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Nasal IL-12p70 DNA Prevents and Treats Intestinal Allergic Diarrhea[1]

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OVA-induced allergic diarrhea occurs as a consequence of over-expression of Th1 inhibitory IL-12p40 monomers and homodimers in the large intestine, establishing a dominant Th2-type environment. In this study, we demonstrate that intranasally administered murine IL-12p70 naked DNA expression plasmids resulted in the synthesis of corresponding cytokine in the large intestinal CD11c$^+$ dendritic cells, leading to the inhibition of Ag-specific Th2-type response for the prevention of allergic diarrhea and the suppression of clinical symptoms including OVA-specific IgE Ab synthesis. The nasal IL-12p70 DNA treatment proved effective even after the establishment of allergic diarrhea. These results suggest that the mucosal administration of naked IL-12p70 DNA plasmid should be considered as a possible preventive and therapeutic treatment for Th2 cell-mediated food allergic diseases in the intestinal tract. ***The Journal of Immunology,* 2005, 174: 7423–7432.**

Allergic diseases of the respiratory and gastrointestinal tracts, including pollinosis, asthma, and allergic diarrhea, respectively, are generally characterized by hyperresponsiveness stemming from the accumulation of eosinophils, mast cells, and mononuclear cells in mucosal tissues along with an elevation of Ag-specific IgE. This hyperreaction is caused by a defect in the immunological balance between Th1 and Th2 lymphocytes, leading to an increase in Th2 cells. The Th2 cytokines IL-4, IL-5, and IL-13 have been shown to be key mediators of the pathology of these allergic diseases (1–3). In our previous study, large intestinal IL-4-producing Th2 cells induced by systemic priming followed by oral challenge with OVA were shown to play a major pathologic role in the development of allergic diarrhea (4). Furthermore, mast cells, IgE and FcεR were also shown to contribute to the induction of this experimental Th2 intestinal allergy (5). Intestinal allergic inflammation was also induced in the small intestine by the eosinophils, eotaxin, and IL-5 associated with a pathologic Th2 environment in mice treated with OVA in enteric-coated beads (6). Our most recent study elucidated the molecular and cellular pathologic mechanisms underlying Th2 cell-mediated intestinal allergic disease by showing that locally produced IL-12p40 contributes to the generation of a dominant Th2-type environment in the large intestine of mice with allergic diarrhea (7).

Dendritic cell (DC)[3]-derived heterodimeric structure, a p35 and a p40 subunit of IL-12 (IL-12p70), is known to promote Th1 cell differentiation and to stimulate the production of IFN-γ from Th1 cells and NK cells (8–10). Production of IL-12 by activated macrophages and DCs also results in the secretion of monomeric and homodimeric IL-12p40 that antagonize IL-12p70 bioactivity via binding to the β1 subunit of the IL-12R (11–13). IL-12p40 transgenic mice have been shown to be more susceptible to the malaria infection due to the reduced Th1 responses (14). These findings, together with our previous observation that large intestinal IL-12p40 contributes to the generation of Th2 cell-mediated allergic disease, suggest that the mucosal IL-12 balance between p70 and p40 might be a key regulator for the Th1 and Th2 networks associated with the mucosal immune compartment (7). Because it is located on chromosome 5q with its allergy-associated cytokine gene cluster of IL-4, IL-5, and IL-13 (15), the IL-12p40 gene in particular can be considered as one of the potent regulators of Th2 cell induction.

Although IL-12p40 can shift the delicate balance existing between the Th1 and Th2 networks more toward the Th2 environment, IL-12p70 is a well-known Th1 inducer with potential for use in treating Th2-dominant diseases such as asthma (16). IL-12 has also been shown to inhibit IL-4 production in bulk cultures of peripheral blood leukocytes from allergic patients and thus to markedly suppress IL-4-mediated IgE production (17, 18). In addition, IL-12 prevents the differentiation of bone marrow cells into eosinophils in a murine model of asthma (19). The i.p. delivery of IL-12 has been shown to inhibit murine Ag-induced eosinophilic inflammation airway hyperresponsiveness and IgE production (20). In a clinical study of allergic asthma, s.c. administration of rIL-12 resulted in a significant decrease in the number of circulating eosinophils, however only minor effects were seen on histamine-associated airway hyperresponsiveness (16). These studies collectively suggest that IL-12 may be an effective suppressor of

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[3] Abbreviations used in this paper: DC, dendritic cell; Flt3, Fms-like tyrosine kinase-3; AFC, Ab-forming cell; DAPI, 4′,6′-diamidino-2-phenylindole; NALT, nasopharynx-associated lymphoid tissue; CLN, cervical lymph node; MLN, mesenteric lymph node; IVIS, in vivo imaging system.

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allergen-induced airway hyperreaction and of atopic asthma-associated inflammation.

Gene therapy using IL-12 may also have a role to play in the development of vaccines administered by mucosal or systemic routes and in disease modification (21, 22). Gene therapy has recently been used both experimentally and clinically as a tool for developing new vaccines or for halting the progression of immunological diseases. Nasal TGF-β DNA plasmid has been shown to be effective in mitigating the severity of ocular and intestinal inflammatory diseases (23, 24) The administration of Fms-like tyrosine kinase-3 (Flt3) ligand DNA has also been shown to increase the number of activated lymphoid DCs and to thereby induce Ag-specific mucosal and systemic Ab responses (25). Although the mucosal delivery of naked DNA specific for regulatory molecules has been shown to be effective for the modulation of immune responses, the fate of the mucosally delivered naked DNA and its efficacy in inducing and regulating immune responses at distant sites remains unknown. In the current study, we demonstrate that nasal IL-12p70 DNA administration results in the expression of the corresponding protein in large intestinal DCs, which promote the shift to a Th1-type cell response at the disease site for the prevention and treatment of Th2-mediated allergic diarrhea.

Materials and Methods

Mice

BALB/c mice were purchased from CLEA Japan. All mice were 6–7 wk of age at the beginning of the individual experiments.

Reagents

The plasmid pORF9-IL-12p70 consists of the pORF9 multiple cloning site (pORF9-mcs) vector plus the full-length recombinant murine IL-12p40 and IL-12p35 cDNA gene (InvivoGen). The pORF is an expression vector containing the hybrid elongation factor 1α/human T cell leukemia virus promoter and the ampicillin-resistant gene. The plasmid DNA was purified using the EndoFree Plasmid Mega kit (Qiagen). pORF-mcs empty plasmid was used as control vector. For use in a time kinetics study, pIRES2-EGFP plasmid was purchased from BD Biosciences. Allophycocyanin anti-CD11b (M1/70, rat IgG2b), allophycocyanin anti-CD11c (HL3, hamster IgG), and allophycocyanin anti-B220 (RA3-6B2, rat IgG2a) were purchased from BD Pharmingen. Anti-IL-12p70 Ab was obtained from R&D Systems and was biotinylated for immunohistochemical analysis. Streptavidin-PE and Alexa Fluor 660-donkey anti-goat IgG were obtained from Molecular Probes. Anti-LYVE (lymphatic vessel endothelial hyaluronan receptor 1) (26) was obtained from Santa Cruz Biotechnology.

Induction of allergic diarrhea

For the induction of allergic diarrhea, we used a previously established protocol (4, 7). Briefly, on the first day of the experiment (day 0), mice were primed by s.c. injection of 1 mg of OVA in CFA (Difco). One week after the systemic priming (day 7), mice were repeatedly challenged with 50 mg of OVA by oral route three times per week for several weeks (4). Within 1–2 h after the tenth administration with OVA, the mice were sacrificed and analyzed.

Nasal IL-12p70 DNA treatment

In vivo nasal treatment was performed as previously described (25). Briefly, BALB/c mice were nasally administered with 50 μg of purified IL-12p70 plasmid or pORF vector (with empty plasmid used as a control) twice a week for the duration of the experiment. Nasal DNA treatment was started at the time of oral challenge to examine preventive effects. In experiments designed to elucidate the therapeutic potential of nasal IL-12 DNA, mice with ongoing allergic diarrhea were treated with nasal IL-12 DNA.

ELISA for OVA-specific IgE Abs in serum

To assess OVA-specific IgE Ab levels in serum, a sandwich ELISA system was adopted as described earlier (4). End-point titers of OVA-specific IgE Abs were expressed as the reciprocal $\log_2$ of the last dilution that showed a level of absorbance 0.1 higher than that of the sera of nonimmune background mice. Total IgE was also analyzed by a sandwich ELISA system that used anti-mouse IgE (R35-72; BD Pharmingen) to capture mAb and biotin anti-mouse IgE (R35-92; BD Pharmingen) for use as the detection mAb (4).

Isolation of mononuclear cells and the Ag-specific Ig production assay

To isolate mononuclear cells from small and large intestines, we used an enzymatic dissociation method (4, 7). Mononuclear cells were incubated in 96-well nitrocellulose plates (Millititer HA; Millipore) precoated with OVA (1 mg/ml) for 4 h at 37°C with 5% CO_2 in air. OVA-specific Ab-forming cells (AFCs) were detected by addition of peroxidase-labeled anti-mouse IgM, IgG, IgG1, IgG2a, IgG2b, and IgA Abs (Southern Biotechnology Associates) and visualized by the reaction of 3-amino-9-ethylcarbozole (Moss). OVA-specific AFCs were automatically counted by using the KS ELISPOT compact (Carl Zeiss).

Ag-induced cytokine analysis

For the detection of IL-4- and IFN-γ-producing $CD4^+$ T cells, intracellular FACS analysis was performed (27). Briefly, mononuclear cells isolated from large intestine or spleen of empty vector-treated allergic mice, IL-12p70 DNA-treated mice, or healthy control mice were incubated with OVA (1 mg/ml) at 37°C for 4 days and were then reacted with 10 μg/ml monensin for the last 5 h of culture. The cells were washed with PBS (pH 7.2) after removal of dead cells using Ficoll gradient separation and fixed with 4% paraformaldehyde in PBS for 20 min at room temperature. Fixed cells were washed, suspended in staining buffer (PBS containing 2% FCS and 0.01% NaN_3), and incubated with 1 μg/ml PE anti-IL-4 mAb and FITC anti-IFN-γ (BD Pharmingen) in staining buffer containing 0.1% saponin (Sigma-Aldrich) in the presence of 5 μg/ml anti-FcγRII/III (2.4G2; BD Pharmingen) for 30 min at 4°C. After washing three times with staining buffer containing saponin, the cells were stained with 1 μg/ml allophycocyanin anti-CD4 mAb (BD Pharmingen) in staining buffer without saponin for 20 min at 4°C. Following a final washing, the cells were analyzed with a FACSCalibur (BD Biosciences) using the CellQuest software.

