority of cases, both serum IgA1 and IgA2 are either deficient or are present in low levels (<50 mg/100 ml). Although rare, selective deficiencies of IgA1 or IgA2 subclasses, due to the deletion of $C\alpha 1$ or $C\alpha 2$ genes, have been described (184). It is well recognized that the majority of IgA-deficient individuals are asymptomatic presumably due to alternative compensatory presence of S-IgM in external secretions that functionally substitutes for the deficient S-IgA. However, it appears that in comparison to normal individuals, patients with IgA deficiency have a higher incidence of recurrent infections, especially in the upper respiratory tract, allergic diseases, autoimmune disorders, and malignancies, particularly intestinal adenocarcinomas (184). Absence or low levels of S-IgA Abs to microbial and food Ags may result in higher rates of absorption of such Ags from mucosal surfaces, induction of higher levels of corresponding Abs in plasma, and formation of circulating immune complexes. Although S-IgM may replace S-IgA in deficient patients, it appears that S-IgM does not fully substitute for the IgA-associated functions. This may be partly ascribed to the anti-inflammatory nature of IgA manifested by its inability to activate C with potential inflammatory consequences. In contrast, both IgM and IgG are potent C activators, and it has been demonstrated that the formation of immune complexes composed of protein Ags and IgM or IgG within mucosal tissues leads to local damage and increased absorption of bystander Ags. Diminished functional substitution of S-IgA with S-IgM is also apparent in frequency of viral and bacterial infections, and responses to vaccines (184).

HIV-1 Infection and the Mucosal Immune System

Mucosal tissues of the genital and intestinal tracts are the most important portals of entry of HIV (124). Epidemiological studies indicate that worldwide ~80%–90% of HIV infections are acquired by mucosal routes through heterosexual and homosexual intercourse and the vertical transmission route *in utero*, during delivery or by breast feeding (124). Further, application of SIV on the surfaces of vagina, penile urethra, or nasopharyngeal lymphoid tissues was sufficient to infect rhesus monkeys (123).

Several mucosal cell types may be involved in the initial uptake of HIV and SIV (123,124). In animal models, specialized M cells found in the intestinal PPs, in analogous lymphoepithelial structures of the rectum, and also in tonsils are capable of internalization of HIV/SIV and presumably passing the virus to adjacent infectable cells including T cells, MΦ and DCs (123,124). Human intestinal and oral EC lines and primary IECs internalize HIV and are infectable *in vitro* due to the expression of HIV receptors/coreceptors (CD4, galactosyl-ceramide, and CC-chemokine receptors, mainly CCR5 and to a lesser extent CXCR4) on their surfaces (185). However, direct *in vivo* evidence for the presence of HIV in enterocytes is not available. In rhesus macaques vaginally exposed to cell-free SIV, Langerhans cells dispersed in the stratified squamous vaginal epithelium were the first cells that were infected (185). Previous studies indicate that SIV and HIV primarily targets and destroys mucosal $CD4^+$ cells perhaps due to the selective expression of chemokine receptors (123,124). Isolated mucosal MΦ are less permissive for HIV infection than phenotypically distinct blood monocytes probably due to the reduced expression of HIV coreceptors (123,124). In addition to the initial HIV infection sites, mucosal tissues, especially gastrointestinal sites, are

involved in the chronic activation of the systemic immune system, a hallmark of progressive HIV infection. A recent study demonstrated that chronically HIV-infected individuals and rhesus macaques infected with SIV showed increased amounts of LPS in the blood, which was presumably derived from commensal microbiota in the GI tracts (186). It was also shown that the microbial translocation from gastrointestinal mucosa to systemic compartments correlated with chronic activation through innate and acquired immune system, providing evidence for chronic systemic immune activation in HIV infection mediated by GI mucosa.

