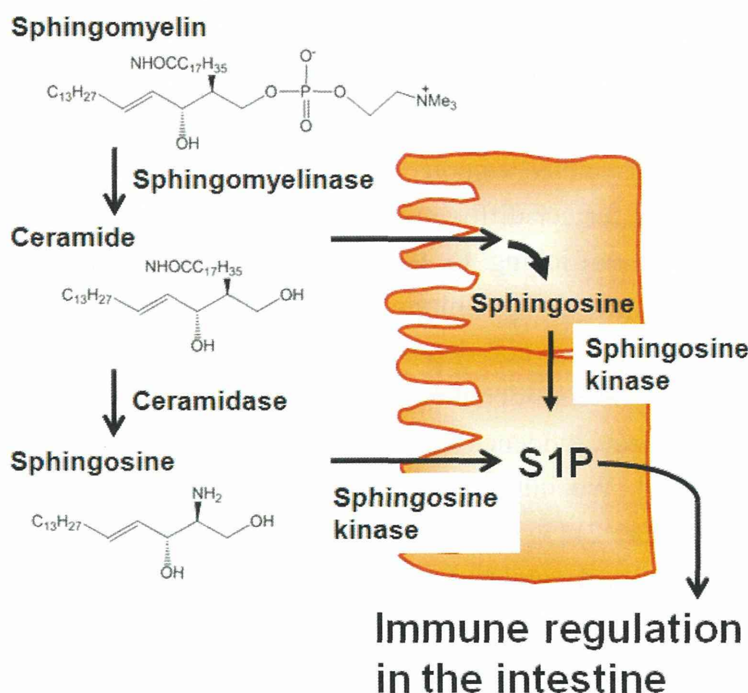
the blood, and germfree rats have comparable levels of sphingolipids in the intestine to conventional specific pathogen-free (SPF) rats [18]. Therefore, it is plausible that a source of sphingolipids in the intestine could be daily consumed diet. Adult humans ingest around 0.3 to 0.4 g sphingolipids per day, especially sphingomyelin from meat, milk, egg, and fish [19]. Dietary sphingomyelin is not directly absorbed, but is first degraded into ceramide and sphingosine [20,21] by alkaline sphingomyelinase and ceramidase, respectively, which are expressed on the apical membranes of epithelial cells [22,23]. Because epithelial cells express several key enzymes (e.g., sphingosine kinase) in the production of S1P from ceramide and sphingosine [23,24], it is possible that epithelial cells obtain ceramide and sphingosine from dietary sphingomyelin to produce S1P (Figure 1), thereby regulating intestinal immune responses and the associated intestinal immune diseases. Consistent with this, several studies showed that the incidence and severity of intestinal inflammation was changed by the uptake of dietary sphingomyelin [25,26] and the enzymatic activity of sphingomyelinase [27] and sphingosine kinase [28]. In addition, it was reported that dietary cholesterol inhibits the intestinal absorption of sphingolipids [29], implicating that cholesterol-rich Western diets may affect the availability of S1P precursors and consequently interfere with S1P-mediated intestinal immunity.

Figure 1. Dietary sphingolipids in epithelial-cell S1P production. Dietary sphingomyelin is degraded into ceramide and subsequently sphingosine by alkaline sphingomyelinase and ceramidase, respectively, which are expressed on the apical membranes of epithelial cells. In the epithelial cells, absorbed ceramide is metabolized into sphingosine. Together with absorbed sphingosine, sphingosine kinase metabolizes sphingosine into S1P, which then participates in immune regulation in the intestine.



3. Regulation of S1P Metabolism by Dietary Materials

In addition to dietary lipids, other dietary materials are also involved in the regulation of S1P metabolism. For instance, S1P lyase, a key enzyme to degrade S1P and thus keep optimal S1P low

concentration, requires vitamin B6 as a co-factor [7]. Thus, administration of vitamin B6 antagonist impaired S1P lyase activity, which consequently led to the defect of lymphocyte trafficking caused by inappropriate S1P gradient [7]. Similar effect was noted in 2-acetyl-4-tetrahydroxybutylimidazole (THI), a component of caramel food colorant III used in food products. THI inhibits S1P lyase and thus, like treatment with vitamin B6 antagonist, prevents normal lymphocyte trafficking [7]. These findings led to the use of THI for the treatment of immune diseases [30–32].

4. S1P Regulates Innate and Acquired Phases of Intestinal IgA Responses

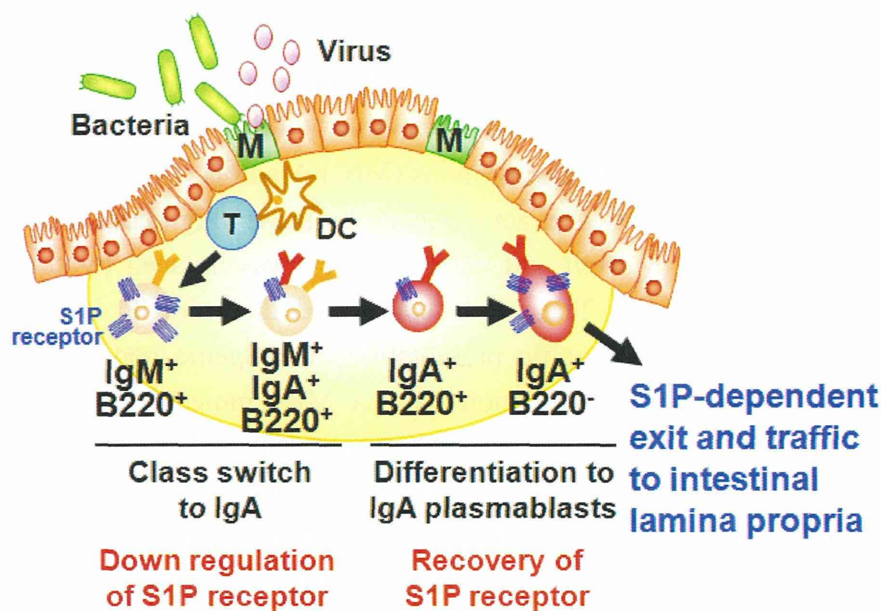
IgA is the most frequently observed antibody isotype in the intestinal compartments and provides the first line of defense against pathogenic microorganisms invading through mucosal tissues. Therefore, the induction of appropriate IgA responses is a logical strategy for the development of oral vaccines [33]. Since IgA antibody is one of the major arms of the mucosal immune system in the digestive tract, which covers a large surface area, the intestinal IgA is originated from several induction sites including Peyer's patches (PPs), isolated lymphoid follicles, and the peritoneal cavity [34].

A well characterized gut-associated lymphoid tissue (GALT) is PPs. PPs act as induction sites for the initiation of IgA responses against T-cell-dependent antigens [35]. PPs are covered with a specialized epithelium known as follicle associated epithelium (FAE) containing antigen-sampling M cells, which are responsible for the uptake and transport of antigens from the intestinal lumen to antigen-presenting cells such as dendritic cells (DCs) (Figure 2) [36]. Then, DCs capture antigens from the M cells, process and present them to T cells. It has been shown that the formation of PP DC-T cell clusters provide both cellular and molecular environment for the generation of IgA committed B cells in PPs [34]. In this pathway, some of the activated T cells differentiate into follicular helper T cells to help the antibody class switching of B cells in the germinal centers [34]. Because of the unique cytokine environment (e.g., TGF-β, IL-4, and IL-21) and continuous stimulation by commensal bacteria in the intestine, PPs have been shown to equip with efficient molecular and cellular environment for the spontaneous and continuous B cell class switching from IgM to IgA [34,35]. After class switching to IgA, B cells further differentiate into IgA plasmablasts and then migrate out from the PPs for their subsequent trafficking to the intestinal lamina propria, where they terminally differentiate into plasma cells producing dimeric (or polymeric) forms of IgA. This process mainly contributes to the development of T cell-dependent antigen-specific immune responses. Thus, the PP-mediated induction pathway is considered to be a major arm of the acquired IgA response [34].

Our investigation provided new evidence that S1P regulated the B cell trafficking in the PPs for the intestinal IgA production [37]. We initially found that S1P1 expression in B cells changes during differentiation in the PPs (Figure 2) [37]. High levels of S1P1 expression were detected in IgM^+ naive B cells, and expression was down-regulated when B cells started class switching to IgA. The low expressions of S1P1 allowed newly class-switched IgA^+ B cells to retain in the PPs for the sufficient differentiation into the IgA^+ plasmablasts. S1P1 expression was restored on the IgA^+ plasmablasts, resulting in their emigration from the PPs. Mice treated with FTY720, an immunosuppressant inducing S1P1 downregulation [38], show selective accumulation of IgA^+ plasmablasts in the PPs, leading to the disturbance of continuous delivery of IgA committed B cells from the PPs to the lamina propria of intestine. Consequently, the decrease of same population in the intestinal lamina propria was noted,

which associated with the reduction of intestinal antigen-specific IgA responses against orally immunized protein antigen [37].

Figure 2. Sequential changes in S1P1 expression during B-cell differentiation in Peyer's patches. Dendritic cells (DC) take the antigens transported by M cells from intestinal lumen and present them to T cells for their activation. Through the interaction with T cells and DCs, IgM^{+} naive B cells show class-switch from IgM to IgA. During this process, S1P1 is expressed at high levels in IgM^{+} naive B cells and downregulated on B cells class-switching from IgM to IgA and subsequently recovered on IgA^{+} B220^{-} plasmablasts, resulting in their emigration from the Peyer's patches and traffic into the intestinal lamina propria.