Immunoprecipitation and Western blot analyses

For the detection of IL-12p70 heterodimers comprised of IL-12p40 and IL-12p35, large intestinal tissue extracts were prepared as previously described with minor modifications (7). Large intestines were removed, minced in cold PBS with protease inhibitor (Complete Mini; Roche Diagnostics), homogenized and incubated to allow cytokine release from the tissue. After centrifugation and the measurement of their protein concentrations, intestinal tissue extracts were precleared with protein G-Sepharose beads (Pharmacia Biotech), incubated with anti-IL-12p40, and mixed with protein G-Sepharose beads. The beads were washed and then subjected to SDS-PAGE under nonreducing conditions. After electrophoresis, proteins were transferred to a polyvinylidene difluoride microporous membrane (Immobilon; Millipore) and the membrane was reacted with biotinylated anti-IL-12p70 (48110.11, rat IgG1; R&D Systems) and then incubated with a biotin-streptavidin complex (ABC-AP kit; Vector Laboratories). The signal was visualized using a nitroblue tetrazolium/5-bromo-4-chloro-3-indolyl phosphate substrate kit (Bio-Rad). The anti-IL-12p70 mAb has no cross-reactivity with recombinant murine IL-12p40.

Immunohistochemical analysis

Following extensive washing, large intestines were fixed in 4% paraformaldehyde-PBS and treated with sucrose gradient, before freezing in OCT embedding medium as previously described with minor modifications (7). For IL-12p70 immunostaining, cryosections were subjected to Ag retrieval using 10 mM citric buffer (pH 6.0) for 5 min at 98°C. Slides were then blocked with anti-FcγRII/III (2.4G2; BD Pharmingen) and incubated with biotin anti-IL-12p70 and goat anti-LYVE for 16 h at 4°C. The sections were then treated with Alexa Fluor 660 anti-goat IgG (Molecular Probes) and Streptavidin-PE (Molecular Probes). For surface marker staining, serial sections were incubated with allophycocyanin anti-CD11b (M1/70; BD Pharmingen) or allophycocyanin anti-CD11c (HL3; BD Pharmingen). Counter staining was performed using 4′,6′-diamidino-2-phenylindole (DAPI; Sigma-Aldrich).

In vivo monitoring of protein expression following nasal GFP-DNA administration

For assessing the time kinetics of the protein expression induced by nasal DNA, 50 μg of GFP plasmid was nasally administered to normal BALB/c mice. Then, mice were subjected to in vivo imaging system (IVIS) analysis for 20 s using the Xenogen IVIS CCD camera system at 0, 3, 6, 9, 12, 18,

and 24 h after nasal administration (28). After ventral images were taken, the mice were sacrificed and various tissues removed for ex vivo image analysis using the IVIS. The image data of GFP expression was analyzed by LivingImage software (Xenogen) and the GFP intensity from selected areas was quantified.

Nasal GFP-DNA administration and sample analysis

For the time kinetics study of corresponding protein expression following nasal GFP DNA treatment, mice were sacrificed at the indicated time and then analyzed for GFP expression by FACS or subjected to immunohistochemical analysis. Mononuclear cells from nasal passages, spleen, cervical lymph node (CLN), submandibular glands, and mesenteric lymph node (MLN) were isolated, fixed in 4% paraformaldehyde, stained for cell surface markers such as B220, CD4, CD11b, and CD11c and then analyzed for GFP expression using the FACSCalibur. Nasopharynx-associated lymphoid tissue (NALT) and large intestine were fixed in 4% paraformaldehyde-PBS and treated with sucrose gradient, before being frozen in OCT-embedding medium. For lymphoid vessel staining using anti-LYVE (Santa Cruz Biotechnology) (26), cryosections were subjected to Ag retrieval using 10 mM citric buffer at pH 6.0 for 8 min at 98°C. Slides were then blocked with anti-FcγRII/III (2.4G2; BD Pharmingen), incubated with goat anti-LYVE or control goat IgG, and then treated with Alexa Fluor 660 anti-goat IgG. Counter staining was performed using DAPI (Sigma-Aldrich), and immunohistochemical analysis was performed using confocal laser scanning microscopy (Leica).

Analysis of large intestinal cells

As soon as mice developed diarrhea after the final oral OVA challenge, they were sacrificed and cells were isolated from the large intestine as described earlier. Cytospin slides were prepared and then stained with Diff-Quik (Sysmex) for the morphological analysis of differential cell populations including mononuclear cells, eosinophils, basophils, and neutrophils.

Statistical analysis

Statistical analyses were performed by the two-sample nonparametric Welch test with a significance level of $p < 0.01$ or $p < 0.05$ for IgE levels. Values for cytokine synthesis and IgA, IgG, and IgG1 AFCs in the samples between IL-12p70 and control plasmid-treated mice were analyzed by using Student's t test at values of $p < 0.01$.

A

Immunization	Nasal treatment	Incidence of OVA-induced diarrhea (%)	
SC/PO	None	22/22	100
	Vector	14/14	100
	IL-12p70 plasmid	0/14	0
SC only	None	0/12	0

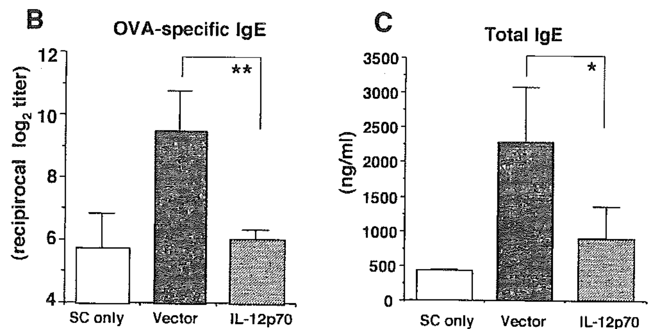

FIGURE 1. Inhibition of allergic diarrhea disease by nasal treatment with IL-12p70 naked DNA plasmid. *A*, The incidence of allergic diarrhea was reduced in mice treated nasally with IL-12p70 DNA when compared with mice treated with empty vector plasmid. Allergic disease was induced in these mice by s.c. immunization and then repeated oral challenge (SC/PO) with OVA. *B*, OVA-specific IgE Abs are reduced in the serum of allergic diarrhea-afflicted mice treated nasally with IL-12p70 DNA. *C*, Total IgE Abs are reduced in the serum of allergic diarrhea-afflicted mice treated with naked IL-12p70 DNA. The data are expressed as the mean ± SD and are representative of five independent experiments. Statistical differences between IL-12p70 DNA- and empty vector-treated mice (**, $p < 0.01$ and *, $p < 0.05$) are indicated.

Results

Nasal IL-12p70 DNA prevents allergic diarrhea

In a previous study, we showed that large intestinal IL-12p40 led to a pathologic Th2-dominant environment conducive to the development of OVA-induced allergic diarrhea (7). Because IL-12p70 is one of the most effective Th1-inducing cytokines (8), we sought to examine whether artificially introduced IL-12p70 DNA could prevent the development of allergic diarrhea by up-regulation of IL-12p70 expression of the disease site. Mice nasally treated with the IL-12p70 plasmid did not develop allergic diarrhea, whereas mice given the empty vector developed severe disease as that observed in OVA-induced diarrhea mice (Fig. 1*A*). Thus, mice receiving the empty vector served as a control for the remainder of the investigation. In addition, elevated OVA-specific IgE Abs were detected in the serum of diarrhea-afflicted mice treated with the control vector DNA, whereas the mice nasally treated with IL-12p70 DNA showed only low OVA-specific IgE Abs (Fig. 1*B*). Furthermore, nasal treatment of IL-12p70 DNA reduced the level of total serum IgE Abs (Fig. 1*C*). These results demonstrate that nasal IL-12p70 plasmid prevented the development of OVA-induced allergic diarrhea and reduced both Ag-specific and total IgE responses. Based on these findings, we hypothesized that nasal treatment with IL-12p70 DNA down-regulates the Th2 environment and thus inhibits the development of allergic diarrhea.

Reduction of large intestinal IgA and IgG production by nasal administration of IL-12p70 DNA

To assess nasal IL-12p70 DNA effects on Th1/Th2 responses, we next examined OVA-specific IgM, IgG, IgG1, IgG2a, IgG2b, and IgA Ab responses in large intestinal lamina propria mononuclear cells using the ELISPOT assay. We found that the numbers of Th2-associated OVA-specific IgA and IgG1 AFCs in large intestinal lamina propria mononuclear cells were significantly reduced in the mice treated nasally with IL-12p70 DNA when compared with those of mice treated with the empty vector DNA (Fig. 2*A*, *left*). As we reported earlier (4), OVA-specific IgG2a, IgG2b (data not shown), and IgM AFCs were not detected in the large intestine of all groups of mice examined. In spleen, no significant differences were detected in the numbers of OVA-specific IgA, IgG, and IgG1 AFCs between IL-12p70-treated and control mice (Fig. 2*A*, *right*). As we have shown previously (4), Ag-specific Ab responses are not induced in the small intestine of mice suffering from allergic diarrhea. Thus, OVA-specific IgA, IgG, and IgG1 Ab responses were not detected in the small intestine of either IL-12p70 DNA-treated or control vector-treated mice (data not shown). Although the exact role of OVA-specific IgA and IgG Ab responses in the large intestine of mice with allergic diarrhea still needs to be elucidated, these findings further support the notion that nasal administration of IL-12p70 DNA can inhibit the generation of locally enhanced Th2 cell-mediated OVA-specific Ab responses in mice suffering from allergic diarrhea.

