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 ($CT\text{-}B_5$ or $LT\text{-}B_5$) 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 $G\alpha$. 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 β1 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 (σ1) protein (44,59). Subsequent studies demonstrated that mucosal immune responses were significantly increased by mucosal immunization by coupling a reovirus-derived σ1 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 $C\alpha1$ or $C\alpha2$ 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

recently reported that S1P, a lipid mediator, contributes to the regulation of migration of pathogenic CD4 T and mast cells from the systemic immune compartment into the large intestine and inhibition of S1P-mediated pathway results in the inhibition of development of allergic diarrhea (205).

Celiac Disease

Celiac disease is another related disorder of mucosal immunity, which is characterized by small-intestinal mucosal injury in response to the dietary ingestion of gluten (29). Gluten is a proline- and glutamine-rich protein that is found in wheat, rye, and barley. Pathologic features of celiac disease include increased numbers of IELs and less extensive villous atrophy and crypt hypertrophy (29). IELs from patients with celiac disease preferentially include NK-like cells, which recognize stress-induced MICA molecules expressed on IECs (29). This process has been considered to be mediated by IL-15. IL-15 induces increased expression of MICA and subsequent interaction between MICA and its receptor, NKG2D, and up-regulates gluten-specific CTL activity in the small intestine (206). Gluten-free diet currently is the only accepted therapy for celiac disease, and various immunological approaches such as blocking IL-15 and treatment with IL-10 have been examined (29). However, in terms of quality of life, it is doubtful that this treatment will be effective with so many potential side effects. Thus, it is essential to identify the gluten-specific pathogenic immunocompetent cell population for the development of novel strategies to selectively delete the specific pathogenic populations.

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ARTICLE

Id2-, RORγt-, and LTβR-independent initiation of lymphoid organogenesis in ocular immunity

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The eye is protected by the ocular immunosurveillance system. We show that tear duct–associated lymphoid tissue (TALT) is located in the mouse lacrimal sac and shares immunological characteristics with mucosa-associated lymphoid tissues (MALTs), including the presence of M cells and immunocompetent cells for antigen uptake and subsequent generation of mucosal immune responses against ocularly encountered antigens and bacteria such as *Pseudomonas aeruginosa*. Initiation of TALT genesis began postnatally; it occurred even in germ-free conditions and was independent of signaling through organogenesis regulators, including inhibitor of DNA binding/differentiation 2, retinoic acid–related orphan receptor γt, lymphotoxin (LT) α1β2–LTβR, and lymphoid chemokines (CCL19, CCL21, and CXCL13). Thus, TALT shares immunological features with MALT but has a distinct tissue genesis mechanism and plays a key role in ocular immunity.

Abbreviations used: AID, activation-induced cytidine deaminase; CALT, conjunctiva-associated lymphoid tissue; CT, cholera toxin; FAE, follicle-associated epithelium; FDC, follicular DC; GC, germinal center; HE, hematoxylin and eosin; HEV, high endothelial venule; Id2, inhibitor of DNA binding/differentiation 2; ILF, isolated lymphoid follicle; LT, lymphotoxin; LTi, lymphoid tissue inducer; MAdCAM-1, mucosal addressin cell adhesion molecule 1; MALT, mucosa-associated lymphoid tissue; NALT, nasopharynx-associated lymphoid tissue; NIK, NF-κB–inducing kinase; NP, nasal passage; pLN, peripheral LN; PNA, peanut agglutinin; PNAd, pLN addressin; PP, Peyer's patch; ROR, retinoic acid–related orphan receptor; TALT, tear duct–associated lymphoid tissue; TLR, Toll-like receptor; TRAF, TNF receptor–associated factor; TRANCE, TNF-related activation-induced cytokine; UEA, *Ulex europaeus* agglutinin; VCAM-1, vascular cell adhesion molecule 1.