HIV-1–specific Abs become detectable in sera shortly after infection. In all seropositive individuals, these Abs are of the IgG isotype; IgA Abs are present less frequently and occur at much lower levels. Extensive studies of external secretions, including tears, saliva, nasal, intestinal, and vaginal washes; semen; cervical mucus; milk; fecal extracts; and urine yielded often controversial results with respect to the presence and isotypes of HIV-1–specific Abs (123,124). Differences in the collection procedures, processing of samples, dilutions of some secretions by washing fluids, and methodologies used for Ab detection may account for some of these discrepancies. Surprisingly, HIV-1–specific Abs of the IgG isotype are dominant in all secretions despite the overwhelming levels of total IgA and route of infection (systemic or mucosal) (123,124). For example, in human milk, intestinal fluid, and saliva, in which IgA represents ~98%–99% and IgG only ~1% of total Igs, HIV-1–specific Abs are present mainly in the IgG isotype. In external secretions of individuals with HIV-1–specific IgA Abs, there is a pronounced restriction to the IgA1 subclass (187). Absence or presence of levels of HIV-1–specific IgA Abs in external secretions is not due to a defect in the production of total IgA or unresponsiveness to viral Ags: IgA Abs to, for example, influenza virus are readily detectable in secretions of HIV-1–infected individuals (188). The mechanisms involved in this diminished responsiveness to HIV-1 but not the influenza virus in the S-IgA isotype have not been clarified. The site of original infection and the presence of effective mucosal inductive sites in the upper respiratory tract but not in the genital tract may play a role. Initial reports (188) of the selective occurrence of HIV-1–specific Abs in secretions of HIV-1–exposed but seronegative individuals have not been confirmed in other studies. Studies concerning the presence of CTLs in mucosal tissues of HIV-infected individuals are rather limited mainly due to the unavailability of tissues to perform extensive analyses. The progressive decline of immune functions in long-term HIV-1–infected and untreated individuals also compromise the mucosal immune system. An increased incidence of infections with mucosal opportunistic pathogens, including viruses, bacteria, fungi and protozoa, and of mucosal neoplasms has been observed.

IBD

IBD represents a chronic, relapsing, and remitting inflammatory condition of the GI tract that is manifest as one of the two, usually distinct but significantly overlapping, clinical entities, ulcerative colitis (UC) and Crohn's disease (CD). Increasing evidence suggests that IBD is a dysregulated mucosal immune response to components of the normal commensal luminal microbiota in a genetically susceptible host that is further modified by a variety of environmental factors. The majority of these insights have come through a variety of animal models of IBD, including those that occur spontaneously and those that are induced by administration of exogenous agents, gene targeting through knockout or transgenic approaches, or transfer of cells into immunodeficient animals (189,190).

The incidence of IBD is reduced when bacterial colonization is eliminated through germ-free conditions or reduced such as through antibiotic administration, suggesting that the intestinal microflora is involved in the development of IBD (189,190). However, not all bacteria are equal in this regard. For example, some groups of organisms are known to trigger colitis in genetically susceptible animal strains, such as the ability of *Bacteroides vulgatus* to stimulate colitis in HLA-B27 transgenic rats (191). However, some groups of organisms are able to prevent colitis such as *Lactobacillus sp.*, which are considered to be probiotics and are known to prevent colitis in genetically susceptible hosts such as IL-10 knockout mice (192). Consistent with the protective role of MyD88 signaling in the intestine as mentioned earlier (22), it is now clear that probiotics mediate their anti-inflammatory effects at least partially through TLR9-mediated CpG signaling (131). Thus, TLR9$^{-/-}$ mice showed impaired inhibitory function of probiotics or CpG against experimental colitis. Further, methylated probiotic DNA, calf thymus DNA, and DNase-treated probiotics had no effect on the inhibition of colitis (131).

In addition, colitis in mouse models appears to be triggered by a subset of protein Ags that largely activate effector T cells as manifested by the evidence of private and, to a lesser extent, public TCR motifs in bacterially driven disease models such as the CD45RB^{high} transfer model in *scid/scid* mice consistent with observations in humans (189,190). Interestingly, the response in the involved IBD intestine is associated with T cell activation and production of Th1 (IFN-γ), Th2 (IL-4), and regulatory cytokines (IL-10 and TGF-β). This is consistent with the concept that the final common pathway of excessive Th1 or Th2 cytokine production that underlies the pathogenesis of these IBDs is achieved by either excessive Th1 or Th2 effector T cells or ineffective counterbalance of effector T cells by regulatory subsets of cells that secrete anti-inflammatory cytokines such as IL-10 and TGF-β. This has placed significant emphasis on defining the regulatory subsets of cells

involved in blocking disease pathogenesis and has allowed for drawing significant similarities between and insights from mechanisms previously related to the study of oral tolerance. Indeed, oral tolerance has been shown to be effective in the prevention of IBD through production of these regulatory cytokines in animal models (132). Additionally, a recent study reveals evidence of a genetic defect in oral tolerance in IBD patients (193). As such, the role of Treg cells in intestinal homeostasis has been gained from studies using the T cell transfer colitis model. In this model, cotransfer of $CD4^+$ $CD45RB^{low}$ T cells together with $CD4^+$ $CD45RB^{high}$ T cells prevents the pathology due to the IL-10– and TGF-β–dependent activity of Treg cells (139). In addition to Treg cells, the iNKT cell type has also been shown to inhibit IBD (194). Thus, an amelioration of IBD symptoms was noted in mice treated with αGalCer, but not αManCer, via the IL-10 production by iNKT cells.