Suppression of large intestinal Th2 cytokine by nasal IL-12p70 DNA

To directly confirm decreased Th2-type responses in the large intestine after nasal IL-12p70 treatment, we next examined Ag-induced cytokine production of the large intestinal $CD4^+$ T cells using intracellular FACS analysis. Nasal IL-12p70 DNA treatment decreased the number of IL-4-producing cells in large intestinal

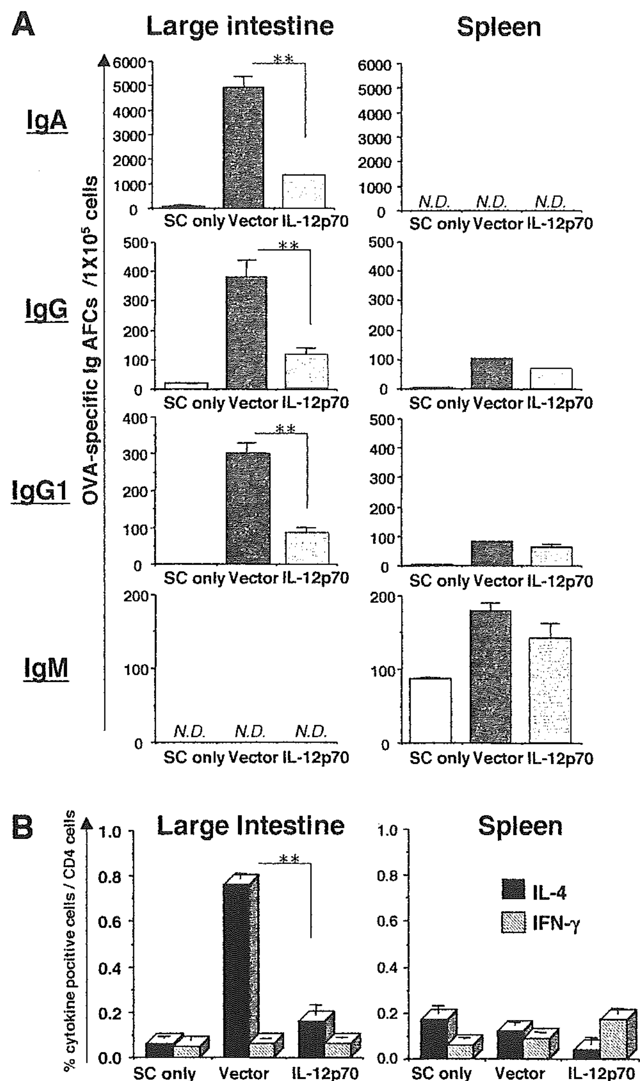

FIGURE 2. Reduction of IgA and IgG hyperresponsiveness in large intestinal B cells and of Th2 cells responses by nasal administration of IL-12p70 DNA. *A*, The frequency of OVA-specific IgA, IgG, IgG1, and IgM AFCs in the large intestine of mice treated with IL-12p70 DNA or empty vector plasmid. *B*, OVA-specific IL-4 and IFN-γ production were analyzed by intracellular staining using FACS analysis. In vivo treatment with IL-12p70 DNA reduced the predominant Th2-type Ag-specific responses by large intestinal mononuclear cells isolated from diarrhea-afflicted mice. The mononuclear cells isolated from the large intestine (1.0×10^6 cells/well) or spleen were cultured with OVA (1 mg/ml) for 4 days. After incubation, cells were harvested and subjected to intracellular staining with anti-IL-4 and IFN-γ. The data are expressed as the percentage of cytokine-positive cells in large intestinal $CD4^+$ T cells. The data are expressed as the mean ± SD and are representative of four independent experiments. Statistical differences between IL-12p70 DNA- and empty vector-treated mice (**, $p < 0.01$) are indicated. N.D., Not detected.

$CD4^+$ T cells when compared with the empty vector-treated mice (Fig. 2*B*, *left*). No difference in the frequency of IFN-γ-producing $CD4^+$ Th cells was seen between the mice treated with nasal IL-12p70 DNA and control vector DNA (Fig. 2*B*, *left*). In contrast, when splenic $CD4^+$ Th cells were examined, no major changes were observed between IL-12p70 DNA-treated and control vector-treated mice (Fig. 2*B*, *right*). However, nasal IL-12p70 treatment did reduce the number of IL-4-producing cells and increase the number of IFN-γ-producing cells in splenic $CD4^+$ Th cells (Fig. 2*B*, *right*). A similar pattern of changes was observed when culture supernatants from the various groups of IL-12p70 DNA- and empty vector-treated mice were examined (data not shown).

Nasal GFP DNA resulted in protein expression in NALT, spleen, and intestine

To directly confirm that the nasal administration of DNA resulted in protein expression in the large intestine, we initially used GFP plasmid DNA for the visualization of corresponding protein expression in vivo. When we used the IVIS to analyze the GFP distribution in vivo following nasal administration of GFP plasmid DNA, the intense fluorescence expression was observed in areas associated with the nasopharynx and CLN as of 3 h after nasal administration (Fig. 3*A*). A high intensity of GFP expression was observed in the intestinal region 9 h after nasal administration (Fig. 3*A*). In addition, we analyzed ex vivo levels of GFP expression in various tissues isolated from mice that had been given nasal GFP plasmid. The spleen and lymph nodes expressed significant GFP beginning at 3 h after administration and continuing up to 18 h (Fig. 3*B*). In mice administered with the GFP gene, GFP expression was particularly strong in CLN. We also analyzed GFP expression levels in the lung, liver, and nasal cavity of mice given nasal IL-12p70 DNA; however, levels of GFP expression were below levels of detection (data not shown). Our effort to analyze GFP expression in the small and large intestine was hampered by autofluorescence, making it difficult to obtain reproducible data.

Next, we assessed whether nasal GFP plasmid would lead to the induction of corresponding protein expression in NALT because the tissue has been shown to be a primary site for the initial uptake of nasal Ags (29). GFP^+ cells were detected under the epithelium of NALT, but not until 12 h after nasal administration (Fig. 4*A*). These GFP^+ cells were examined by costaining them with red fluorescence-conjugated anti-CD11c Ab (Fig. 4*A*, *right*). These data suggest that nasal administration with GFP plasmid resulted in the expression of the corresponding protein in NALT DCs. Because GFP expression had already been noted in other tissues distant from NALT, it can be assumed that some of the administered gene might rapidly pass through NALT without protein expression in the lymphoid tissue.

To chronologically assess the GFP-expressed DCs located in various tissues after nasal GFP plasmid, mice were sacrificed at predetermined time points (0, 1, 3, 6, and 12 h and 1, 2, 3, 4, 5, and 7 days) and mononuclear cells from various lymphoid tissues were analyzed for the presence of GFP^+ DCs at the single cell level. When mononuclear cells from nasal passages, spleen, CLN, MLN, and submandibular glands were examined, we detected some GFP^+ cells in the DC fraction. However, the frequency was not high enough to meet the minimum limits of reliable FACS analysis. For example, we were able to detect GFP^+ $CD11c^+$ cells in MLN at the frequency of ~0.1%. In contrast, it was possible to detect sufficient numbers of GFP-expressing DCs in spleen due to the quantity of naturally occurring $CD11c^+$ cells. Thus, the levels of $CD11c^+$ cells expressing GFP were detected in spleen as early as 3 h, peaked at 12–24 h, and gradually decreased beginning 96 h after nasal administration (Fig. 4*B*). In contrast, CD11b, B220, and $CD4^+$ cells did not express GFP in spleen (data not shown).

To directly confirm GFP expression in intestinal tissues, we next analyzed large intestines for fluorescence expression using immunochemical analysis. GFP^+ cells appeared in large intestines from 6 h to 4 days after nasal administration of GFP plasmid (Fig. 4*C*). High magnification analysis revealed that GFP^+ cells were located in nearby lymphoid vessels (Fig. 4*C*, *right*). Most GFP^+ cells in the large intestines were $CD11c^+$ (data not shown). These findings directly demonstrate that nasal delivery of naked DNA resulted in