Mucosa-associated lymphoid tissues (MALTs), including nasopharynx-associated lymphoid tissue (NALT) and Peyer's patches (PPs), are gateways for the uptake of inhaled and ingested antigens from the lumen of the aerodigestive tract, and are considered to be the sites of induction of mucosal immune responses (Mestecky et al., 2003; Kiyono and Fukuyama, 2004). The ocular surface leading to the lacrimal sac and nasolacrimal duct also forms an interface with the outside environment. In fact, it has been proposed that conjunctiva-associated lymphoid tissue (CALT), together with tear duct–associated lymphoid tissue (TALT), organizes eye-associated lymphoid tissue to create mucosal surveillance and a barrier in the eye

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region of humans (Knop and Knop, 2000, 2001). Past investigations have focused on the identification and characterization of CALT (Gomes et al., 1997; Chodosh et al., 1998; Knop and Knop, 2000, 2005; Giuliano et al., 2002; Cain and Phillips, 2008). Mice and rats do not possess CALT, whereas other mammals (e.g., cats, dogs, and humans) do develop CALT (Chodosh et al., 1998). Rat conjunctivae, lacrimal glands, and harderian glands contain immunocompetent cells (e.g., $CD4^+$ and $CD8^+$ cells; Gomes et al., 1997); however, the immunocompetent cells do not form any organized microarchitecture at the conjunctiva and are thus diffusely located. Tears contain cytokines (e.g., IL-1, IL-4, IL-6, and TGF-β), antimicrobial peptides (e.g., lactoferrin and defensin), and secretory IgA; these secretions are an important arm of mucosal innate and acquired immunity, and respond to antigens that contact and invade the eye (Allansmith et al., 1985; Kijlstra, 1990; Gupta et al., 1996; Haynes et al., 1998; Nakamura et al., 1998; Uchio et al., 2000). Tear flow does not just provide mucosal protection at the ocular surface; it also connects the ocular surface with the nasal cavity via the tear duct, suggesting that tear flow is integral to regulating the homeostasis of the oculonasal mucosal barrier. On the other hand, the eye is considered to be an immune-privileged site, because the microenvironment of the eye is regulated by several complex aspects of the immune system (Stein-Streilein and Taylor, 2007). However, little information is currently available about the immunological nature of the eye-associated lymphoid tissue system—particularly the regulation of the tissue genesis of TALT and its immunological functions—despite the fact that TALT develops in humans (Knop and Knop, 2000, 2001).

Organogenesis of secondary lymphoid tissues, such as PPs and peripheral LNs (pLNs), is dependent on inflammatory cytokines, the release of which is mediated by lymphotoxin (LT) β receptor (LTβR) signals during the embryonic period (Mebius, 2003). The basis of LT-mediated lymphoid organ development at a molecular level was first shown in genetically manipulated *Lt*$\alpha^{-/-}$ mice, which lack PPs and pLNs (De Togni et al., 1994). Injection of an agonistic LTβR antibody into *Lt*$\alpha^{-/-}$ mice during a limited period in embryogenesis regenerates pLNs (Rennert et al., 1998). The physiological ligand of LTβR is a membrane-bound form of LTα1β2 produced by $CD3^-CD4^+CD45^+$ lymphoid tissue inducer (LTi) cells expressing IL-7Rα (Mebius et al., 1997). IL-7Rα–mediated signals trigger LTi cells to produce LTα1β2, and *Il-7r*$\alpha^{-/-}$ mice do not form PPs (Adachi et al., 1998b; Honda et al., 2001). In addition, deficiency of either inhibitor of DNA binding/differentiation 2 (Id2) or the retinoic acid–related orphan receptor (ROR) γt gene results in a lack of PPs and pLNs because the differentiation of $CD3^-CD4^+CD45^+$ LTi cells is impaired (Yokota et al., 1999; Sun et al., 2000; Eberl et al., 2004). These facts indicate the importance of inflammation-related cytokines, as well as Id2- and RORγt-subordinated $CD3^-CD4^+CD45^+$ LTi cells, in the organogenesis of lymphoid tissues, including PPs and pLNs (Kiyono and Fukuyama, 2004). However, NALT does not follow the general biological rule of the dependence of embryonic genesis on inflammatory cytokines (Fukuyama et al., 2002; Harmsen et al., 2002). NALT organogenesis, which occurs postnatally, is independent of LTβR-mediated signals and RORγt but does require Id2 (Fukuyama et al., 2002; Harmsen et al., 2002).