In the IgA production pathway in the gut, peritoneal B cells are an additional source of intestinal IgA [39]. A number of peritoneal B cells belong to a unique B-cell subset, termed as B1 cells, which produces antibodies against T-cell-independent antigens such as lipids and polysaccharides. Because these T-cell-independent antigens are conserved in various microorganisms, B1-cell-derived antibodies undiscriminatingly react to commensal and pathogenic bacteria and prevent their attachment and invasion into the host. This reaction is opposite to antibody responses against protein antigen mediated by PP B cells, which show rigid specificity against microorganisms. Therefore, it has been considered that B1-cell-derived IgA is categorized as to be innate-type antibodies that recognize a wide range of microorganisms in the intestine [39].

Trafficking of peritoneal B1 cells into the intestine requires S1P-mediated signaling [40]. Like B cells in the PPs, peritoneal B1 cells identically expressed S1P1. Thus, trafficking of peritoneal B cells into the intestine and consequent production of intestinal IgA are diminished by treatment with FTY720, mainly because of the inhibition of B1 cell emigration from the parathymic lymph nodes, which drain to the peritoneal cavity [40]. This impaired trafficking in FTY720-treated mice was associated with the decreased IgA responses against phosphorylcholine (a T-cell-independent antigen) induced by oral immunization with heat-killed *Streptococcal pneumoniae* [40].

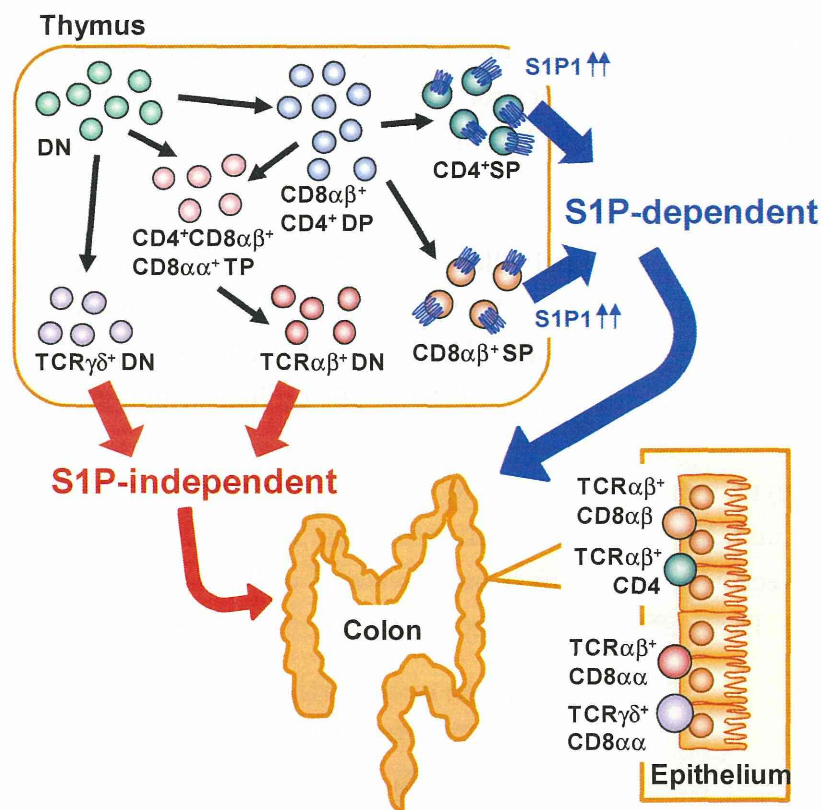
We also found that S1P-mediated regulation of peritoneal B-cell trafficking requires crosstalk with stromal cells in the peritoneal cavity [41]. This interaction mediated by adhesion molecules (e.g., ICAM-1 and VCAM-1) on stromal cells and the expression is regulated by NFκB-inducing kinase (NIK). Therefore, NIK-mutant aly/aly mice show decreased sensitivity to FTY720 in the regulation of peritoneal B-cell trafficking due to the impaired expression of adhesion molecules although peritoneal B1 cells in aly/aly mice expressed comparable levels of S1P1.

5. Distinct S1P Dependency of Trafficking of Intraepithelial T-Lymphocytes in the Gut

Large numbers of lymphocytes are also present in the intestinal epithelium and called as intraepithelial lymphocytes (IELs) [42]. IELs are mostly T cells, but unlike in conventional T cells observed in the systemic compartments (e.g., spleen) which predominantly express the αβ T-cell receptor (αβTCR), in the IEL subset there is an abundance of T cells expressing the γδ T cell receptor (γδTCR) in addition to $\alpha\beta TCR^+$ T cells [42]. αβTCR recognizes peptide antigen presented via major histocompatibility complex (MHC) molecules, whereas γδTCR recognizes non-classical MHC molecules such as MHC class I chain-related proteins (MIC) A and B (MICA/B) in human and Rae-1 in mouse [43]. Unlike MHC molecules that act as ligand by presenting peptide antigen, non-classical MHC molecules act as a ligand by itself and the expression was induced by stress (e.g., infection, tumors, or chemical treatment) [44]. Thus, it is considered that αβTCR is involved in acquired immunity through the activation by specific presentation of antigenic peptides, whereas γδTCR is involved in innate immunity by the ligation of non-classical MHC molecules [42]. A distinctive pattern of CD8 expression has also been noted in IELs. Conventional $\alpha\beta TCR^+$ T cells express CD8 as a heterodimer of α and β (CD8αβ). In contrast, some IELs uniquely express CD8 as a homodimer (CD8αα) [42]. A previous study identified a unique precursor of CD8αα IELs in the thymus [45]. In the thymus, $CD4^-$ $CD8^-$ double-negative thymocytes differentiate into $CD4^+$ $CD8^+$ double-positive thymocytes and then further differentiate into single-positive thymocytes expressing either CD4 or CD8. $CD8\alpha\beta^+$ IELs are derived mainly from $CD8^+$ single-positive thymocytes expressing αβTCR. $CD8\alpha\alpha^+$ IELs, however, originate from double-negative thymocytes expressing either αβTCR or γδTCR that have themselves differentiated from unique $CD4^+$ $CD8\alpha\alpha^+$ $CD8\alpha\beta^+$ triple-positive thymocytes (Figure 3) [45].

S1P has been involved in the regulation of cell trafficking of different subsets of IELs originated from thymus. We found that each type of IEL shows a different dependency on S1P in its trafficking from the thymus to the intestine, especially in the colon (Figure 3) [46]. When mice were treated with FTY720, decreased numbers of $CD8\alpha\beta^+$ IELs were observed. In contrast, the numbers of $CD8\alpha\alpha^+$ IELs were barely affected. These data suggest that, in the colonic epithelium, $CD8\alpha\beta^+$ IELs are S1P dependent and $CD8\alpha\alpha^+$ IELs are S1P independent. Consistent with this finding, $CD8^+$ single-positive thymocytes—the precursors of $CD8\alpha\beta^+$ IELs—express high levels of S1P1 [8], whereas no S1P1 expression has been noted on double-negative thymocytes, the precursors of $CD8\alpha\alpha^+$ IELs [46]. These findings suggest that S1P1 expression was different in different subsets of thymic precursors of IELs and provide versatile immunological pathways in the intestine.

Figure 3. Distinct dependency on S1P in T-cell trafficking into the colonic epithelium. In the thymus, $CD4^-$ $CD8^-$ double-negative (DN) thymocytes differentiate into $CD4^+$ $CD8^+$ double-positive (DP) thymocytes and then into single-positive (SP) thymocytes expressing either CD4 or CD8 and αβTCR. These SP thymocytes express high levels of S1P1 and migrate out from the thymus and into the colon in an S1P-dependent manner. DN thymocytes express TCRαβ or TCRγδ. DN thymocytes expressing TCRαβ are derived from $CD4^+$ $CD8\alpha\alpha^+$ $CD8\alpha\beta^+$ triple-positive (TP) thymocytes differentiated from DN or DP thymocytes. Little or no S1P1 expression is noted in the DN thymocytes expressing TCRαβ or TCRγδ, so traffic to the colonic epithelium proceeds in an S1P-independent manner.