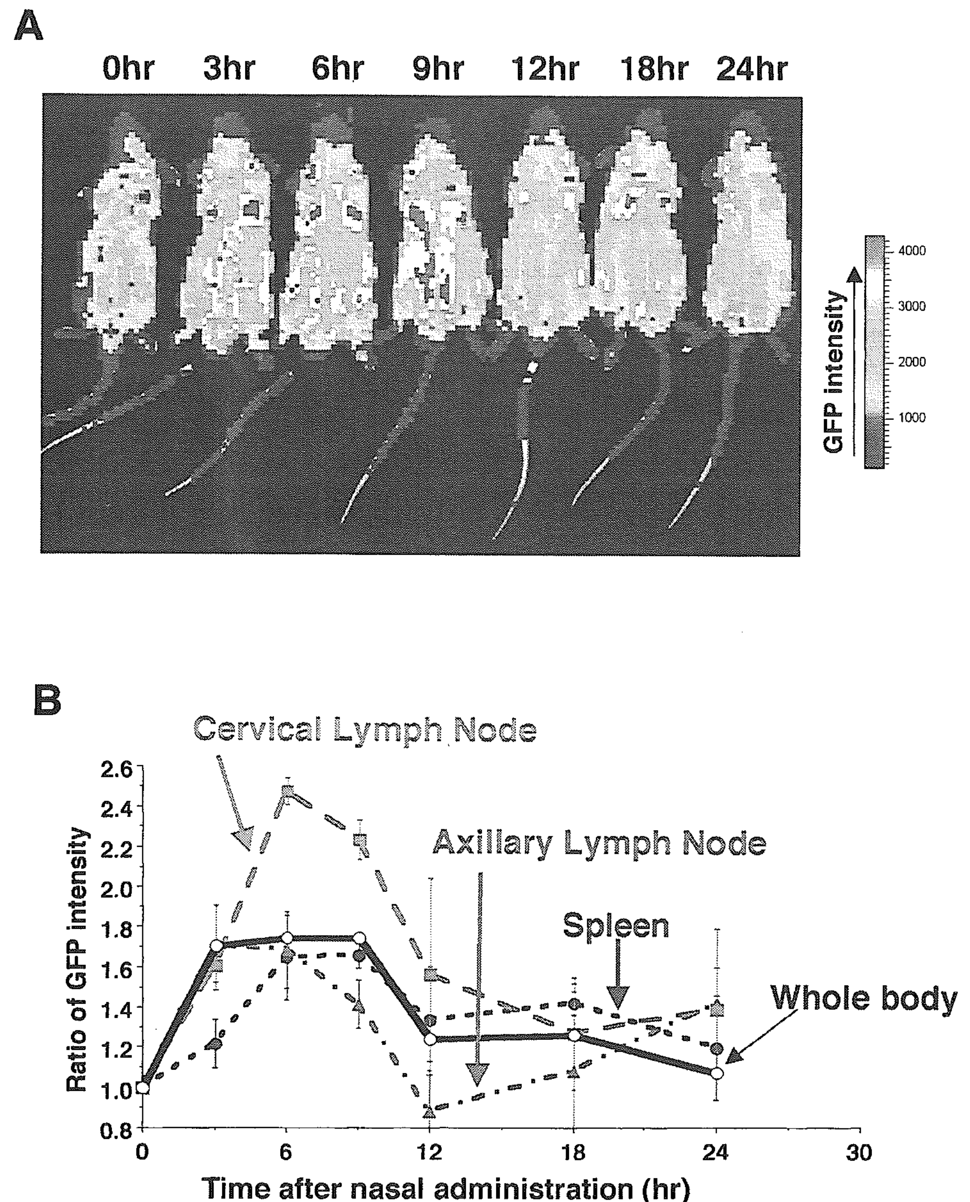

FIGURE 3. Analysis of green fluorescence expression in different tissues following nasal administration of GFP DNA in vivo using IVIS. GFP DNA plasmids were administered nasally for the purpose of analyzing the tissue distribution of GFP expression. GFP expression was detectable in vivo beginning 3 h after nasal administration (*A*). Tissues from sacrificed mice were examined ex vivo for GFP levels using IVIS. The GFP expression levels were quantified by Living Image software (*B*).

the expression of the corresponding protein in a distant mucosal compartment (e.g., large intestine).

Nasal IL-12p70 DNA led to the protein expression in large intestine

To directly demonstrate that nasal IL-12p70 DNA administration induces IL-12p70 expression at the site of disease development, we used immunohistochemical analysis to assess the cytokine expression in the large intestine of mice nasally treated with IL-12p70 DNA. When large intestines were examined, IL-12p70-producing cells were located in nearby lymph vessels (Fig. 5*A*). In contrast, large intestinal cells of mice treated with vector DNA only did not contain any cells expressing IL-12p70 (Fig. 5*A*). Further analysis of the IL-12p70 expressed by large intestinal mononuclear cells revealed that these cells were costained with green fluorescence-coupled anti-CD11c (Fig. 5*C*). These data suggest that nasal administration of IL-12p70 DNA resulted in IL-12p70 protein expression in large intestinal DCs. An identical finding was also observed using nasal GFP DNA treatment (Fig. 4). Furthermore, to finally confirm that IL-12p70 is expressed in mice treated nasally with IL-12p70 DNA, we examined IL-12p70 protein expression by Western blot analysis. Extracts of large intestine from mice nasally treated with IL-12p70 DNA expressed a high intensity band corresponding to IL-12 (Fig. 5*B*). However, only a faint band corresponding to residual IL-12p70 was detected in large intestinal extracts from mice treated with the empty vector. These results indicate that nasal IL-12p70 DNA treatment leads to IL-12p70 expression in large intestinal cells including DCs, accounting for the inhibition of pathologic Th2 reactions and thus for the prevention of allergic diarrhea.

Nasal IL-12p70 DNA administration cures ongoing OVA-induced allergic diarrhea

Once we had established that nasal IL-12p70 DNA prevented allergic diarrhea, we examined whether nasal IL-12p70 DNA could alter the disease condition in mice with existing allergic diarrhea. Severe allergic diarrhea was first induced in systemically primed mice by 10 oral challenges with OVA. When mice were given nasal IL-12p70 DNA, the diarrhea was completely cured after three doses (Fig. 6, *A* and *B*). As one might expect based on our

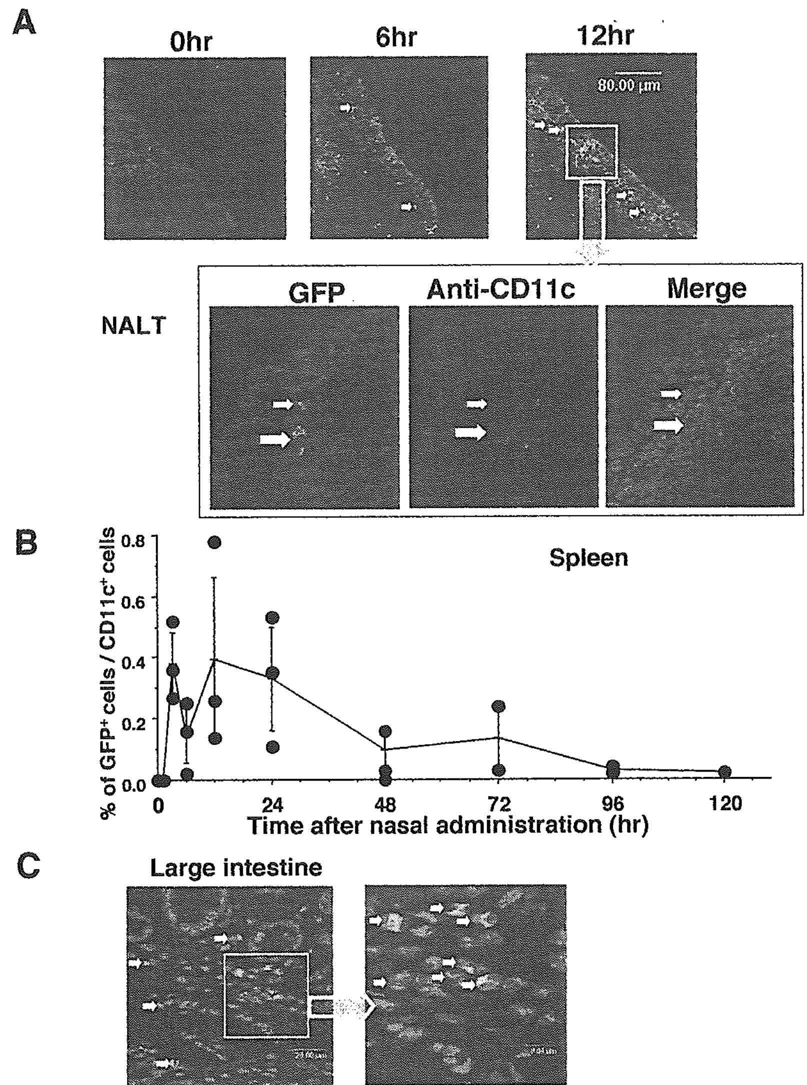

FIGURE 4. Expression fo GFP by CD11c$^+$ cells in systemic (spleen) and mucosal (NALT and large intestine) tissues following nasal administration of GFP DNA. *A*, At 6 and 12 h after nasal administration of GFP DNA, GFP and CD11c double-positive cells were observed in NALT. The arrows point to double-positive cells. *B*, Time kinetic studies using FACS analysis determined the frequency of GFP$^+$ cells in the spleen of mice nasally treated with GFP DNA. GFP$^+$ cells were preferentially detected in splenic CD11c$^+$ cells from 3 h to 3 days after nasal DNA administration. GFP$^+$ cells were not found in B220-positive and CD11b$^+$ populations. *C*, Nasal administration of GFP DNA resulted in the expression of the corresponding protein in the large intestines. They appeared from 6 h to 3 days after nasal DNA administration. GFP$^+$ cells were located near lymphatic vessels, which are indicated in red. Enlargement (*right*) of the *inset* in the white square (*left*).

previous findings (4), a large number of basophils and eosinophils were seen in the large intestine of mice suffering from allergic diarrhea (Fig. 6*C*, *top*). The nasal treatment with IL-12p70 DNA removed these basophils and eosinophils from the large intestine (Fig. 6*C*, *bottom*). Furthermore, the large intestinal hyperresponse of Th2-associated IgA, IgG, and IgG1 AFCs were also significantly decreased by the nasal IL-12p70 DNA treatment (Fig. 6*D*). Thus, nasal administration with IL-12p70 DNA proved to be effective at inhibiting ongoing large intestinal allergic reaction.

Discussion

Food allergies in humans are caused by hypersensitivity to food allergens and can result in severe diarrhea (30). The OVA-induced Th2 cell-mediated allergic diarrhea model, which involves systemic priming followed by oral challenge, is a useful and adequate model for the investigation of mechanisms that result in food allergies (4, 7). Our most recent study showed that local accumulations of IL-12p40 are a major triggering factor for the creation of an aberrant Th2 environment in the large intestine, one which is conducive to the induction of allergic diarrhea (7). In the current study, our findings demonstrate that nasal IL-12p70 DNA treatment prevents the development of IL-12p40-mediated OVA-induced Th2-dominant allergic disease by inducing IL-12p70 production in the large intestinal tract. Not surprisingly, the inhibition of pathologic Th2 cell responses was seen in the large intestine of mice nasally treated with IL-12p70 DNA. Based on the results obtained by in vivo image and immunohistochemical analyses, we suggested that nasal deposition of naked IL-12p70 DNA resulted in the expression of the corresponding protein at distant sites including large intestine. This finding was further substantiated by results showing that nasal administration of naked GFP plasmid DNA resulted in the expression of GFP-positive DCs but not of macrophages and B cells in the intestinal tract, NALT, and spleen. Although the efficacy of the nasal administration of naked DNA was previously reported using Flt3 ligand and TGF-β DNA plasmid (23, 25), our study is the first to show that expression of nasal DNA occurs in distant mucosal compartments at the single cell level (e.g., IL-12p70-producing large intestinal DCs) and to link that expression to the prevention and treatment of intestinal allergic diseases. Taken together, these data show that noninvasive nasal administration with naked DNA plasmid results in the expression of the corresponding protein in both the mucosal and systemic lymphoid tissues and so may offer a new avenue of therapy for mucosa-associated diseases. Specifically, nasal administration of naked IL-12p70 DNA should be given consideration as a new tool in preventing and treating allergic diseases associated with the distant mucosal tissues.