In this study, we provide evidence that TALT develops independently of organogenesis regulators, a finding that distinguishes TALT genesis from that of other lymphoid organs. In addition, we found that TALT plays a central role in the induction of antigen-specific immune responses against ocularly encountered antigens.

RESULTS

Identification of TALT in mice

TALT develops in the human tear duct (Knop and Knop, 2000, 2001; Paulsen et al., 2000, 2003), but to our knowledge no information is currently available on TALT in mice. To identify organized lymphoid tissue in the mouse tear duct and to elucidate its immunological and developmental features, we first examined the anatomy of this duct in mice. To visualize the position of the tear duct, we administered hematoxylin to mice in eye drops. We clearly observed the location of the tear duct where it connects the ocular surface to the nasal cavity (Fig. 1 A). In coronal and horizontal views, we were able to identify TALT in both the left and right side of the lacrimal sac in C57BL/6 WT mice (Fig. 1, B and C).

Postnatal development of TALT

The genesis of each type of lymphoid tissue occurs within a given time window: for example, PPs develop during late embryogenesis and NALT develops postnatally (Fukuyama et al., 2002; Mebius, 2003; Kiyono and Fukuyama, 2004). The initiation of intestinal isolated lymphoid follicles (ILFs) also occurs after birth, and the genetic background (e.g., whether the mouse is of the C57BL/6 or BALB/c strain) influences the postnatal time of initiation of tissue genesis (Hamada et al., 2002). To determine when TALT genesis is initiated and to evaluate the influence of genetic background on tissue genesis, we took tissue samples from both C57BL/6 and BALB/c mice at various pre- and postnatal stages for histological analysis. No sign of mononuclear cell accumulation was observed at embryonic day (E) 18 or postnatal day (D) 5 in C57BL/6 or BALB/c mice (Fig. 1 D and Fig. S1). In contrast, we detected accumulation of mononuclear cells at D10 in both C57BL/6 and BALB/c mice, indicating that the TALT development was initiated between D5 and D10, and that the different genetics of the two strains did not influence TALT organogenesis (Fig. 1 D and Fig. S1). To support the data, the initial appearance of mononuclear cells was noted at D7 in both C57BL/6 and BALB/c mice (Fig. 1 D and Fig. S1). Furthermore, pLN addressin (PNAd)–positive high endothelial venules (HEVs) developed at D10 but not at D5 and D7 (Fig. 1 E). These findings suggest that TALT develops postnatally, as does NALT. Unlike in the genesis

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of other lymphoid tissues (Mebius, 2003), expression of vascular cell adhesion molecule 1 (VCAM-1) was not observed at the TALT anlage (unpublished data).

To determine which cell population initially migrates to the TALT anlage, we analyzed the tissue genesis site at D5, D7, and D10 by confocal microscopy. The D5 anlagen did not