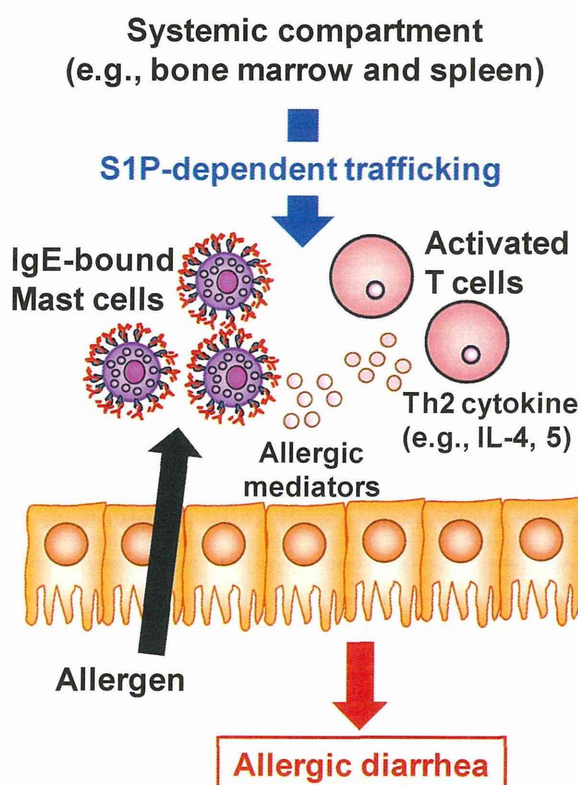


6. S1P-Mediated Regulation in the Development of Intestinal Immune Diseases

Accumulating evidence has revealed the pivotal role of S1P in the development of inflammatory diseases such as autoimmune type 1 diabetes, rheumatoid arthritis, and multiple sclerosis [5]. FTY720 prevents the egress of autoreactive lymphocytes from the lymph nodes into the peripheral circulation and subsequent across the blood–brain barrier into the central nerve system and thus has recently been approved as an oral therapy for multiple sclerosis [47]. In addition to being involved in these immune diseases at the systemic immune compartments, S1P is involved in the development of intestinal immune diseases including food allergies and intestinal inflammation [5]. The number of patients with food allergies has increased not only in children but also in adults; the development of effective preventive and therapeutic strategies for food allergies is therefore required to improve patients' quality of life. Using the ovalbumin-induced murine food-allergy model developed by our group [48], we examined the molecular and cellular mechanisms underlying the development of food allergies and found that, in allergic mice, activated T cells migrate into the colon, where they produced high

amounts of Th2 cytokines such as IL-4 and IL-5 [48]. We demonstrated that the trafficking of pathogenic T cells from the systemic compartments into the colon was mediated by S1P (Figure 4) [49]. Indeed, activated T cells in the colon of allergic mice expressed S1P1 and their infiltration into the colon and subsequent production of Th2 cytokines (e.g., IL-4 and IL-5) were inhibited by the treatment with FTY720 [49]. In addition, the infiltration of mast cells, effector cells in the development of food allergy, into the colon was also prevented in the FYT720-treated mice [49]. As a mechanism of FTY720-mediated inhibition of mast cell infiltration, it was likely that FTY720 directly and indirectly prevented the mast cell infiltration into the colon. Direct effect of FTY720 was predicted by results that mast cells expressed S1P1 and their *in vitro* migration was inhibited by FTY720 [49]. Indirect effect is mediated by activated T cells producing Th2 cytokines which enhanced the proliferation and recruitment of mast cells [50]. Thus, inhibition of activated T cell trafficking into the colon by FTY720 resulted in the reduced recruitment and/or proliferation of mast cells. Taken together, involvement of S1P in the trafficking of both pathogenic T cells and mast cells is a potential target for prevention and treatment of food allergies.

Figure 4. S1P mediates intestinal allergy by regulating pathogenic T and mast cell infiltration into the colon. In murine food allergy model, systemically sensitized T cells migrate into the colon upon the oral challenge with same allergen. This trafficking is mediated by S1P and thus treatment with FTY720 resulted in the inhibition of activated T cell trafficking into the colon. In the colon, these activated T cells produced high amounts of Th2 cytokines such as IL-4 and IL-5 for promotion of mast cell recruitment and proliferation. In addition, mast cell itself expresses S1P1. Therefore, FTY720 treatment directly and indirectly (Th2 cytokine from activated T cells) decreases the numbers of mast cells in the colon. These effects lead to the inhibition of allergic diarrhea.



Similarly, several lines of evidence have demonstrated that the FTY720 treatment prevents the development of intestinal inflammation [51–53]. For example, in a spontaneous colitis model in interleukin-10-deficient mice, administration of FTY720 suppressed the infiltration of pathogenic T cells producing interferon-γ [51]. Infiltration of the colon by pathogenic T cells was also inhibited by treatment with FTY720 in both a dextran sulfate sodium (DSS)-induced colitis model and a T-cell transfer model in mice [52,53]. Although S1P regulates the activation of several inflammatory cells via modulation of the signaling of certain innate receptors such as toll-like receptors, TNF receptor, and protease-activated receptor 1, and S1P itself is produced by activated inflammatory cells [4], collectively these findings suggest that S1P–S1P1 axis participates mainly in the development of intestinal immune diseases at the stage of pathogenic cell trafficking into the colon.

7. Conclusion

It is clear from past and current studies that S1P plays an important role in the regulation of the immune system of the gut in both healthy and disease states. In general, S1P is derived from sphingomyelin and is produced mainly by platelets, erythrocytes, and endothelial cells in the body. However, in the intestine, it is likely that epithelial cells contribute most to the production of S1P. Most importantly, S1P produced by epithelial cells seems to originate from dietary sphingolipids, especially sphingomyelin. Thus, elucidation of the complex networks established by dietary lipids will create a new era in nutrition-based mucosal immunology and should provide a new strategy against intestinal immune diseases.

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Conflict of Interest

The authors declare no conflict of interest.

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Peaceful Mutualism in the Gut: Revealing Key Commensal Bacteria for the Creation and Maintenance of Immunological Homeostasis

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Quantitative and qualitative aspects of commensal bacteria determine the active and quiescent status of host immunity. In a recent *Science* paper, Atarashi et al. (2011) identify *Clostridium* clusters IV and XIVa as indigenous commensal bacteria that induce regulatory T cells for the creation and maintenance of immunological homeostasis.

The intestinal tract of mammals is home to 10^{13} to 10^{14} commensal bacteria composed of hundreds of species that benefit the host by supplying nutrients, metabolizing otherwise indigestible food, and preventing colonization by pathogens. Additionally, immune system development requires interactions with commensal bacteria (Hill and Artis, 2010). Because commensal bacteria commonly produce ligands of innate immunity, it was thought that unspecified commensal bacteria indiscriminately induced immune system development. However, accumulating evidence has indicated that individual species of commensal bacteria play specific roles in determining the immunological balance in the mucosal and systemic compartments. In a recent issue of *Science*, Honda and colleagues identified a cluster of indigenous commensal bacteria that are key to maintaining quiescent immunity (Atarashi et al., 2011).

Recent advances in genetic analyses of the composition of commensal bacteria led to the discovery that changes in microbial composition accompany alterations in the quality of host immunity and occasionally underlie immune diseases such as inflammatory bowel diseases (IBD) (Hill and Artis, 2010). These findings straightforwardly led to works addressing the puzzling question of how specific species of commensal bacteria regulate particular immune responses. One example of recent success in this area is the identification of segmented filamentous bacteria (SFB) as inducers of active immunity. Several groups, including Honda's, showed that SFB efficiently induce effector T cells, especially Th17 cells observed predominantly in the gut, where they provide protective immunity against intestinal infection (Gaboriau-Routhiau et al., 2009; Ivanov et al., 2009).

In addition to immunosurveillance against harmful pathogens, the gut immune system mediates quiescent immunity (or tolerance/unresponsiveness) against harmless and beneficial nonself materials such as dietary antigens and commensal bacteria. Among multiple immunoregulatory pathways, regulatory T (Treg) cells play pivotal roles in achieving quiescent immunity. Like Th17 cells, Treg cells are abundantly present in the gut, which is explained at least partly by the function of the vitamin A metabolite retinoic acid that is produced by gut-associated dendritic cells (Mucida et al., 2009). Although probiotic strains could also induce Treg cells in the gut (Kwon et al., 2010), whether and how indigenous commensal bacteria induce Treg cells remained unclear.

In their recent *Science* paper, Honda's group extends their studies and identifies *Clostridium* clusters IV and XIVa (also known as the *Clostridium leptum* and *coccoides groups*) as among the indigenous commensal bacteria inducing colonic Treg cells. Atarashi et al. (2011) demonstrated that only a few Treg cells were present in the colon of germ-free mice but increased to normal levels in specific pathogen-free (SPF) mice by colonization with commensal bacteria originating from SPF mice. By eliminating bacteria using antibiotics and chemical reagents, together with information about prominent commensal bacteria in the colon, they identified gram-positive and spore-forming *Clostridia* as candidate commensal bacteria that induce colonic Treg cells. Direct evidence was obtained from gnotobiotic mice that were generated by colonization with *Clostridium* clusters IV and XIVa. Intriguingly, the induction of Treg cells by commensal bacteria was observed specifically in the colon, whereas Treg cells in the small intestine were normally present in germ-free mice (Atarashi et al., 2011). The physiological functions of the small and large intestines differ substantially, and the small intestine is specialized to digest and absorb dietary materials. Treg cells in the small intestine increase after weaning (Atarashi et al., 2011), raising the possibility that materials in the diet and/or breast milk may regulate the induction of Treg cells in the small intestine.