Mucosal Allergies

The majority of allergic immunologic diseases are of mucosal origin, and their clinical manifestations are locally expressed. The diseases tend to mainly affect the upper respiratory and GI tracts. Numerous anti-allergic drugs have been developed, but these drugs do not achieve a permanent cure for the allergic diseases. There is a general belief that this increase is due to "cleanliness" in our environment so that exposure to allergens can more often result in hypersensitivity and not classical immunity to infections like tuberculosis, measles, or hepatitis A (195). Asthma is the most common of the severe atopic diseases, which also include allergic rhinitis. The three hallmarks of asthma include: 1) variable airflow obstruction, 2) airway hyperresponsiveness (AHR), and 3) airway inflammation (196). However, hypersensitivity in the GI tract emanates from ingestion of large amounts of food Ags, including cow's milk proteins, eggs, and peanuts (197). It is generally agreed that Th2-type responses, characterized by the enhanced production of IL-4, IL-5, IL-9, and IL-13, and IgE-mediated Ab responses to common inhaled and fed allergens are major factors in the development of both asthma and food allergy (196,197). Therefore, inhibition of allergen-specific Th2 cells and blocking Th2 differentiation among new responses are reasonable goals for disease manipulation. The former is achieved, for example, by treatment with anti-IL-4 or IL-13 mAbs or soluble cytokine receptors, and the latter was mediated by blocking the interaction between CD28 and CD80/CD86, inhibiting ICOS function, or IL-12 expression (197). In addition, new drugs targeting Th2 effector molecules include PPAR agonists (e.g., cyclopentenone, prostaglandins, and thiazolidinediones) (198). The treatment with PPAR agonists inhibited GATA-3, a Th2-specific transcription factor, and decreased Th2-driven IgE production and inhibited asthma. Further, a Th1-dominant environment is also achieved by TLR-mediated stimulation because these triggers tend to induce Th1 responses as mentioned above. Thus, some TLR ligands (e.g., CpG oligodeoxynucleotides) have been established as agents effective against mucosal allergy in animal models, and clinical trials have been initiated (131). Similarly, blocking IgE activity resulted in the inhibition of allergic responses by suppression of inflammatory cell (e.g., mast cells and basophils) migration and degranulation (199).

Induction of tolerance is another strategy against mucosal allergy (132). Induction of tolerance by repeated subcutaneous injection of allergen has been used in clinical practice for nearly 100 years and is successful in selected allergic patients sensitive to a limited number of allergens. In addition to subcutaneous injection, oral or nasal administration has been employed to induce tolerance in animal models and clinical trials against various allergens such as pollen and house dust (132). In this context, a rice-based vaccine system expressing multiple T cell epitopes has been developed to induce oral tolerance and inhibit allergy (200). In addition to allergy, mucosal tolerance has been applied to other diseases, including EAE, arthritis, diabetes, myasthenia gravis, transplantation, and others (132).

As mentioned earlier, Treg cells play an important role in the induction of mucosal tolerance (132,139). The relationship between Treg cells and mucosal allergy was implicated by reports that the ratio of allergen-specific effector Th2 cells and Treg cells may be linked to the pathogenesis of food allergy (201). Among several types of Treg cells (e.g., naturally arising Treg cells, Tr1, and Th3), allergen-specific IL-10–producing Treg cells display strong immunosuppressive potential (201). Consistent with this, several studies suggested that IL-10 levels were inversely correlated with the severity of human allergic disease (201). However, recent studies revealed a novel pathway mediated by naturally arising Treg cells to inhibit asthma via an IL-10–independent pathway (202), suggesting that mucosal allergies can be treated by the versatile Treg cell system.

The recruitment of effector cells at the sites of allergen exposure is a target for treating mucosal allergy (197). This could be achieved by interfering selectively with the system of adhesion molecules regulating the trafficking of Th2 cells and inflammatory cells (e.g., eosinophils, basophils, and mast cells). For example, blocking the adhesion molecule LFA-1 has been shown to be effective in the inhibition of airway inflammation in animals and in allergic patients (197). Chemokines are other target molecules in preventing allergic inflammation (197). A recent study demonstrated that Th17 cells reduced AHR by down-regulation of CCL11/eotaxin and CCL17/TARC production in the lungs (203). In addition, several other chemokines (e.g., CCL17, CCL1, and CXCL12) have been considered to facilitate preferentially the development of Th2 inflammation (204). Using a food allergy model, it was