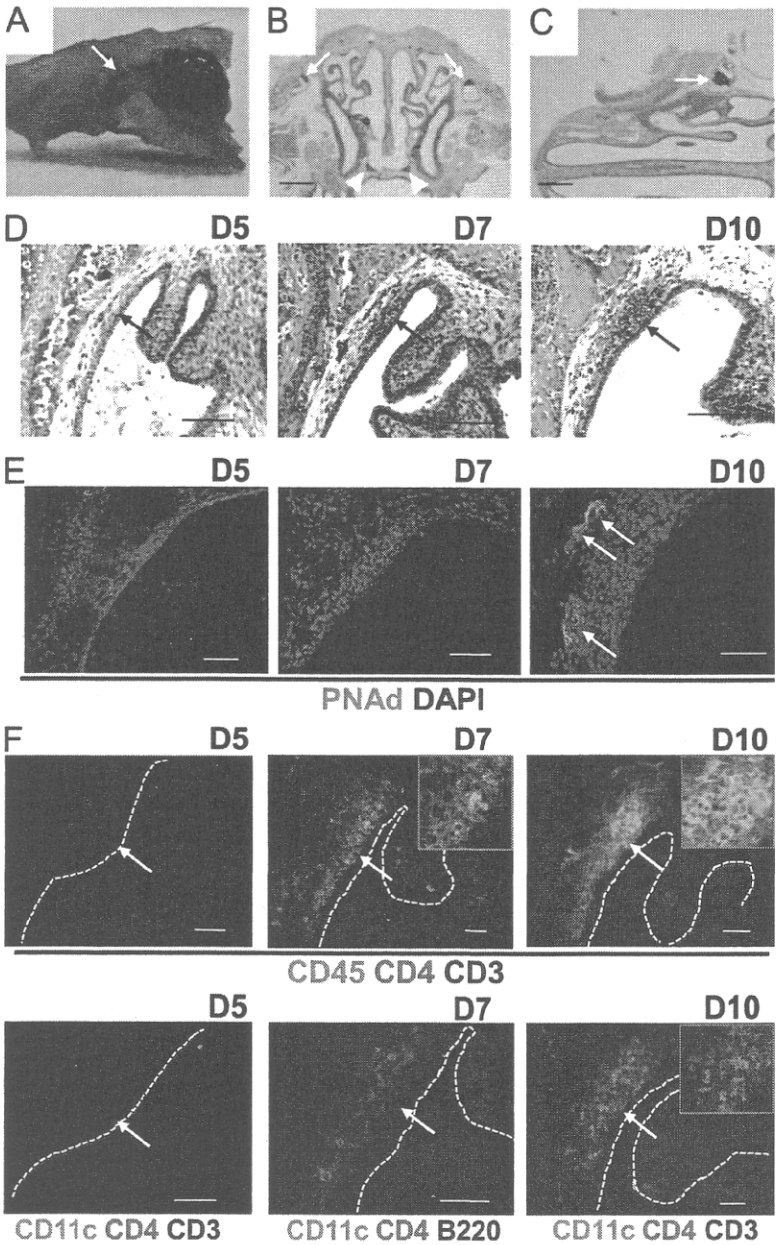

Figure 1. Postnatal development of TALT. (A–C) 10 µl of hematoxylin solution was added to the ocular surface of 8-wk-old C57BL/6 mice to visualize the tear duct (A, arrow). Coronal (B) and horizontal (C) paraffinized sections of the head were stained with HE. Arrows and arrowheads indicate TALT and NALT, respectively (n = 3 mice/group). Bars, 1 mm. (D) Paraffinized tissues of heads from D5, D7, and D10 C57BL/6 mice were examined by HE staining. Arrows indicate the site of TALT genesis (n = 5 mice/group). Bars, 100 µm. (E and F) Head tissue of C57BL/6 mice was examined by confocal microscopy with the indicated antibodies at D5, D7, and D10. Arrows indicate $PNAd^+$ HEVs (E) and the site of TALT genesis (F). Some magnified pictures are shown in F (insets). Dashed lines indicate the edge between the TALT epithelium and tear duct lumen. These data are representative of at least three independent experiments (n = 5 mice/group). Bars, 50 µm.