Atarashi et al. also showed that an artificial increase in *Clostridium* in neonatal SPF mice resulted in the attenuation of intestinal inflammation in adulthood, which is potentially related to the lower levels of *Clostridium* clusters IV and XIVa in IBD patients (Frank et al., 2007). These regulatory effects were mediated by the preferential induction of Treg cells that produced IL-10 and expressed high levels of cytotoxic T-lymphocyte antigen

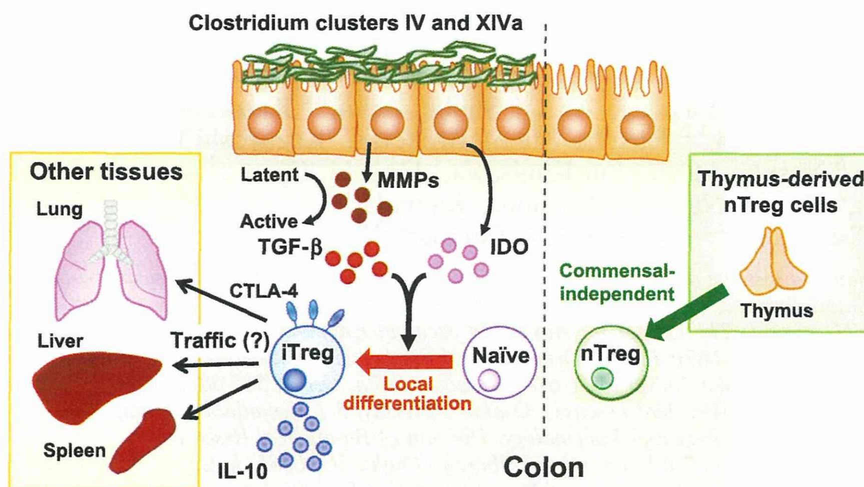

Figure 1. Induction of IL-10-Producing-Induced Treg (iTreg) Cells through the Interaction between Indigenous *Clostridium* Species and Epithelial Cells

After weaning, *Clostridium* clusters IV and XIVa become prominent in the colon, where they form a thick layer on the epithelium. *Clostridium* clusters IV and XIVa promote the production of matrix metalloproteinases (MMPs) from epithelial cells to convert TGF-β from the latent to the active form. Together with indoleamine 2,3-dioxygenase (IDO) produced by epithelial cells, the active form of TGF-β converts non-Treg cells into induced Treg (iTreg) cells that produce IL-10 and express high levels of CTLA-4. The locally differentiated iTreg cells prevent inflammatory and allergic responses in the gut and presumably other remote tissues. In contrast, thymus-derived naturally occurring Treg (nTreg) cells do not require stimulation by commensal bacteria.

4 (CTLA-4) (Figure 1). Interestingly, colonization with *Clostridium* resulted in the specific increase of IL-10-producing Treg cells at distant tissues, such as the spleen and lung, and inhibited allergic responses. These data suggest that T cells educated by commensal bacteria may move from the gut to remote tissues, where they determine the T cell-mediated immunological balance. This idea is plausible based on recent findings that Th17 cells induced by gut-resident SFB have pathogenic roles in the development of arthritis (Wu et al., 2010) and that probiotic-induced Treg cells accumulate at inflammatory sites of various tissues (Kwon et al., 2010).

Investigating the mechanisms of *Clostridium*-mediated induction of Treg cells, Atarashi et al. showed that *Clostridium* formed a thick colonizing layer on the epithelium where it enhanced the release of the active form of TGF-β and indoleamine 2,3-dioxygenase (IDO) from epithelial cells (Atarashi et al., 2011) (Figure 1). The TGF-β pathway was mediated by increasing the gene transcription of matrix metalloproteinases that converted latent TGF-β into the active form. Therefore, colonization with *Clostridium* preferentially converts non-Treg cells into Helios-negative induced Treg cells with little effect on Helios-positive thymus-derived naturally occurring Treg cells. A recent study demonstrated that a mixture of probiotic strains, including *Lactobacillus* and *Bifidobacterium*, enhanced the production of TGF-β and IDO from dendritic cells and consequently induced Treg cells (Kwon et al., 2010), similar to the effects of *Clostridium* on epithelial cells. Interestingly, Atarashi et al. (2011) demonstrated that colonization with a mixture of three *Lactobacillus* strains was not sufficient to induce colonic Treg cells, suggesting that the generation of a bacterial community in which bacteria respond to each other's metabolic products and establish a niche among commensals is important to create an environment that facilitates the induction of Treg cells. Another major unresolved question is the function of *Clostridium* in the induction of colonic Treg cells. Atarashi et al. mention that pattern-recognition receptors were not involved in this pathway, in contrast to the Toll-like receptor 2-dependent conversion of Treg cells induced by polysaccharide A by the human commensal *Bacteroides fragilis* (Round and Mazmanian, 2010). Collectively, these findings suggest that there are versatile pathways in the commensal bacteria-mediated induction of Treg cells, and thus it is important to examine not only bacteria-host interactions but also the role of the bacterial community in the establishment of immunological mutualism. The role of dietary materials (e.g., fatty acids, vitamins, and carbohydrates) in the three-way communications with the host and commensal bacteria is an additional fascinating subject (Maslowski and Mackay, 2011). These future studies will facilitate our understanding of how our immune system mutually evolves with commensal bacteria to achieve the protective but still homeostatic immunity in the intricate environment of the gut, and will also lead to novel strategies to prevent and treat inflammatory, allergic, and infectious diseases.

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Interleukin-1 Family Cytokines as Mucosal Vaccine Adjuvants for Induction of Protective Immunity against Influenza Virus[▽]

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A safe and potent adjuvant is needed for development of mucosal vaccines against etiological agents, such as influenza virus, that enter the host at mucosal surfaces. Cytokines are potential adjuvants for mucosal vaccines because they can enhance primary and memory immune responses enough to protect against some infectious agents. For this study, we tested 26 interleukin (IL) cytokines as mucosal vaccine adjuvants and compared their abilities to induce antigen (Ag)-specific immune responses against influenza virus. In mice intranasally immunized with recombinant influenza virus hemagglutinin (rHA) plus one of the IL cytokines, IL-1 family cytokines (i.e., IL-1α, IL-1β, IL-18, and IL-33) were found to increase Ag-specific immunoglobulin G (IgG) in plasma and IgA in mucosal secretions compared to those after immunization with rHA alone. In addition, high levels of both Th1- and Th2-type cytokines were observed in mice immunized with rHA plus an IL-1 family cytokine. Furthermore, mice intranasally immunized with rHA plus an IL-1 family cytokine had significant protection against a lethal influenza virus infection. Interestingly, the adjuvant effects of IL-18 and IL-33 were significantly decreased in mast cell-deficient *W/W[v]* mice, indicating that mast cells have an important role in induction of Ag-specific mucosal immune responses induced by IL-1 family cytokines. In summary, our results demonstrate that IL-1 family cytokines are potential mucosal vaccine adjuvants and can induce Ag-specific immune responses for protection against pathogens like influenza virus.

Because most pathogenic viruses, including influenza virus, enter through a mucosal surface (18), preventing infection at the viral entry site by inducing mucosal immunity should be an effective strategy for combating such pathogens. A key aspect of mucosal immunity is production of secretory immunoglobulin A (sIgA), as well as induction of cytolytic T lymphocytes (CTLs) against epithelium-transmitted pathogens (5, 21). Therefore, it is important to develop mucosal vaccines that induce effective immune responses at mucosal surfaces (31).

However, protein subunit antigens (Ags) generally evoke only a weak or undetectable adaptive immune response when administered intramucosally (1). Therefore, to produce effective mucosal vaccines, it is necessary to develop an appropriate mucosal vaccine adjuvant (34). Cholera toxin (CT) and *Escherichia coli* heat-labile enterotoxin are known potent mucosal vaccine adjuvants and have been used in nonclinical experimental systems (9, 27). However, their clinical application as nasal adjuvants had to be discontinued because of side effects such as Bell's palsy (29). Therefore, mucosal vaccine adjuvants with high efficacy and safety for clinical application continue to be urgently required.

Cytokines are key molecules that trigger the innate and adaptive immune responses (including maturation of Ag-presenting cells, differentiation of Th1 and Th2 cells, and induction of cytotoxic natural killer [NK] cells and CTLs), resulting in protective layers against virus infection (11, 41, 43). Therefore, cytokines are promising vaccine adjuvants for enhancing the immune response against infectious pathogens. At present, more than 30 members of the interleukin (IL) cytokine/IL receptor family have been identified and found to be involved

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in regulating and maintaining homeostasis of the immune system (3, 14). Specific IL cytokines have been used as vaccine adjuvants to enhance primary and memory immune responses against some cancers and infectious diseases (2, 6). However, there has been no comparative study of IL cytokines as mucosal vaccine adjuvants.

Recently, it was pointed out that identification of the cellular targets of vaccine adjuvants is an important issue (12). Dendritic cells (DCs) are responsible for Ag uptake and presentation to naive T cells and represent a key target for adjuvant activity (22, 33). Recent reports have demonstrated that other accessory cells, such as mast cells (MCs) and NKT cells, act as immunosensors to initiate and modulate innate and adaptive immune responses (16, 40). It has been reported that MCs contribute to the induction of an adaptive immune response or accessory function and that the synthetic Toll-like receptor 7 ligand imiquimod acts as a mucosal vaccine adjuvant in an MC-dependent manner (19). However, it is still not clear whether MCs are promising cellular targets for cytokine adjuvants in mucosal vaccines.

In this study to develop effective and safe mucosal vaccine adjuvants, we identified promising cytokines with mucosal vaccine adjuvant activity by screening 26 different IL cytokines. We also investigated the mucosal and systemic immune responses induced by these cytokines in normal and MC-deficient mice. The IL-1 family cytokines (IL-1α, IL-1β, IL-18, and IL-33) were found to be effective mucosal vaccine adjuvants for induction of protective sIgA and CTL immunity against influenza virus. In addition, the adjuvant activities of IL-18 and IL-33 were MC dependent.