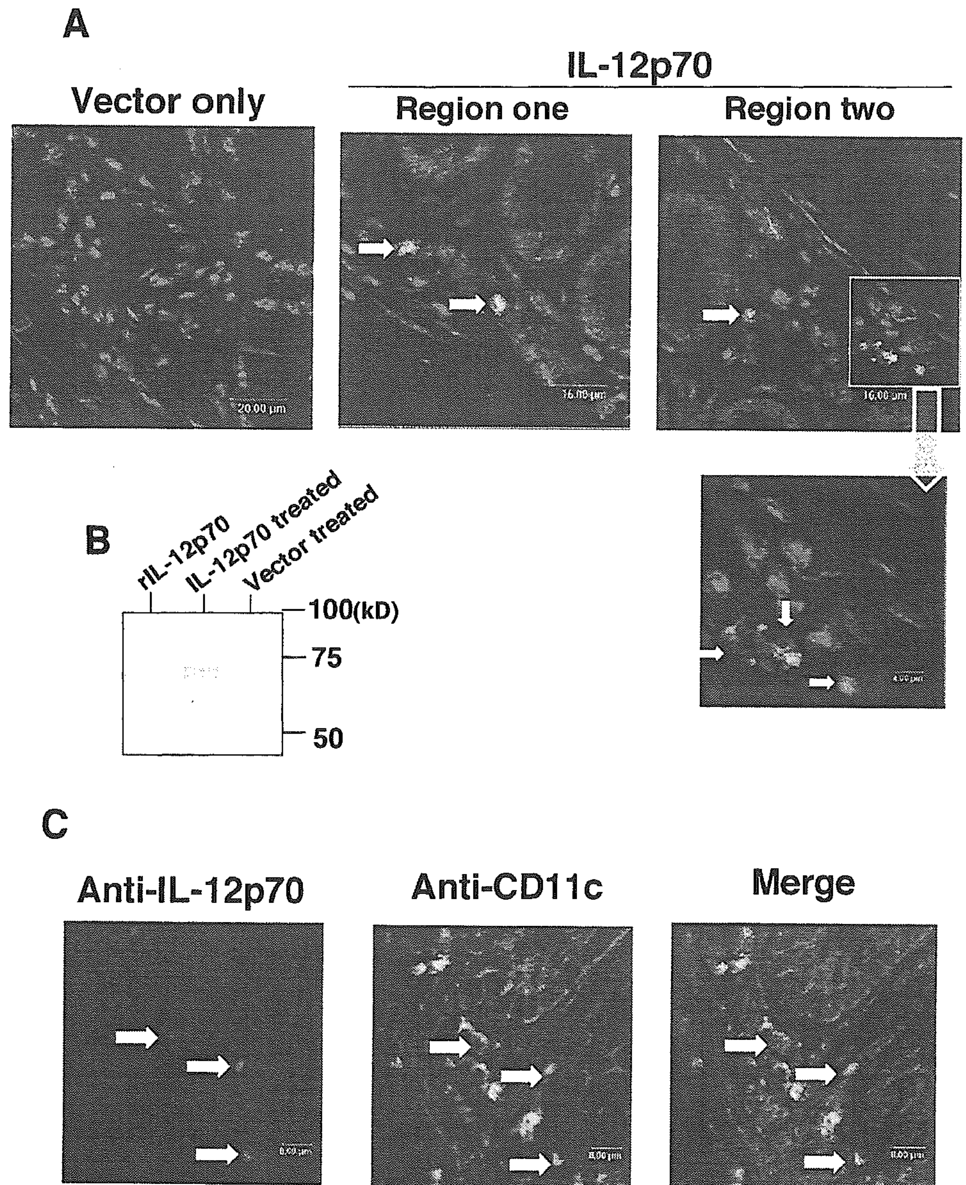

FIGURE 5. Induction of IL-12p70 protein in the large intestine by nasal administration of IL-12p70 DNA. Anti-IL-12p70 mAb used did not cross-react with the IL-12p40 molecule. *A*, IL-12p70-positive cells were detected near large intestinal lymphatic vessels after nasal IL-12p70 DNA treatment. *B*, The IL-12p70 protein was also detected in large intestinal extracts using immunoprecipitation and blotting analysis. Recombinant murine IL-12p70 (rIL-12p70) protein (*lane 1*) was used as a positive control for the detection of IL-12p70 (*B*). *C*, IL-12p70-producing cells were stained with fluorescence-conjugated anti-CD11c in the large intestine.

The IL-12 protein treatment has been shown to be effective in inhibiting allergic asthma-associated airway hyperreaction and in blocking the associated eosinophil accumulation in the lung and the elevation of allergen-specific IgE (19, 20, 31). Oral IL-12 treatment inhibited peanut allergic reactions by reducing the release of histamine and peanut-specific serum IgE and IgG1 levels (32). These results suggest that IL-12 might be a useful immunotherapeutic agent for the control of respiratory mucosa-associated allergic diseases because these clinical symptoms have been shown to result from the development of aberrant Th2-type responses (4, 7). It is also reported that the IL-12 gene therapy using cationic liposome or virus as the DNA delivery vehicle is effective in controlling allergic asthma (22, 33, 34). Thus, the i.v. injection of IL-12p70 DNA plasmid mixed with liposome achieved high protein expression in the lung (35, 36). Systemic IL-12 gene therapy resulted in the down-regulation of airway inflammation by suppressing the secretion of eotaxin in the lung tissue (33). When the effects of systemic IL-12p70 DNA and protein administration were compared, the half-life of the circulating IL-12p70 protein produced was much longer after gene transfer than after IL-12 protein injection, and no negative side effects were seen (21). Other groups have reported that local intratracheal or nasal administration of the IL-12p70 gene prevented the development of respiratory allergic disease (22, 34). These elegant studies demonstrated the efficacy of the recombinant vaccinia virus vectors designed to deliver the IL-12-encoding gene to respiratory tissues for the treatment of allergic airway disease (34). The vaccinia virus vector system allowed the restricted expression of IL-12p70 locally in the airway but not systemically. Our present study further demonstrates the attractiveness of mucosal gene therapy for the prevention and treatment of mucosa-associated immunological diseases. The current study adds a new dimension to the efficacy of the nasal delivery of IL-12p70-specific DNA by showing that it allows the expression of the corresponding protein at distant mucosal sites, presumably via APCs, namely DCs. Considering possible application of our findings to clinical setting, one must realize a fact that murine IL-12p40 homodimer has been shown to bind to IL-12R with high affinity (37), whereas human IL-12p40 homodimer possesses a somewhat weaker binding affinity than mouse IL-12p40 (13).

We could not detect the elevation of IL-12p70 levels in serum (data not shown), but the presence of IL-12p70-positive DCs were noted in the large intestine and spleen. This finding strongly suggests a possibility that the selective expression of IL-12p70 by DCs may lead to the effective delivery of the protein perhaps made

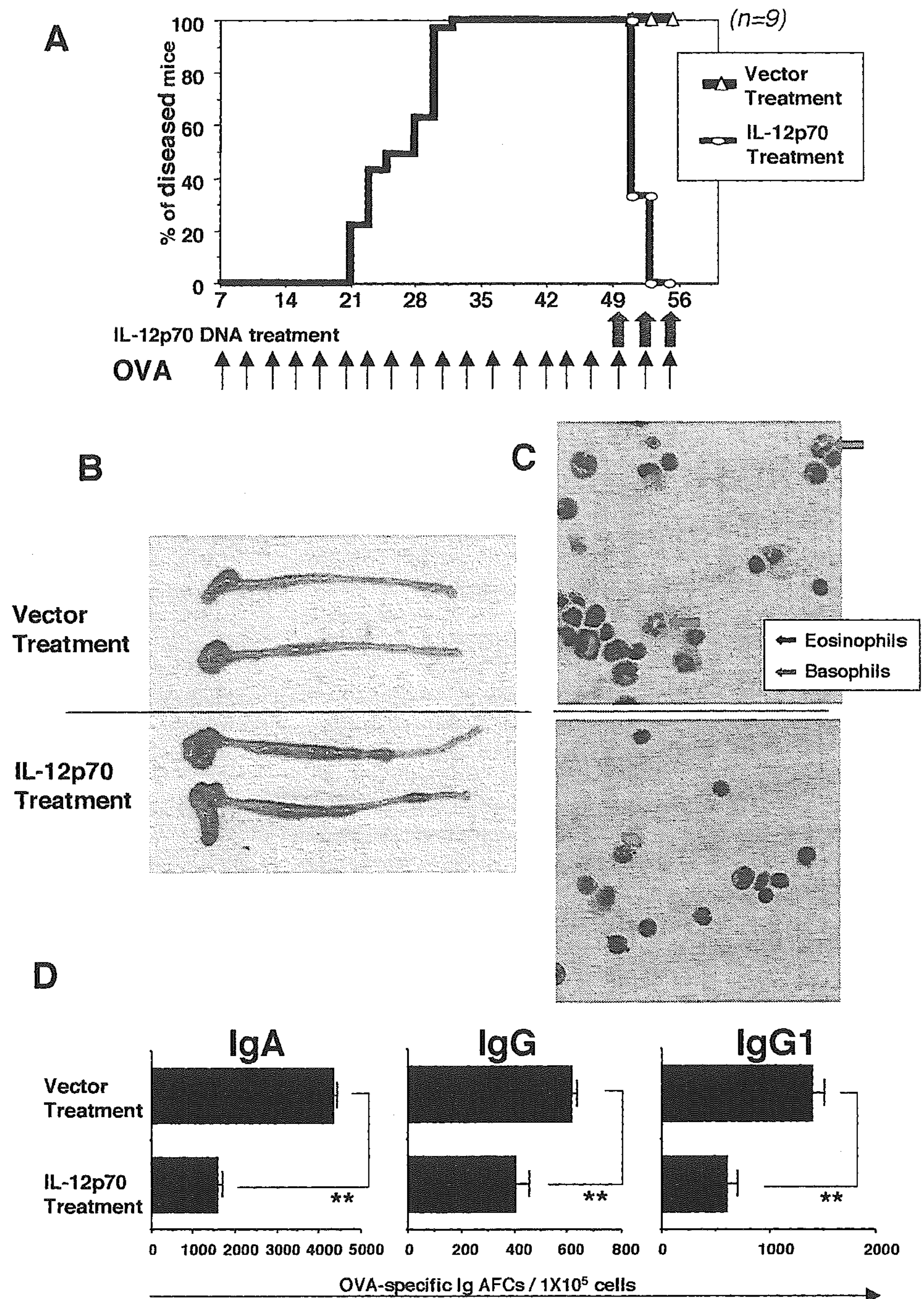

FIGURE 6. Nasal IL-12p70 DNA treatment cured allergic diarrhea. *A*, Frequency of allergic diarrhea decreased after IL-12p70 DNA treatment ($n = 9$ per group). *B*, The symptoms of diarrhea were completely inhibited after three treatments with IL-12p70 DNA. *C*, Infiltration of eosinophils and basophils into the large intestine was blocked by the IL-12p70 DNA administration. The red arrows point to eosinophils and the blue arrows point to basophils. *D*, Results of the ELISPOT assay used to determine the OVA-specific Ig responses in the large intestinal mononuclear cells. The data are expressed as the mean ± SD and are representative of three independent experiments. Statistical differences between IL-12p70 DNA- and empty vector-treated mice (**, $p < 0.01$) are indicated.