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contain any $CD45^+$ cells, whereas the D7 TALT anlage possessed $CD45^+$ cells (Fig. 1 F). $CD3^-CD4^+CD45^+$ cells have been shown to be LTi cells (Mebius, 2003). Among the $CD45^+$ cells in the D7 TALT anlage, we identified $CD3^-CD4^+CD45^+$ cells and $B220^+$ B cells (Fig. 1 F, D7). $CD11c^+$ DCs were not found at D5 and D7. At D10, $CD11c^+$ DCs and increased numbers of $CD3^-CD4^+CD45^+$ cells were found in the TALT (Fig. 1 F). Because $B220^+$ B cells were among the first cells to migrate at the TALT anlage (Fig. 1 F, D7), we examined B cell–deficient *Igh6*$^{-/-}$ mice for the development of TALT. The TALT genesis occurred normally, even in the B cell–deficient condition (Fig. S2 A). Further, TALT also developed in T cell–deficient *Tcrβ*$^{-/-}$, *Tcrδ*$^{-/-}$ mice (Fig. S2 B). These findings show that B and T lymphocytes and DCs are dispensable for the initiation of TALT development.

Organogenesis of TALT does not require microbial stimulation

TALT has been identified in only 30–40% of humans examined (Paulsen et al., 2000, 2003; Knop and Knop, 2001), raising the possibility that environmental conditions, including microbial infections and allergic responses, are involved in the initiation of TALT development. In addition, our finding that TALT develops postnatally also points to the possible involvement of microbial stimulation in TALT genesis. However, we detected TALT in mice deficient in Toll-like receptor (TLR) signals, including *Tlr2*$^{-/-}$, *Tlr4*$^{-/-}$, and *MyD88*$^{-/-}$ mice (Fig. S2, C–E). Further, we found that germ-free mice developed TALT (Fig. 2 A). Thus, TALT organogenesis is most likely independent of microbial stimulation.

TALT development is independent of organogenesis regulators

We next determined the molecular requirements for TALT development. PPs and pLNs are not present in alymphoplasia (*aly/aly*) mice, which carry a null mutation of NF-κB–inducing kinase (NIK), resulting in a failure to transmit LTβR-mediated signals (Shinkura et al., 1999). However, our histological analysis showed that the TALT structure was preserved in *aly/aly* and *Ltα*$^{-/-}$ mice, although it was smaller than in WT TALT (Fig. 2, A and B; and Fig. S3). In addition, TALT was even observed in *Il-7rα*$^{-/-}$ mice, although the size was again small (Fig. 2 B and Fig. S3). Thus, the initiation of TALT development mediated by inducer cells was independent of the IL-7R and LTα1β2–LTβR–NIK pathway signaling cascades, whereas the maturation process of accumulating lymphocytes required the cytokine signaling cascade, as is the case with other lymphoid organs (Mebius, 2003; Kiyono and Fukuyama, 2004).

Lymphoid chemokines, including CXCL13, CCL19, and CCL21, play important roles in the migration of LTi cells to the sites of tissue genesis (Honda et al., 2001; Luther et al., 2003; Fukuyama et al., 2006). We therefore examined the involvement of these lymphoid chemokines in TALT genesis. The TALT structure was preserved in *Cxcl13*$^{-/-}$ mice, although they lacked some pLNs and had reduced numbers of PPs (Fig. 2 B; Ansel et al., 2000). TALT also developed well in *plt/plt* mice (Fig. 2 B), which carry null mutations of both the *Ccl19* and *Ccl21* genes (Nakano et al., 1998). Furthermore, the initiation of TALT formation was maintained in triple mutant (*Cxcl13*$^{-/-}$ *plt/plt*) mice (Fig. 2 B), confirming that these lymphoid chemokines are not required for the initiation of TALT organogenesis, although the TALT was smaller in the lymphoid chemokine–null condition than in WT (Fig. S3). These results supported the observation that an NIK-mediated pathway is required to recruit large numbers of lymphocytes to TALT.