MATERIALS AND METHODS

Cytokines and Ags. CT was purchased from List Biological Laboratories (Campbell, CA). Twenty-six types of mouse recombinant IL cytokines (IL-1α, IL-1β, IL-2, IL-3, IL-4, IL-5, IL-6, IL-7, IL-9, IL-10, IL-11, IL-12, IL-13, IL-15, IL-17, IL-18, IL-19, IL-20, IL-21, IL-22, IL-23, IL-27, IL-28A, IL-28B, IL-31, and IL-33) were purchased from R&D Systems (Minneapolis, MN). Baculovirus-expressed recombinant influenza virus hemagglutinin (rHA) derived from influenza virus A/New Caledonia/20/1999 (Protein Sciences, Meriden, CT) was used as the vaccine Ag.

Mice and immunization protocols. Female BALB/c mice and MC-deficient (WBB6F1 *W/W^v^*) and congenic littermate control (WBB6F1 WT) mice were purchased from Japan SLC (Hamamatsu, Japan) and used at 6 weeks of age. All animal experimental procedures used in this study were performed in accordance with our institutional guidelines for animal experiments. Mice were immunized intranasally with rHA alone (1 μg/mouse), rHA (1 μg/mouse) plus CT (1 μg/mouse), or rHA (1 μg/mouse) plus one of the IL cytokines (0.1 μg, 0.3 μg, or 1.0 μg/mouse) on days 0 and 28.

Sample collection. Fourteen days after the final immunization, plasma and mucosal secretions (nasal washes, saliva, vaginal washes, and fecal extracts) were obtained as previously described (24).

Detection of Ab responses by ELISA. rHA-specific antibody (Ab) levels in plasma and mucosal secretions were determined by enzyme-linked immunosorbent assay (ELISA) as previously described (24). Briefly, ELISA plates were coated with 2 μg rHA/ml of 0.1 M carbonate buffer and incubated overnight at 4°C. The plates were then incubated with blocking solution (Block Ace; DS Pharma Biomedical, Osaka, Japan) at 37°C for 2 h. Diluted plasma or mucosal secretions were added. After incubation at 37°C for 2 h, the coated plates were washed with phosphate-buffered saline (PBS)–polyoxyethylene sorbitan monolaurate (Tween 20; Wako Pure Chemical, Tokyo, Japan) and incubated with horseradish peroxidase (HRP)-conjugated goat anti-mouse IgG solution to detect IgG in plasma or with a biotin-conjugated goat anti-mouse IgA detection Ab (Southern Biotechnology Associates, Birmingham, AL) solution to detect sIgA in mucosal secretions, at 37°C for 2 h. For detection of sIgA, the plates were incubated with HRP-coupled streptavidin (Zymed Laboratories, South San Francisco, CA) for 1 h at room temperature. After incubation, a color reaction was developed with tetramethylbenzidine (Moss, Inc., Pasadena, MD), stopped with 2 N H_2SO_4, and measured as the optical density at 450 to 655 nm ($OD_{450-655}$) in a microplate reader.

Multiplex cytokine assay. Splenocytes from immunized BALB/c, WBB6F1 *W/W^v^*, or WBB6F1 WT mice were harvested 14 days after the final immunization and stimulated *in vitro* with 10 μg rHA/ml. After 72 h, culture supernatants from *in vitro* unstimulated and rHA-stimulated cells were analyzed by a Bio-Plex multiplex cytokine assay (Bio-Rad Laboratories, Hercules, CA) according to the manufacturer's instructions. Samples were analyzed on a Luminex 100 analyzer (Luminex, Austin, TX). The mean concentrations of cytokines in supernatants from rHA-stimulated cells were calculated relative to those in unstimulated cells.

IFN-γ ELISPOT assay. Splenocytes from immunized mice were harvested 14 days after the final immunization and stimulated at a cell density of 1×10^7 cells/ml with a mixture of two H-2K^d-restricted class I HA peptides, $HA_{240-248}$ (IYSTVASSL) and $HA_{462-470}$ (LYEKVKSQL) (MBL, Nagoya, Japan), at a final concentration of 10 μg total peptide/ml complete RPMI (25). After 24 h of incubation at 37°C, plates were washed, and gamma interferon (IFN-γ)-producing cells were measured by use of an enzyme-linked immunospot (ELISPOT) assay kit (BD Biosciences, San Diego, CA) according to the manufacturer's instructions.

Tetramer assay. Splenocytes from immunized mice were harvested 14 days after the final immunization and used as effector cells to determine $HA_{240-248}$-specific CTL responses. Splenocytes (7×10^6 cells) were added to wells in a 24-well plate, followed by addition of 1 ml of medium containing a CTL epitope peptide ($HA_{240-248}$; IYSTVASSL) at a final concentration of 1 μg/ml. After incubation at 37°C for 2 days, medium containing human recombinant IL-2 (rIL-2) (Shionogi Co., Osaka, Japan) was added to each well of CTL effector cells, to a final concentration of 10 U human rIL-2/ml. Effector cells were stained for tetramers after restimulation for 7 days. For analysis, 1×10^6 cells were treated with purified anti-mouse CD16/CD32 Ab (Fc-γ III/II receptor Ab; BD Biosciences Pharmingen, San Diego, CA) and then stained with phycoerythrin (PE)-conjugated H-2K^d-$HA_{240-248}$ peptide tetramer (MBL, Nagoya, Japan) for 20 min at room temperature. Fluorescein isothiocyanate (FITC)-conjugated CD8α (clone KT15; MBL, Nagoya, Japan) was added for an additional 20 min. Cells were analyzed with a FACS Canto flow cytometer (BD Biosciences Pharmingen). Data analysis was done with FlowJo (TreeStar, Eugene, OR) software.

Histopathological analysis. BALB/c mice were immunized intranasally with rHA (1 μg/mouse), with or without IL-1α, IL-1β, IL-18, or IL-33 (1 μg/mouse), on days 0 and 28. Fourteen days after the final immunization, the heads of the mice were severed from the bodies and placed in fixative solution (4% paraformaldehyde). The samples then were sectioned and stained with hematoxylin and eosin (H&E) or Luna stain and examined for pathological changes under a light microscope. Histopathological examination was performed by the Applied Medical Research Laboratory (Osaka, Japan).

Influenza virus infection *in vivo*. To examine the prophylactic effect of IL cytokine treatment against influenza virus, mice were immunized intranasally on days 0 and 28 with 1 μg PR8 HA vaccine (inactivated-product vaccine with influenza virus A/Puerto Rico/8/34) (Charles River, North Franklin, CT)/mouse plus 1 μg CT or IL-1 family cytokine/mouse. Fourteen days after the final immunization, mice were fully anesthetized by intraperitoneal injection of pentobarbital, and each was infected by intranasal application of 25 μl PBS containing 256 hemagglutinating units (HAU) of influenza virus A/PR/8/34 (H1N1) (kindly provided by the Research Institute for Microbial Diseases of Osaka University, Osaka, Japan) per mouse. This procedure produced upper and lower respiratory tract infections.

Statistical analysis. All results are expressed as means ± standard errors of the means (SEM). Differences were compared using Bonferroni analysis of variance (ANOVA).

RESULTS

Comparative analysis of rHA-specific Ab responses induced by 26 different IL cytokines. One potential advantage of successful mucosal immunization is the possibility of eliciting both systemic IgG and mucosal sIgA Ab responses against invading pathogens. Therefore, in this study, we tested 26 different IL cytokines (IL-1α, IL-1β, IL-2, IL-3, IL-4, IL-5, IL-6, IL-7, IL-9, IL-10, IL-11, IL-12, IL-13, IL-15, IL-17, IL-18, IL-19, IL-20, IL-21, IL-22, IL-23, IL-27, IL-28A, IL-28B, IL-31, and IL-33)