in limited quantifies to Ag-specific CD4$^+$ T cells via the cognate cell-to-cell interaction, and thus obviating elevated serum levels to elicit therapeutic response as was seen in serum isolated from nasally treated mice. This result is of specific significant in light of well-documented systemic toxicities associated with parentally administrated IL-12. Thus, it is well known that systemic injection of IL-12p70 protein shows efficacy in suppressing tumors in both mice and humans (38–40). However, the results of the initial human clinical trials using systemic treatment with rIL-12 protein were discouraging due to dose-dependent toxicity (41, 42). To overcome the obstacle posed by such toxicity, the IL-12p70 DNA was substituted for the IL-12 protein in a murine study focused on the suppression of tumors (43). The study showed that the IL-12 gene therapy proved to be as efficient as the IL-12p70 protein therapy, while inducing far fewer toxic side effects such as weight loss, splenomegaly, and elevated IFN-γ levels in serum. Similarly, the mice treated nasally with IL-12p70 in the murine intestinal allergy model showed no such side effects in this study (data not shown). It has been shown that systemic administration of IL-12p70 plasmid DNA had therapeutic effects against murine tumors (44–46). The antitumor activity induced by administration of naked IL-12p70 DNA was associated with the augmentation of tumor-specific CTLs (46) or the prevention of tumor angiogenesis (45). In terms of clinical applications for the control of human diseases including cancer and allergies, IL-12p70 DNA treatment may be better than IL-12p70 protein treatment in that it avoids unnecessary toxic side effects while remaining fully capable of controlling disease.

In this study, we were able to show the dynamic chronologic expression and migration of the nasally introduced naked DNA

from at the site of introduction to the distant large intestinal tract. Nasal deposition of the naked IL-12p70 or GFP DNA resulted in the expression of the gene in NALT DCs. Subsequently, DCs specifically expressing the corresponding protein were also noted in CLN, spleen, and the large intestine. Using the IVIS, we noted steady accumulation of green fluorescence in the region associated with the nasal cavity, including the CLN and distant spleen following the nasal naked GFP DNA. Another recent report used IVIS to show that nasal administration of streptococci transfected with a bioluminescent gene induced bioluminescent signals in nasal tissues, particularly in NALT, and that these signals were observed very early and peaked 1 h following nasal treatment (28). It was also demonstrated that M cells located in the NALT epithelium were the primary entry point for streptococci invasion and that luminescence was subsequently observed in the systemic tissues such as the spleen and lymph nodes of the nasally treated mice (28). To this end, our results also showed the early expression of IL-12p70 or GFP gene-expressing DCs in NALT after nasal delivery, though the level of expression was low. In another report, i.v. and intratracheal IFN-γ gene deliveries were examined in mice with allergen-induced airway hyperresponsiveness (47). Although both routes of gene delivery resulted in the expression of IFN-γ, the former route was much more effective in inducing protein expression in the lung.

In our study, nasal deposition of expression plasmid DNA resulted in more protein expression in spleen and intestine than lung. The expression of IL-12p70 in both spleen and intestine may account for the effectiveness of the nasal IL-12p70 treatment. We thus demonstrated previously that the systemic priming in spleen was essential for the development of large intestinal allergic diarrhea following oral challenge (4). Furthermore, it was also shown that systemically primed splenic OVA-specific $CD4^+$ T cells preferentially migrated into the large intestine (48). These findings suggest that some of the pathologic Ag-specific $CD4^+$ T cells originate from the spleen and thus the expression of IL-12p70 in the spleen may provide an opportunity to alter Th2 dominance. Of course, the high expression of IL-12p70 at the site of Th2 hyperresponse will lead to the inhibition of IL-12p40-mediated pathologic Ag-specific $CD4^+$ T cell induction in the large intestine. Although our current findings suggest that nasal gene therapy with IL-12p70 is effective in the prevention of IL-12p40-mediated and Th2 cell-mediated allergic diarrhea, one cannot exclude a possibility that swallowed DNA due to possible spill over of nasally administrated IL-12p70 to esophagus may contribute the expression of the corresponding protein in the intestine. Thus, our ongoing experiments also aim to assess the efficacy of orally administered IL-12p70 DNA in controlling gastrointestinal allergic diseases.

Inasmuch as our recent and separate study showed that the formation of IL-12p40 over IL-12p70 is a major pathologic factor in the creation of the dominant Th2 environment in the large intestine, which is the most conducive to the development of allergic diarrhea (7), an obvious approach was to use the IL-12p35 gene in an attempt to alter the dominant p40 to p70 formation in mice suffering from intestinal allergies. For IL-12p70 protein expression, it is necessary to have the same cell expression in both IL-12p40 and IL-12p35 (10). Thus, it was logical to also test the feasibility of IL-12p35 DNA nasal administration. When the p35 DNA was nasally administered, it did not prevent the development of allergic diarrhea (data not shown). Furthermore, we were unable to find any increase in IL-12p70 expression in any of tissues tested in the p35-treated mice. Although we cannot explain why the p35 treatment failed, it is possible that most of the large intestinal DCs obtained from allergic diarrhea-afflicted mice were already programmed to form the homodimeric p40 and thus cannot be altered even via the exogenous deposition of the p35 gene. In contrast, the delivery of the intact p70 gene may effectively induce naive DCs to express the heterodimeric gene and subsequently to synthesize protein. Furthermore, the introduction of the IL-12p70 gene forcefully induces protein expression even by DCs already committed to the expression of p40 homodimers.

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Disclosures

The authors have no financial conflict of interest.

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A marvel of mucosal T cells and secretory antibodies for the creation of first lines of defense

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Abstract. The mucosal immune system acts as a first line of defense against bacterial and viral infections while also playing a crucial role in the establishment and maintenance of mucosal homeostasis between the host and the outside environment. In addition to epithelial cells and antigen-presenting cells (dendritic cells and macrophages), B and T lymphocytes form a dynamic mucosal network for the induction and regulation of secretory IgA (S-IgA) and cytotoxic T lymphocyte (CTL) responses. This review seeks to shed light on the pathways of induction and regulation of these responses and to elucidate the role they simultaneously play in fending off pathogen invasion and maintaining mucosal homeostasis.

Key words. Secretory IgA; cytotoxic T lymphocyte; $\gamma\delta$IEL; M cell.

Introduction

Intact or injured sites of the respiratory and digestive tracts represent major entry sites for pathogens from the lumen via inhalation and digestion, respectively. Several physical and biological barriers associated with the innate immune system protect these sites from invasion and help to maintain their mucosal homeostasis. The first line of defense is offered by a barrier structure made up of epithelial cells (ECs) joined firmly by tight junction proteins such as occludin, claudins and zonula occludens [1, 2]. In addition, the attachment and penetration of pathogenic microorganisms to mucosal sites are impeded physically by brush-border microvilli and a dense layer of mucin at the apical site of the EC, and biologically by the production of antimicrobial peptides such as a β-defensin [3]. Additionally, Paneth cells secrete biological defensive molecules, including lysozyme, type II phospholipase A2, and α-defensins, in response to bacterial infection [4, 5].

In addition to these physical and innate defense systems, mucosal tissues contain immunocompetent cells for adaptive immunity. As drawn in figure 1, numerous populations of T and B lymphocytes, dendritic cells (DCs), macrophages and granulocytes form a mucosal network known as the common-mucosal immune system (CMIS) [6]. The CMIS links inductive and effector tissues and also plays a key role in the induction of antigen-specific immune responses. The primary CMIS inductive site for orally administered antigen is the Peyer's patch (PP) of the gastrointestinal tract, and for nasally administered antigen, the nasopharynx-associated lymphoid tissue (NALT). Isolated lymphoid follicles (ILFs), which are located throughout the intestine, were recently identified and characterized by Dr Ishikawa's and our groups as an additional inductive site for the digestive tract [7]. These different organized lymphoid structures are generally known as mucosa-associated lymphoid tissues (MALTs). Despite variations in organogenesis [7–9], the MALTs share several interesting features associated with their role as inductive tissues. First, MALTs are overlaid by a follicle-associated epithelium (FAE) containing antigen-sampling M (microfold) cells, allowing selective transport of antigens to underlying antigen-presenting cells (APCs) in the inductive tissues. Second, they consist of an assembly of naïve B cells, often including a germinal center, supported by a network of follicular DCs and $CD4^+$ T cells. Upon activation by antigens, B and T cells emi-

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grate from the inductive tissue, circulate through the bloodstream and home to distant mucosal compartments, especially the lamina propria regions of the intestinal, respiratory and reproductive tracts. The diffused lamina propria region and the epithelium have been considered effector sites, where the MALT-originated, immunoglobulin A(IgA)-committed B cells differentiate into IgA plasma cells for the secretion of dimeric or polymeric forms of IgA. Effector tissues contain a variety of T cell subsets, which exhibit helper, regulatory and cytolytic activities and so help to regulate protective immunity at the mucosal surface. Additionally, a unique T cell population, known as intraepithelial lymphocytes (IELs), is located between ECs. IELs have been shown to possess a cytotoxic function against pathogen-infected cells.

Accumulating evidence suggests that the mucosal immune system not only protects from bacterial or viral infection, but also aids in the maintenance of mucosal homeostasis between the host and outside environmental antigens. This review focuses on the cellular and molecular mucosal network for the induction and regulation of mucosal antibody and T cell responses.