We then addressed the involvement of Id2 and RORγt, key transcriptional regulators in the induction of lymphoid organogenesis by $CD3^-CD4^+CD45^+$ LTi cells (Yokota et al., 1999; Sun et al., 2000; Eberl et al., 2004). Surprisingly, TALT formation was preserved in *Id2*$^{-/-}$ and *Rorγt*$^{-/-}$ mice (Fig. 2 B). Consistent with this finding, FACS and confocal microscopy analyses detected $CD3^-CD4^+$ LTi cells in the TALT anlage of *Id2*$^{-/-}$ and *Rorγt*$^{-/-}$ mice as well as in WT mice (Fig. 3, A and B). Furthermore, when we isolated these $CD3^-CD4^+CD45^+$ cells from WT mice and examined the gene expression of the tissue genesis–associated transcription factors by RT-PCR, we found that $CD3^-CD4^+CD45^+$ cells isolated from the TALT anlagen did not express either *Id2* or *Rorγt*, whereas $CD3^-CD4^+CD45^+$ cells isolated from the embryonic intestine, i.e., PP inducer cells, expressed both *Id2* and *Rorγt* (Fig. 3 C). Thus, TALT organogenesis proceeds independently of Id2, RORγt, and LT. Therefore, TALT genesis is quite different from the genesis of other secondary lymphoid tissues, including PPs, pLNs, and NALT (Mebius, 2003; Kiyono and Fukuyama, 2004).

Microarchitecture of TALT

The structure of the MALT epithelium is characterized by the presence of follicle-associated epithelium (FAE; Kiyono and Fukuyama, 2004). Indeed, we were able to divide the epithelial layer of the lacrimal sac into two populations on the basis of its morphological structure (Fig. 4 A): TALT-FAE was characterized by a thin layer of squamous epithelium (Fig. 4 B), whereas the lacrimal sac epithelium had a multilayered and squamous morphology (Fig. 4 C). Interestingly, TALT-FAE lacked mucus-producing goblet cells and cilia (Fig. 4 B), a fact that distinguished this tissue from NALT-FAE, which has some goblet cells (Fig. 4 D). Instead, mucus-producing gland tissue was frequently observed on the conjunctiva (unpublished data). Confocal microscopic analysis of TALT revealed large numbers of $B220^+$ B cells (Fig. 4 E) and $CD11c^+$ DCs in the subepithelial dome region of the FAE (Fig. 4 F), as well as $CD3^+CD4^+$ T helper cells distributed around the B cell follicles (Fig. 4, G and H). These data indicate that TALT is composed of a highly compartmentalized and organized lymphoid structure. In accordance with the finding that neonatal TALT developed $PNAd^+$ HEVs (Fig. 1 E), adult TALT HEVs expressed PNAd but not mucosal addressin cell adhesion molecule 1 (MAdCAM-1; Fig. 4 I and Fig. S4 A); this is similar to the case with NALT HEVs