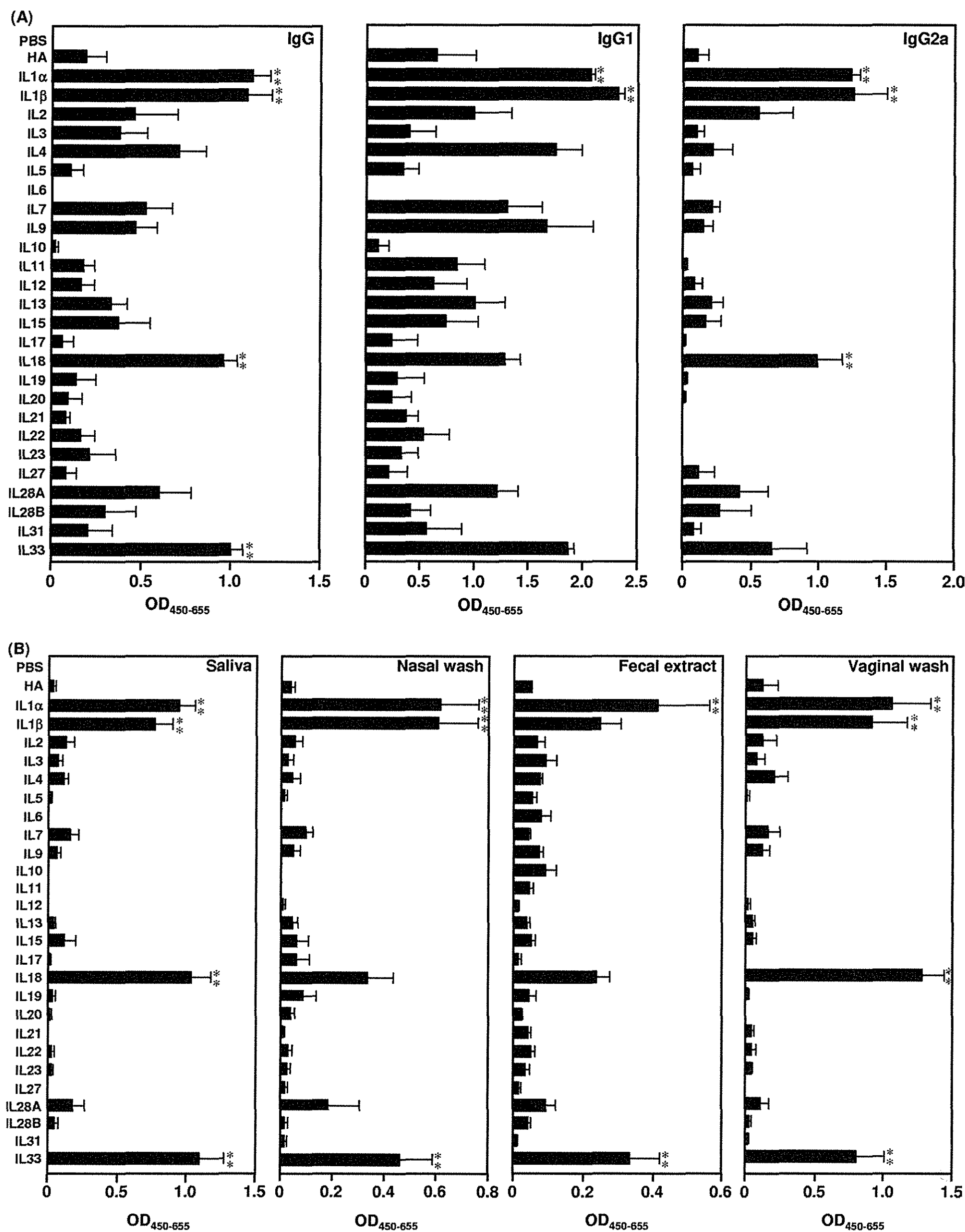

FIG. 1. Ab responses induced by IL-1 family cytokines. BALB/c mice were immunized intranasally at 0 and 28 days with rHA alone or rHA plus each interleukin. (A) Plasma was collected 14 days after the final immunization and analyzed by ELISA for rHA-specific IgG, IgG1, and IgG2a. (B) Saliva, nasal washes, fecal extracts, and vaginal washes were collected 14 days after the final immunization and analyzed by ELISA for rHA-specific sIgA. Data are presented as means ± SEM ($n = 5$). **, $P < 0.01$ compared to the value for the rHA-treated group.

as mucosal vaccine adjuvants. To examine the potential of these IL cytokines as mucosal vaccine adjuvants, BALB/c mice were immunized intranasally with 1 μg rHA plus 1 μg of an IL cytokine on days 0 and 28. Fourteen days after the final immunization, we examined the level of anti-rHA IgG in plasma by ELISA (Fig. 1A). Intranasal immunization with rHA plus 11 of the IL cytokines (IL-1α, IL-1β, IL-2, IL-3, IL-4, IL-7, IL-9, IL-13, IL-15, IL-18, IL-28A, and IL-33) induced higher rHA-specific IgG responses in plasma than those for mice immunized with rHA alone (Fig. 1A). In particular, immunization with rHA plus IL-1α, IL-1β, IL-18, or IL-33, referred to as IL-1 family cytokines, resulted in the highest rHA-specific IgG responses among the IL cytokines. The IgG subclass of the rHA-specific responses was then examined to assess the type of immune response induced by the 26 IL cytokines (Fig. 1A). Plasma Ag-specific IgG subclasses reflect the subset of $CD4^+$ T-helper cells induced by vaccination, with IgG1 and IgG2a corresponding to Th2 and Th1 responses, respectively. Consistent with the rHA-specific IgG responses, intranasal immunization with rHA plus IL-2, IL-3, IL-4, IL-7, IL-9, IL-13, IL-15, or IL-28A generally produced a greater rHA-specific IgG1 subclass response than immunization with rHA alone but a similar IgG2a response to that with rHA alone. In contrast, mice immunized with rHA plus IL-1 family cytokines showed

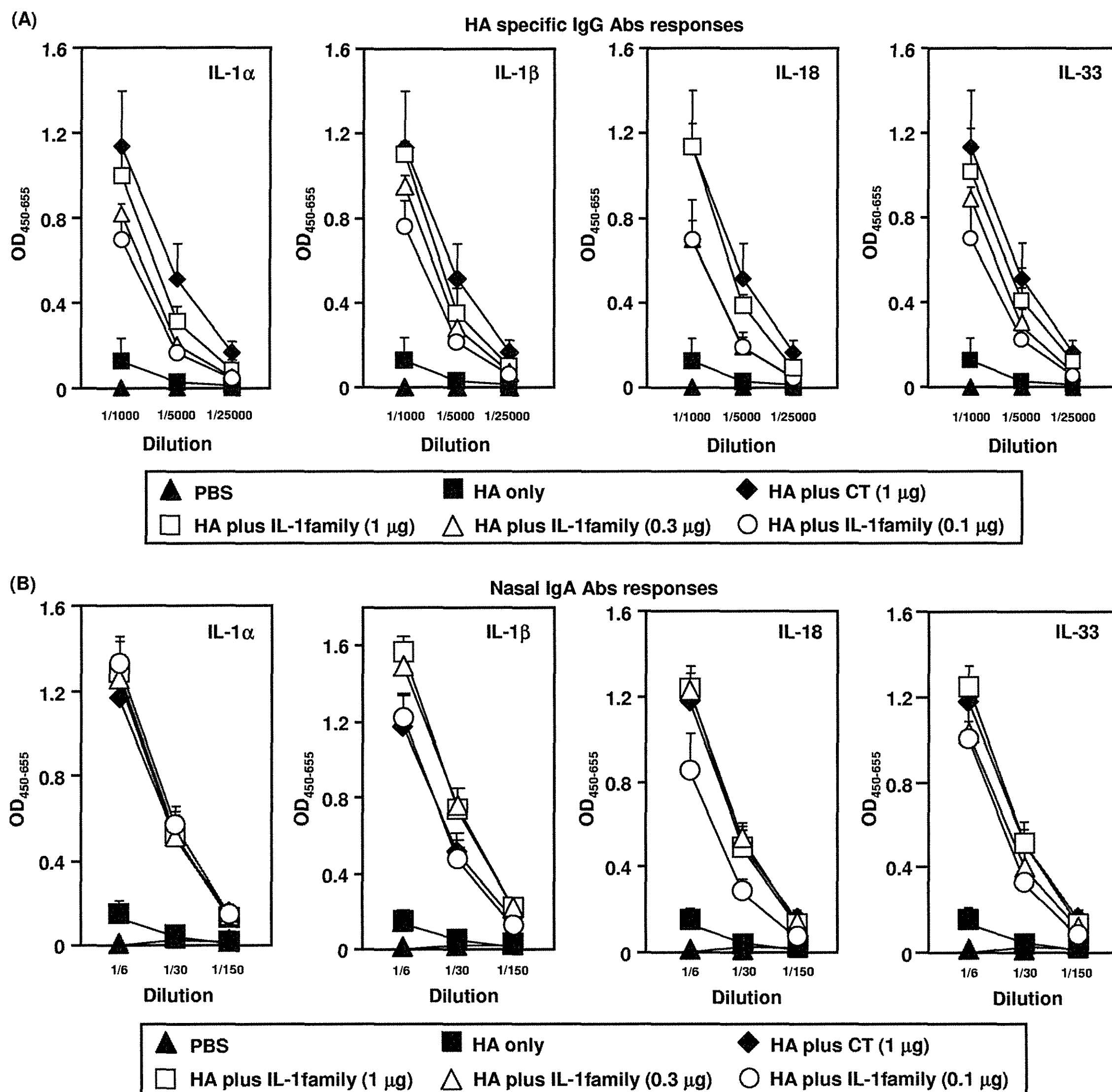

FIG. 2. Dose-response relationship for induction of rHA-specific Ab responses by nasal immunization with rHA plus an IL-1 family cytokine. BALB/c mice were immunized intranasally at 0 and 28 days with rHA alone, rHA plus CT (1 μg/mouse), or rHA plus an IL-1 family cytokine (0.1, 0.3, or 1 μg/mouse). (A) Plasma was collected 14 days after the final immunization and analyzed by ELISA for rHA-specific IgG, at dilutions of 1/1,000, 1/5,000, and 1/250,000. (B) Nasal washes were collected 14 days after the final immunization and analyzed by ELISA for rHA-specific sIgA, at dilutions of 1/6, 1/30, and 1/150. Data are presented as means ± SEM (n = 5).

significantly higher IgG1 and IgG2a Ab responses than those immunized with rHA alone. These results indicate that nasal administration of IL-1 family cytokines has the potential to induce potent rHA-specific systemic IgG Abs, as well as IgG1 and IgG2a Ab responses. We then studied the rHA-specific sIgA response in mucosal secretions (i.e., in saliva, nasal washes, fecal extracts, and vaginal washes) induced by the 26 IL cytokines (Fig. 1B). For these 26 IL cytokines, IL-1 family cytokines induced the highest mucosal sIgA Ab responses in salivary, nasal, fecal, and vaginal mucosal secretions (Fig. 1B). Taken together, these results indicate that nasal immunization with IL-1 family cytokines effectively induced rHA-specific Ab responses in both systemic and mucosal immune compartments, suggesting that IL-1 family cytokines might be effective mucosal vaccine adjuvants.