Antigen uptake, processing and presentation at mucosa

Following oral or nasal administration, foreign antigens follow the sequence of uptake, transport, processing and presentation at the inductive tissues, such as PPs and ILFs, or NALT, respectively. For selective uptake of antigens, the epithelium covering the inductive tissues develops FAE consisting of professional antigen-sampling epithelial cells, known as M cells (fig. 1) [10]. M cells are distinguished from the surrounding ECs by some unique histological and biochemical features, including the lack of brush borders, a limited mucus production and a lower level of degradation activity [11, 12]. Conversely, M cells exhibit a high transcytosis activity and are characterized by a unique pocket structure, where numerous kinds of immunocompetent cells, including DCs, macrophages, T cells and B cells, are located [12, 13]. These unique biological characteristics allow M cells to take up antigens from the lumen into their pocket structures and so selectively transport them to APCs. Hence, mice who lacked PPs because the tissue genesis cytokine cascade of interleukin 7 receptor (IL-7R) and lymphotoxin β receptor (LTβR) had been disrupted showed alternative and/or less ability to take up bacteria and particulate antigens from the intestinal lumen [14, 15]. Once antigens have been taken up from the lumen by M cells and transferred to the M cell pocket, APCs, including DCs, can process the antigens and migrate into the interfollicular areas of the PP, where they present epitopes to T cells [13, 16] (see Iwasaki's review, this issue).

Although FAE-associated M cells at inductive tissues (e.g., PP) are thought to be a major gateway for antigen uptake from the lumen for the initiation of antigen-specific immune responses, an alternative induction pathway may exist for the mucosal immune system, since antigen-specific immune responses have been induced in PP-null mice following oral immunization [15, 17]. At least three different scenarios have been offered regarding this alternative pathway. First, our group has recently identified M cells on intestinal villous epithelium (villous M cells) that is not in the vicinity of PP [14]. Intestinal villous M cells are developed in various PP/ILF-null mice and are capable of taking up bacterial antigens. Thus, villous M cells represent one novel gateway for antigen uptake in the intestine, as well as a possible new site for invasion of pathogenic microorganisms. Second, an M cell-independent pathway is operated by mucosal DCs, which express tight junction-associated proteins (e.g., occludin, claudin 1 and zonula occludens 1) and thus are capable of extending their dendrites between ECs [18]. On a similar note, CD18-expressing phagocytes have been reported to be involved in an M cell-independent pathway for bacterial invasion [19]. By protruding dendrites into the lumen, mucosal DCs located between ECs are able to sample gut antigens and then present them to T and/or B cells [18]. The third pathway for antigen uptake are ECs themselves. Some evidence has shown that ECs could process and then present antigens to T cells via major histocompatibility complex (MHC) class I as well as class II molecules [20]. In addition to sampling a wide variety of foreign antigens, the mucosal immune system must contend with the high number of apoptotic ECs, which result from the rapid turnover of epithelium. Although most of these apoptotic ECs are ceded by the epithelium to the lumen, some of them are potentially immunogenic and can be transported to T cell areas of mesenteric lyrrph nodes (MLNs) by mucosal DCs [21].

Like the intestinal tract, NALT and bronchus-associated lymphoid tissue (BALT) of the respiratory tract have been shown to contain M cells along their epithelium for antigen sampling [22, 23]. Thus, nasal immunization has been shown to be effective for the induction of Ag-specific immune responses. Our previous study showed that nasally administered fusogenic liposome-containing vaccine antigens were effectively taken up by M cells located on the NALT epithelium [24]. The efficacy of NALT-mediated immunity was further demonstrated by the use of σ-1 protein-coupled DNA vaccine [25]. These NALT- and BALT-associated M cells were of course also entry sites for pathogens [26]. Currently, far less is known about the antigen uptake pathways for the respiratory tract than for the intestinal tract, and indeed, it is not yet known whether alternative gateways even exist in the respiratory tract.

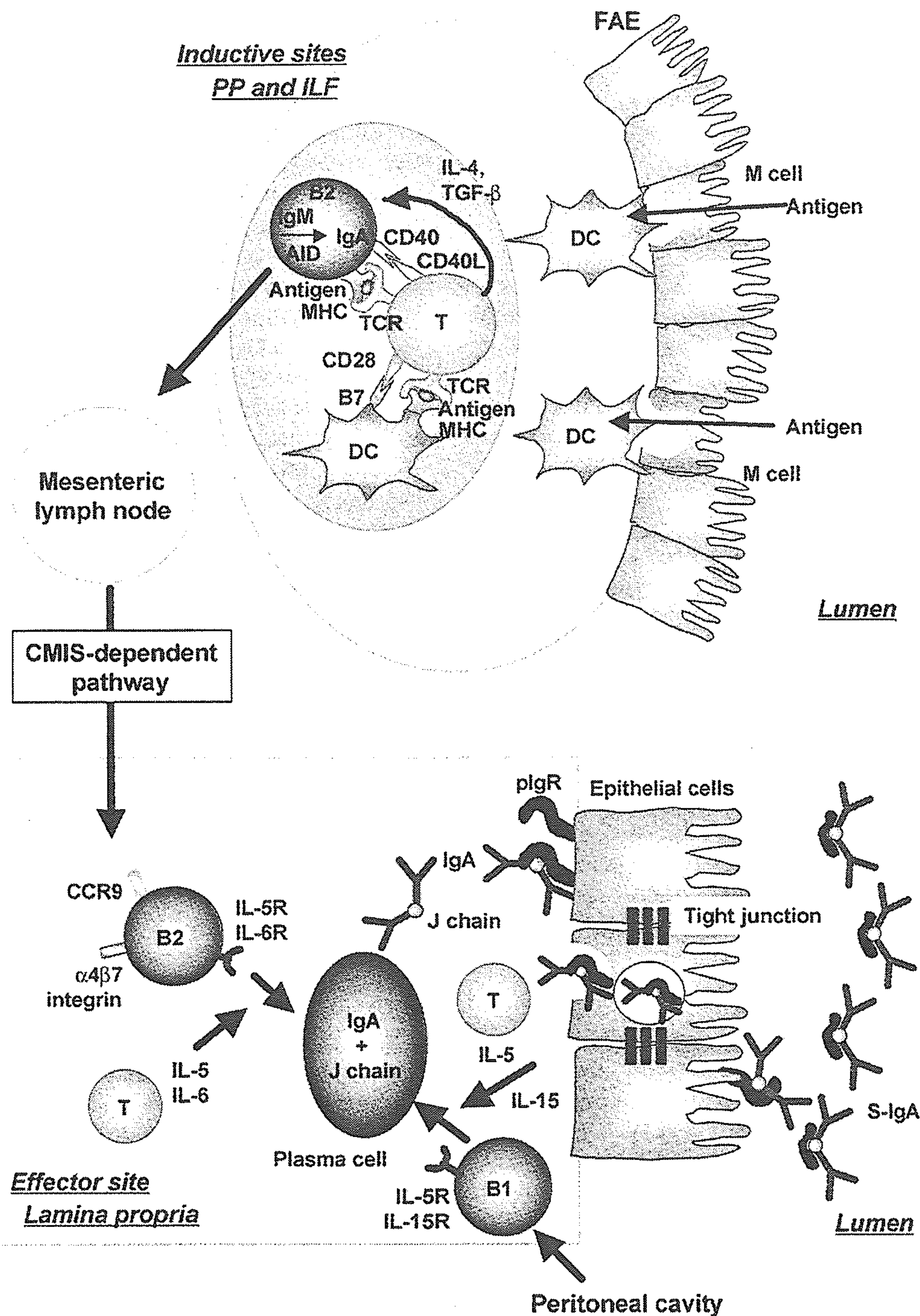

Figure 1. Multistep model to generate secretory IgA (S-IgA) responses in the intestine. In the common-mucosal immune system (CMIS)-dependent pathway, naïve B cells, also known as B2 lymphocytes, are stimulated in a T cell-dependent manner within Peyer's patches (PPs) and isolated lymphoid follicles (ILFs), where several factors (e.g., CD40 and cytokines) induce class-switch recombination from IgM to IgA. The IgA-committed B cells exit through the lymph and home through the thoracic duct and peripheral blood to mucosal effector sites such as the lamina propria of the gut. Intestinal homing is mediated by adhesion molecules and chemokine-mediated interaction. At the effector site, IgA-committed B2 cells receive several signals, resulting in the generation of plasma cells. The plasma cells produce IgA as a dimer joined by a J-chain, and the dimeric form of IgA binds to poly Ig receptor (pIgR) on epithelial cells, is transported across the epithelium and is released in the intestinal lumen as S-IgA, which acts as a first line of defense against pathogens and maintains mucosal homeostasis. Another lineage of B cells, B1 cells, are derived from the peritoneal cavity and act as the other source of intestinal secretory IgA. AID, activation-induced cytidine deaminase; APC, antigen-presenting cell; CCR9, CC-type chemokine receptor; FAE, follicle-associated epithelium; IL-4, interleukin 4; MHC, major histocompatibility complex; TCR, T cell receptor; TGF-β, transforming growth factor β.

Unique B cell network for mucosal IgA production

To provide a first line of defense at the mucosal surfaces of the aerodigestive and reproductive tracts, the mucosal immune system selectively uses IgA as a major isotype of antibody for the formation of secretory IgA (S-IgA). In order to induce the secretory form of IgA, mucosal B cells have to undergo two major molecular and cellular events in the organized inductive and diffused effector tissues interconnected by the CMIS. In PP, for example, a μ to α class switch recombination (CSR) occurs under the influence of transforming growth factor β (TGF-β) and antigen stimulation (fig. 1) [27]. After IgA isotype switching, IgA-committed B cells leave PP, migrate to distant effector tissues such as the intestinal lamina propria, and then, under the influence of IgA-enhancing cytokines such as IL-5 and IL-6, enter the terminal differentiation process to become IgA plasma cells (fig. 1) [28, 29]. Dimeric or polymeric forms of IgA produced by these plasma cells then interact with the poly Ig receptor (pIgR) expressed on the basal membrane of ECs and are transported to the apical membrane, where they form S-IgA [30].