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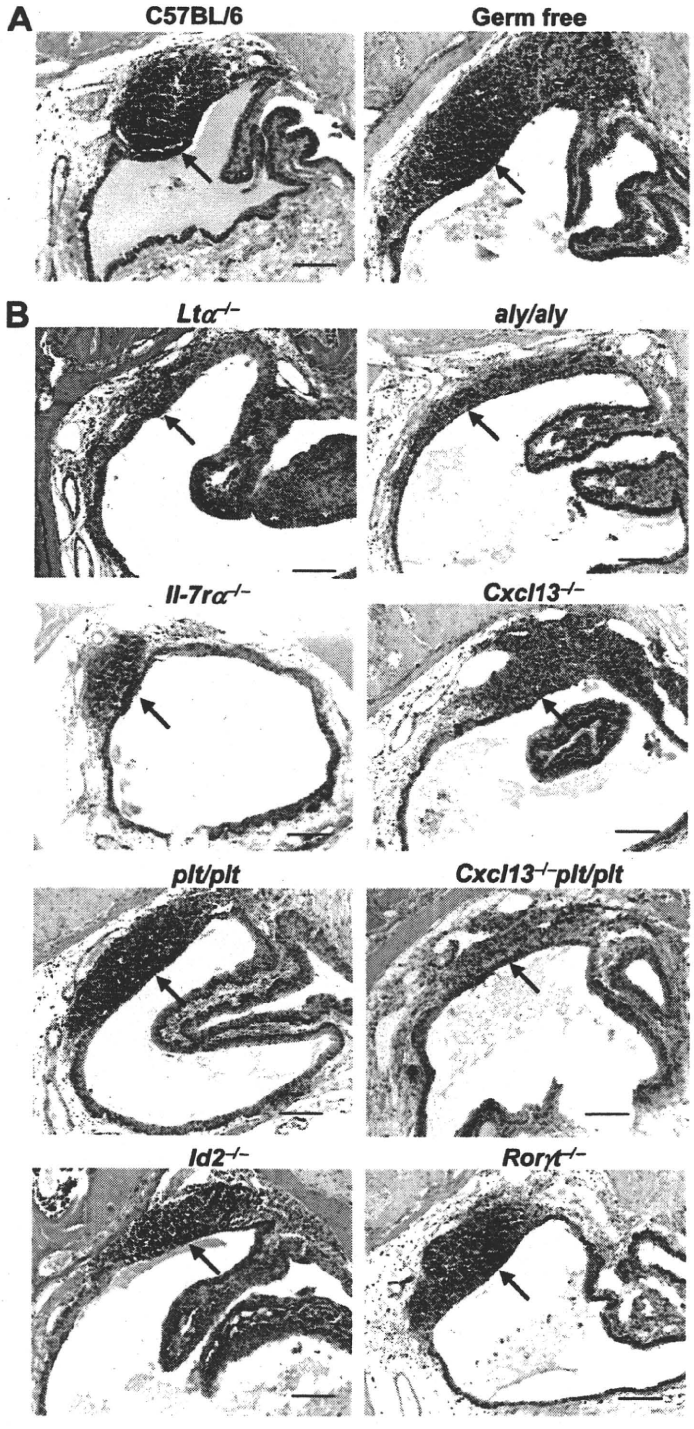

Figure 2. TALT genesis is independent of microbial stimulation and organogenesis-associated molecules. Paraffin-embedded tissue sections were analyzed by HE staining for TALT development. (A) The presence of TALT in germ-free mice, as well as C57BL/6 WT mice, shows that TALT develops independently of microbial stimulation (n = 3 mice/group). (B) Development of TALT in 8-wk-old *Ltα*$^{-/-}$, *aly/aly*, *Il-7rα*$^{-/-}$, *Cxcl13*$^{-/-}$, *plt/plt*, *Cxcl13*$^{-/-}$*plt/plt*, *Id2*$^{-/-}$, and *Rorγt*$^{-/-}$ mice shows that the initiation of TALT development occurs independently of organogenesis-associated molecules. Arrows indicate the presence of TALT. These data are representative of at least three independent experiments per group (n = 5 mice/group). Bars, 100 µm.

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(Fig. 4 J and Fig. S4 B). These observations suggest that cellular trafficking to TALT and NALT is regulated via an L-selectin–PNAd interaction and is distinguishable from gut-trafficking mechanisms, which are dependent on α4β7 integrin/MAdCAM-1 (Kiyono and Fukuyama, 2004). These results indicate that TALT possesses many of the characteristic traits of organized MALT but is distinguished by the lack of goblet cells in its FAE region.

TALT is a site of immunological induction

To investigate the physiological function of TALT, we examined whether TALT takes up ocularly administered antigens. M cells, characterized by the M cell–specific mAb NKM16-2-4$^+$ (Nochi et al., 2007), *Ulex europaeus* agglutinin (UEA) 1$^+$, and wheat germ agglutinin$^-$, were found in the FAE of TALT (Fig. 5 A). Electron microscopic analysis showed that TALT-FAE contained cells bearing the hallmarks of M cells: microvilli and a unique pocket formation with lymphocytes (Fig. 5, B and C). When mice were ocularly dosed with GFP-expressing *Salmonella*, we observed the uptake of *Salmonella* by UEA-1$^+$ M cells (Fig. 5 D) as well as by CD11c$^+$ DCs (Fig. 5 E). Moreover, when mice were ocularly challenged with *Pseudomonas aeruginosa* PAO1, large amounts of *P. aeruginosa* PAO1 were located within the TALT (Fig. 5 F). *P. aeruginosa* PAO1 was not detected in naive mice (Fig. 5 G). Mice ocularly challenged with *P. aeruginosa* PAO1 formed germinal centers (GCs; Fig. 5 H); this is an important immunological event for the initiation of hypersomatic mutations and Ig class switching, which enable the production of memory B cells with high-affinity B cell receptors (Kelsoe, 1996; Shapiro-Shelef and Calame, 2005). In contrast, GC formation was not present in the TALT of naive mice (Fig. 5 I; and Fig. 6, A and B). These data suggest that TALT is a preferential site for the uptake of ocularly encountered antigens/pathogens and for the subsequent induction of antigen-specific B cell responses.