Dose-response relationship of IL-1 family cytokines as mucosal vaccine adjuvants for induction of rHA-specific Ab responses. To determine the dose-response relationship of IL-1 family cytokines as mucosal vaccine adjuvants to induce rHA-specific IgG and sIgA Ab responses, mice were immunized intranasally with rHA plus 0.1, 0.3, or 1 μg of each IL-1 family cytokine (Fig. 2). Immunization with rHA plus the IL-1 family cytokines induced rHA-specific IgG in plasma in a dose-dependent manner. Even rHA plus the lowest dose (0.1 μg) of IL-1 family cytokines induced IgG to levels significantly higher than those induced by rHA alone (Fig. 2A). Importantly, the use of 1 μg of IL-1 family cytokines as an adjuvant resulted in

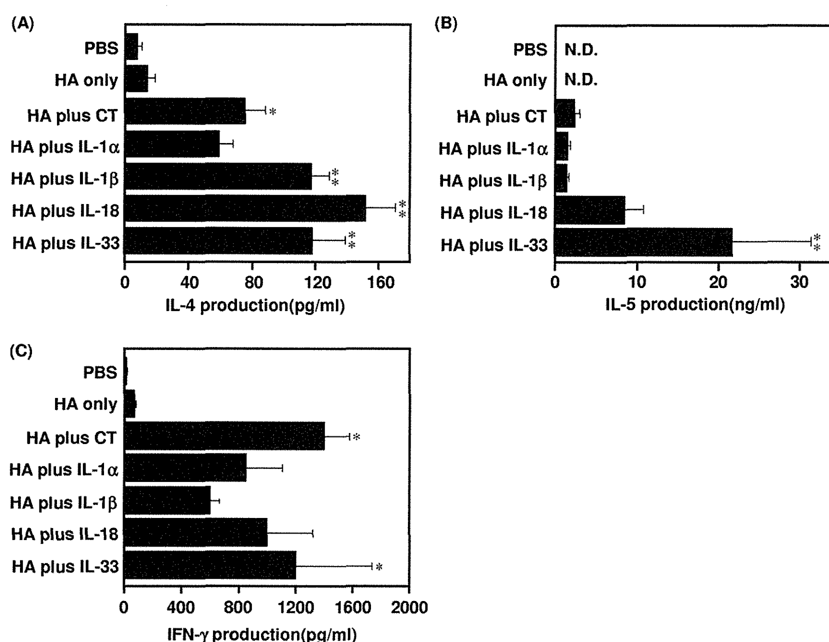

FIG. 3. Cytokine responses induced by nasal immunization with rHA plus IL-1 family cytokines. BALB/c mice were immunized intranasally at 0 and 28 days with rHA alone, rHA plus CT, or rHA plus an IL-1 family cytokine. Fourteen days after the final immunization, splenocytes from each group were cultured with 10 μg rHA/ml. Culture supernatants were harvested after a 3-day incubation and then assayed for rHA-specific IL-4 (A), IL-5 (B), and IFN-γ (C), using a Bio-Plex multiplex cytokine assay. Data are presented as means ± SEM ($n = 5$). *, $P < 0.05$; **, $P < 0.01$ compared to the value for the rHA-treated group. N.D., not done.

strong rHA-specific IgG Ab responses equivalent to those elicited by CT, which is one of the most potent mucosal vaccine adjuvants (Fig. 2A). Furthermore, the level of rHA-specific sIgA induced by rHA plus 0.1 μg of each IL-1 family cytokine in nasal secretions was significantly higher than that induced by rHA alone (Fig. 2B). The level of rHA-specific nasal sIgA induced in mice immunized intranasally with rHA plus 0.3 μg of each IL-1 family cytokine was equivalent to that observed in mice treated with 1 μg CT. Taken together, these results clearly indicate that nasal immunization with an IL-1 family cytokine as a mucosal vaccine adjuvant induced dose-dependent levels of both rHA-specific IgG and sIgA Abs in the mucosal and systemic immune compartments.

Induction of rHA-specific Th1- and Th2-type responses after nasal administration of IL-1 family cytokines as mucosal vaccine adjuvants. To evaluate the ability of IL-1 family cytokines to boost rHA-specific cytokine responses induced by mucosal immunization, splenocytes from mice that had been immunized intranasally with rHA alone, rHA plus CT, or rHA plus an IL-1 family cytokine were restimulated *in vitro* with rHA and then assayed for Th1 (IFN-γ) and Th2 (IL-4 and IL-5) cytokines (Fig. 3). Splenocytes from mice immunized with rHA alone did not show significant cytokine production compared to those from PBS-treated mice. Consistent with the IgG subclass results (Fig. 1A), mice immunized with IL-1 family cytokines had higher levels of IL-4 and IL-5 (Th2-associated sIgA-enhancing cytokines) than mice given rHA alone. In particular, the highest levels of IL-4 and IL-5 were detected in splenocytes of mice immunized with rHA plus IL-18 or IL-33, and these responses were significantly higher than those in splenocytes of mice immunized with CT. It was also noteworthy that IFN-γ, a Th1 cytokine, was induced in mice immunized intranasally with rHA plus an IL-1 family cytokine. Thus, IL-1 family cytokines might induce CTL responses when administered nasally. These results show that as mucosal vaccine adjuvants, IL-1α, IL-1β, IL-18, and IL-33 elicit both Th1- and Th2-type cytokine responses.

***In vivo* CTL induction by nasal immunization with rHA plus IL-1 family cytokines as mucosal vaccine adjuvants.** Virus clearance is known to require strong Th1-polarized immune responses characterized by IFN-γ production and CTL responses in the systemic compartment. To investigate the ability of IL-1 family cytokines to act as mucosal vaccine adjuvants and to induce rHA-specific Th1/CTL immune responses, we measured H-$2K^d$/$HA_{240-248}$ tetramer$^+$ $CD8^+$ T cells (Fig. 4A) and H-$2K^d$/$HA_{240-248}$-specific IFN-γ-secreting cells (Fig. 4B) in splenocytes from mice that had been immunized intranasally with rHA alone, rHA plus CT, or rHA plus an IL-1 family cytokine. The level of H-$2K^d$/$HA_{240-248}$ tetramer$^+$ $CD8^+$ T cells induced by rHA plus IL-1β was found to be similar to that induced by rHA alone, but the level induced by rHA plus IL-1α, IL-18, or IL-33 was significantly greater than that induced by rHA alone (Fig. 4A). Furthermore, the level of functionally active H-$2K^d$/$HA_{240-248}$-specific IFN-γ-secreting cells induced by rHA plus IL-1α, IL-18, or IL-33 was the same as or greater than that in mice intranasally immunized with rHA plus CT (Fig. 4B). Taken together, these results indicate that the IL-1 family cytokines IL-1α, IL-18 and IL-33 induce high-avidity $CD8^+$ CTLs. Therefore, intranasally administered IL-1α, IL-18, and IL-33 might be useful adjuvants for development of an effective mucosal influenza vaccine.

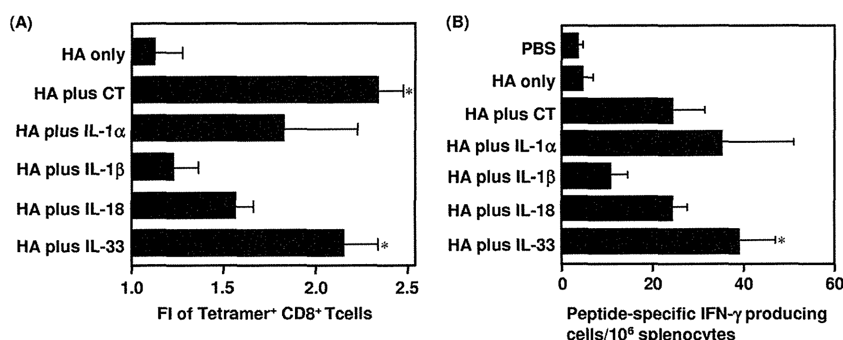

FIG. 4. Measurement of H-$2K^d$/$HA_{240-248}$ tetramer$^+$ $CD8^+$ T cells and H-$2K^d$/$HA_{240-248}$-specific IFN-γ-secreting cells in the spleen after nasal immunization with rHA plus an IL-1 family cytokine. BALB/c mice were immunized intranasally at 0 and 28 days with rHA alone, rHA plus CT, or rHA plus an IL-1 family cytokine. Fourteen days after the final immunization, splenocytes from immunized mice were harvested and stimulated with H-$2K^d$-restricted class I HA peptide at a final concentration of 10 μg total peptide/ml. (A) For detection of H-$2K^d$/$HA_{240-248}$ tetramer$^+$ $CD8^+$ T cells, splenocytes from immunized mice were cultured in medium containing a CTL epitope peptide ($HA_{240-248}$; IYSTVASSL) plus 10 U human IL-2/ml for 7 days, stained for CD8, and analyzed for tetramer-binding cells by flow cytometry. FI, fluorescence intensity. (B) After 24 h of incubation, IFN-γ-producing cells were measured by an ELISPOT assay. Data are presented as means ± SEM (n = 5). *, $P < 0.05$ compared to the value for the rHA-treated group.