Supporting this notion, immunization of the eyes with eye drops containing cholera toxin (CT), a well-known mucosal immunogen, resulted in the formation of GCs with a follicular DC (FDC) network in the TALT (Fig. 6, C and D). Interestingly, ocular immunization also induced GC formation in NALT (Fig. 6, E and F), suggesting that the anatomical

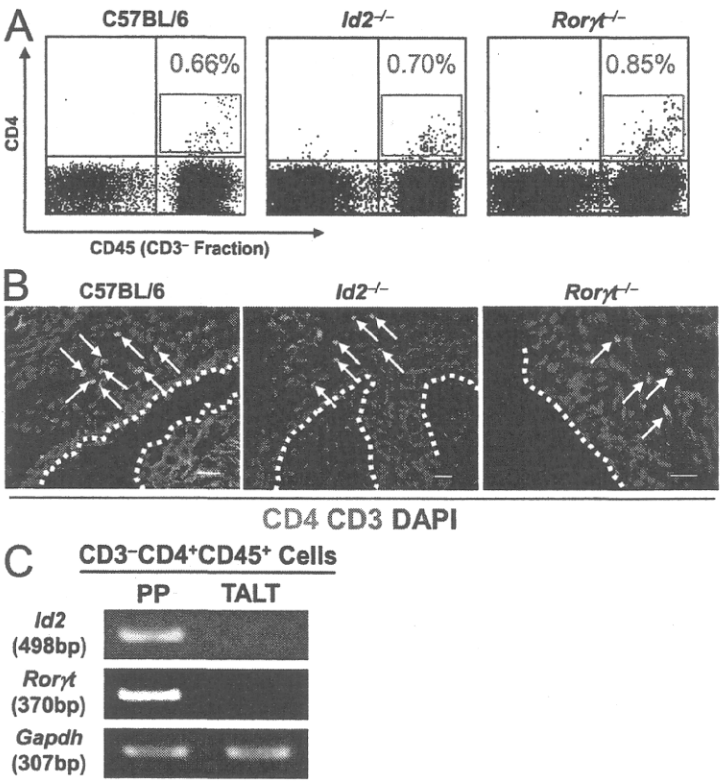

Figure 3. Presence of CD3$^-$CD4$^+$CD45$^+$ cells in the TALT anlagen. (A) Because substantial numbers of CD3$^-$CD4$^+$CD45$^+$ cells were noted in the TALT anlagen of D10 mice, we analyzed mononuclear cells from D10 tear ducts by FACS. Percentages of CD3$^-$CD4$^+$CD45$^+$ cells are shown in red (n = 6 mice/group). (B) Confocal microscopic analysis of the site of TALT genesis at D10. Frozen tissue samples were stained with the antibodies indicated. Arrows point to CD3$^-$CD4$^+$ cells (n = 6 mice/group). Dotted lines indicate the edge between the TALT epithelium and tear duct lumen. Bars, 50 µm. (C) CD3$^-$CD4$^+$CD45$^+$ cells from PP and TALT were isolated from an E17 intestine and D10 tear duct, respectively. Gene expression of *Id2* and *Rorγt* were analyzed by RT-PCR. The expression of *Gapdh* is shown as an internal control. These data are representative of at least three independent experiments (n = 18–20 mice/group).

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