Histopathological changes due to IL-1 family cytokines administered intranasally as mucosal vaccine adjuvants. Although enterotoxin-based adjuvants show strong mucosal immunity-inducing ability, they have significant toxic side effects on the central nervous system due to the presence of a specific receptor, GM1 ganglioside, which is highly expressed in neuronal tissue (39). To evaluate the *in vivo* toxicity of IL-1 family cytokines, histopathological changes in nasal tissues of mice given 1 μg of IL-1 family cytokines were investigated. No histological changes indicative of severe inflammation or membrane barrier disruption were observed in the nasal cavities of mice nasally administrated 1 μg of an IL-1 family cytokine (Fig. 5A). In particular, there was no evidence of massive accumulations of mononuclear cells around the airways and blood vessels or of infiltrates in the nasal tissues for all mice examined. Importantly, mice immunized intranasally with IL-1 family cytokines did not induce the goblet cell hyperplasia observed in patients with asthma and chronic obstructive pulmonary disease. Furthermore, Luna staining revealed that IL-1 family cytokine-treated mice did not develop infiltration of Luna-stained eosinophils into the nasal septum (Fig. 5B). Although further evaluation is required, these results indicate that the toxicity of IL-1 family cytokines is likely to be relatively low.

Antiviral immune response to influenza virus infection in mice after nasal immunization with IL-1 family cytokines as mucosal vaccine adjuvants. To determine the level of protection against viral infection provided by IL-1 family cytokines, BALB/c mice were immunized intranasally with 1 μg PR8 HA alone or with 1 μg of an IL-1 family cytokine on days 0 and 28. The immunized mice were then challenged with 256 HAU of mouse-adapted PR8 virus 14 days after the final immunization. The survival and weight of the infected mice were observed every other day (Fig. 6). All mice in the group receiving PBS alone and 86% of the mice immunized with PR8 HA alone died within 7 days of infection. In contrast, mice immunized intranasally with PR8 HA plus an IL-1 family cytokine showed a marked increase in survival (Fig. 6A). Notably, mice immunized with PR8 HA plus IL-1β or IL-18 had 100% survival 14 days after challenge, though with a slight loss of body weight (Fig. 6B). These results indicate that IL-1 family cytokines are potent nasal vaccine adjuvants for providing protection against viral infection.

Role of MCs in rHA-specific immune responses induced by nasal immunization with rHA plus IL-1 family cytokines. MCs are localized predominantly at the interface between the host and the environment (i.e., skin and mucosal surfaces). Recent reports have demonstrated the importance of IL-18-mediated MC activation for host defense, including innate sensing of pathogens (35) and recruitment of DCs and T lymphocytes to sites of inflammation. These findings prompted us to investigate whether MCs have a significant role in the immune response induced by IL-1 family cytokines as mucosal vaccine adjuvants. Hence, we examined MC-dependent rHA-specific systemic IgG and mucosal sIgA Ab responses induced by IL-1 family cytokine adjuvants. For this study, we compared the induction of specific Ab responses in MC-deficient (W/W^v) and WT mice immunized intranasally with rHA plus an IL-1 family cytokine (Fig. 7A and B). Both WT and W/W^v mice immunized with rHA had only minimal rHA-specific IgG Ab responses. However, rHA plus an IL-1 family cytokine induced significant rHA-specific IgG Ab responses in WT mice. W/W^v mice immunized with rHA plus IL-1α, IL-1β, or IL-33 also had significant rHA-specific IgG Ab responses (Fig. 7A), suggesting that IL-1α, IL-1β, and IL-33 act in an MC-independent manner. In contrast, the rHA-specific IgG Ab response induced in W/W^v mice by IL-18 was considerably lower than that in WT mice (Fig. 7A). Similar results were found for mucosal sIgA Ab responses: a significant response was seen with rHA plus IL-1α, IL-1β, or IL-33 in both WT and W/W^v mice, and a decreased response was seen with rHA plus IL-18 in W/W^v mice compared to WT mice (Fig. 7B). We then compared IL-4, IL-5, IL-2, and IFN-γ production in WT and W/W^v mice immunized with rHA plus IL-1 family cytokines (Fig. 7C). WT mice immunized with rHA plus IL-1 family cytokines showed significantly more rHA-specific IL-4, IL-5, IL-2, and IFN-γ production than did WT mice immunized with rHA alone. In contrast, the responses induced by rHA plus IL-18 were sig-

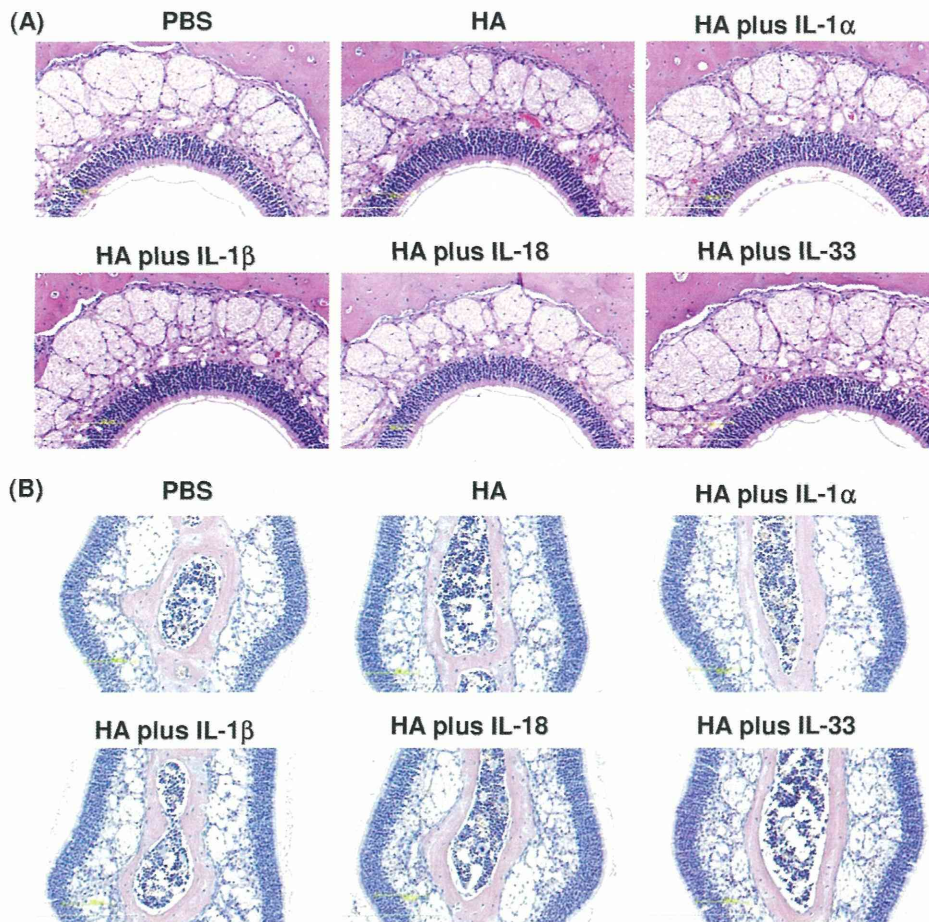

FIG. 5. Histopathological analysis of the nasal cavities of mice immunized intranasally with IL-1 family cytokines. Frontal cross sections of the nasal cavities of mice were taken after two administrations of PBS, rHA alone, or rHA plus an IL-1 family cytokine. Sections were prepared and stained with H&E (A) or Luna stain (B) to assess pathological changes. Overall views of the nasal epithelium (A) and of Luna-stained eosinophils in the nasal septum (B) are shown.

nificantly reduced in *W/W^v^* mice. In addition, although rHA-specific IL-2, IL-4, and IL-5 production in *W/W^v^* mice immunized with rHA plus IL-33 was comparable to that in WT mice, the rHA-specific IFN-γ response was significantly reduced in *W/W^v^* mice. Collectively, these results indicate that MCs have a crucial role in the rHA-specific immune response induced by nasal immunization with rHA plus IL-18. In particular, MCs appear to have an important role in regulating rHA-specific

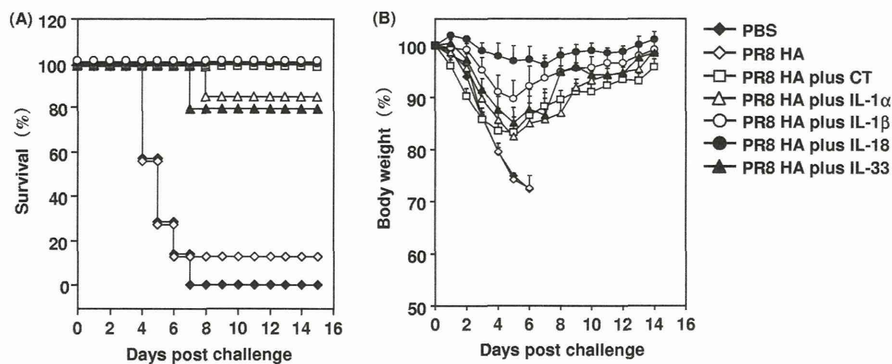

FIG. 6. Protection of BALB/c mice against lethal influenza virus infection by IL-1 family cytokine adjuvants. BALB/c mice were immunized intranasally at 0 and 28 days with rHA alone, rHA plus CT (1 μg/mouse), or rHA plus an IL-1 family cytokine (1 μg/mouse). Fourteen days after the final immunization, mice were intranasally infected with 256 HAU of influenza virus A/PR/8/34. Mice were monitored for survival (A) and weight loss (B) for 14 days after infection. The results are expressed as percent survival (A) and percent initial body weight (B). Data are presented as means ± SEM (n = 4 to 7).