As shown in figure 1, at least three different types of cells have to harmoniously form a mucosal internet for the induction of S-IgA at the diffused effector site: (i) IgA-committed B cells originated in PP, (ii) T helper 2 (Th2)-type cells producing IgA-enhancing cytokines (IL-5 and IL-6) and (iii) ECs expressing pIgR. Once in place, S-IgA antibodies also play a key role in establishing a cohabitant environment with commensal microorganisms in the intestinal tract [31].

Contribution of conventional B cells (B2 cells) to IgA responses

In the mucosal immune system, IgA is produced by two subsets of B cells, namely B1 and B2 cells [32]. For example, the murine intestinal lamina propria region contains equal numbers of B1 and B2 cells committed for IgA [33]. When MALTs such as PPs, ILFs and NALT were examined, the inductive tissues were found to contain numerous B2 cells originating from bone marrow-derived precursor cells. IgA-committed B cell development in these inductive organs seems to depend on antigenic stimulation of germinal centers, where B cells interact with both antigens trapped on follicular DCs and local CD4$^+$ T cells to induce the μ to α isotype CSR and somatic hyper mutation [34]. Similarly, NALT revealed the presence of germinal centers and μ to α isotype switching after antigen stimulation [35, 36]. The CSR in PPs is mediated by the CD40/CD40 ligand and by TGF-β [27, 37]. Also essential to CSR is the interaction between the inducible co-stimulator (ICOS), which is expressed on activated Th cells, and its ligand, ICOS-L, which is constitutively expressed on B cells [38]. Following stimulation by these molecules, multiple transcription factors induce the CSR. For example, an element for binding to Smad, which is a TGF-β-induced transcriptional factor, is located in the $C_{H\alpha}$ promoter region, and this pathway co-operates with acute myeloid leukemia (AML) transcription factors [39]. The discovery of activation-induced cytidine deaminase (AID) has led to a dramatic breakthrough in our understanding of the CSR and somatic hyper mutation in germinal centers [40]. AID is specifically expressed in germinal center B cells and may also exhibit an RNA- or DNA-editing cytidine deaminase activity. Surprisingly, the expression of AID alone induced CSR on artificial substrates in fibroblasts, indicating that AID per se can induce CSR [41, 42]. However, the molecular mechanism by which AID initiates this reaction in B cells and recognizes the specific immunoglobulin loci has yet to be clarified. By AID-mediated CSR together with TGF-β and antigen signaling, IgM$^+$B220$^+$B cells undergo μ-to-α gene rearrangement via the formation of an Iα-Cμ circular transcript. The expression of an Iμ-Cα transcript indicates the completion of the isotype switching for the generation of IgM$^-$IgA$^+$B220$^+$ B cells [40].

The post-switched IgA$^+$ B cells exit PP and NALT and migrate to MLNs and cervical lymph nodes, respectively, where they proliferate further and differentiate into B blasts (fig. 1). The B blasts migrate preferentially into the mucosal effector tissues (e.g., the gut lamina propria and the nasal passage) through the thoracic duct and blood circulation. Accumulating evidence suggests that the IgA$^+$ B cell trafficking to the gut lamina propria is facilitated by changes in the expression of adhesion molecules and chemokine receptors. IgA$^+$ B cells produce $\alpha4\beta7$ integrin that interacts specifically with mucosal vascular addressin cell adhesion molecule 1 (MAdCAM-1) expressed by blood vessels in the lamina propria [43]. Later, CCR9 is selectively expressed on IgA-, but not IgM- or IgG-, committed B cells [44]. The ligand of CCR9 is CCL25, also known as thymus-expressed chemokine (TECK), which is produced dominantly by the intestinal epithelium, determining the selective homing of IgA$^+$ B cells into the intestinal lamina propria [44]. Although the detailed mechanism remains to be investigated, it has been reported that the migration of IgA$^+$ cells from NALT to the nasal passage might be due to the expression of mucosae-associated epithelial chemokine (MEC)/CCL28 [45].

Role of B1 cells in mucosal IgA responses

The peritoneal cavity may be another source of intestinal B cells (fig. 1) [46]. Early research demonstrated that peritoneal cavity-derived B1 cells differ from conventional B2 cells in origin, surface marker expression (e.g.,

B220, IgM, IgD, CD5 and Mac-1) and growth properties [32, 47]. Of note, B1 cells exhibit different V_H repertoires and Ig specificities, and they are thought to be specialized in responding to T cell-independent antigens conserved on common pathogens, such as DNA and phosphatidylcholine. In contrast, the response of B2 cells to most protein antigens requires activation by DCs and Th cells [48–51]. Consistent with this notion, IgA production from B1 cells was noted in MHC class II-deficient mice as well as in T cell receptor (TCR) β and δ chain-deficient mice [52, 53].

B1 and B2 responses have distinct cytokine requirements. Our previous studies demonstrated that, like IL-5, a well-known IgA-enhancing cytokine, IL-15, promotes proliferation and differentiation into IgA-producing cells of B1 but not of B2 (fig. 1) [33, 54]. Indeed, a disruption in the IL-5 receptor gene or treatment with anti-IL-15 antibody resulted in the severe paucity of B1 cells at effector sites such as the intestinal lamina propria and nasal passage but did not affect B2 cell number [33, 54]. A previous report proposed that the homing pathway of B1 cells to the peritoneal cavity depended on the CXCL13 (also known as B lymphocyte chemoattractant, BCL) produced by peritoneal macrophages [55]. Another study using alymphoplasia (*aly*) mice that carried a point mutation in the nuclear factor κB-inducing kinase (NIK) demonstrated a complete absence of B cell population in the intestinal lamina propria of *aly* mice, and a defective migration of peritoneal cells to intestinal effector compartments [56, 57]. These data imply that the NIK-mediated pathway is involved in the B1 cell mucosal migration, which might be dependent on specific but not yet identified chemokine receptors. We previously reported that B1 cells existed in the nasal passages [33], but the actual molecular machinery of B1 cell migration into the nasal passages remains an open question.

Recent results obtained from $AID^{-/-}$ mice suggest an alternative pathway for CSR induction at diffused effector sites (e.g., the intestinal lamina propria), one that does not involve the organized inductive tissues, such as PPs [58]. In this study, stromal cell-derived TGF-β in the intestinal lamina propria was shown to trigger IgM^+B220^+ B cells to undergo μ-to-α CSR and to become IgA-switched B cells. Thus, the intestinal lamina propria might be able to act as both inductive and effector sites. However, the recent discovery of ILFs that are equipped like mucosal inductive sites challenges this hypothesis [7]. Because $AID^{-/-}$ mice were shown to exhibit numerous hyperplasia of ILFs, it is possible that IgA-switching of B cells was triggered within ILFs [59]. In accordance with these observations, the expression of a series of IgA isotype CSR molecules, including AID, the Iα-Cμ circular transcript and the Iμ-Cα transcript, were detected only in the organized tissues (e.g., PPs, ILFs and NALT), and not in diffused effector tissues [34]. Although this finding directly demonstrates that organized lymphoid structures are key to CSR in B2 cells, it does not rule out the possibility that IgA-specific CSR for B1 cells may not occur in the organized lymphoid tissues. In this regard, a majority of B cells belonging to the organized MALT were found to be of B2 lineage, and the diffused lamina propria regions of the aerodigestive tract and peritoneal cavity were observed to be rich in B1 cells [32].

Formation and transport of S-IgA by epithelial cells via pIgR

Two essential steps for the production of IgA antibody in the lumen and secretions have already been outlined: (i) the switching of B cells to IgA at inductive sites (e.g., PPs, ILFs, and NALT) and (ii) the migration of those IgA-committed B cells to effector sites (the intestinal lamina propria and nasal passages). Additionally, IgA production requires the expression of the joining chain (J-chain) and pIgR (fig. 1). The J-chain gene expressed in B cells is a small polypeptide that regulates polymer formation of IgA and IgM, but not that of other types of Ig [60, 61]. J-chain synthesis is tightly regulated at the transcription level. Transcription is induced by antigen recognition, which is dependent on IL-2-induced chromatin remodeling of the J-chain locus and interaction of specific transcription factors with the J-chain promoter [62, 63]. It is interesting to note that the expression of the J-chain has been identified in invertebrates (Mollusca, Annelida, Arthropoda, Echinodermata and Holothuroidea) that lack B cell development in the phylogenic tree [64]. Since mucosa-oriented, IgA-committed B cells produce dimeric or polymeric forms of IgA in the effector tissues, while serum IgA is generally a monomeric form, the expression of the J-chain is essential for the formation of S-IgA.

Similarly, pIgR expressed by the basal membrane of ECs is a prerequisite for the formation and transport of S-IgA [30]. Dimeric or polymeric IgA containing the J-chain shows a high affinity for pIgR, thereby accelerating the internalization and transport of the complex to the apical site via transcytosis [65]. Thus, elevated serum IgA and decreased fecal IgA levels were observed in J-chain knockout mice due to the decreased affinity for pIgR [66, 67]. At the apical site, S-IgA antibodies are produced by endoproteolytic cleavage of the pIgR domain to become secretory components. As in J-chain knockout mice, disruption of the pIgR gene results in a defective transport of IgA into the intestinal lumen and, thus, in the reduction of IgA antibodies in the gut secretions, despite the presence of high numbers of IgA plasma cells in the intestinal lamina propria [68, 69]. The high levels of pIgR constitutively expressed by ECs are regulated at the transcription level by specific transcriptional factors (USF-1 and USF-2) [70, 71]. Additionally, the constitutive expression of pIgR is further upregulated by a group of Th1, Th2 and in-

flammatory cytokines [e.g., interferon, (IFN-γ), IL-4 and tumor recrosis factor (TNF)], indicating that pIgR expression is also involved in increased local IgA production during the course of mucosal injury, such as infection [72]. This evidence further emphasizes the unique mechanism of S-IgA production, whereby PP- or NALT-originated Th1 and Th2 cells as well as IgA-committed B cells form a mucosal intranet together with ECs.

IgA as a mucosal guarding and symbiosis molecule

Several studies have shown that S-IgA is capable of neutralizing viruses and bacteria in cultures and of protecting the host from pathogenic microorganisms in vivo. For example, IgA derived from the saliva of mice nasally immunized with fimbriae prevented the adhesion of *Porphyromonas gingivalis* to ECs, which resulted in the subsequent inhibition of inflammatory cytokine produc-

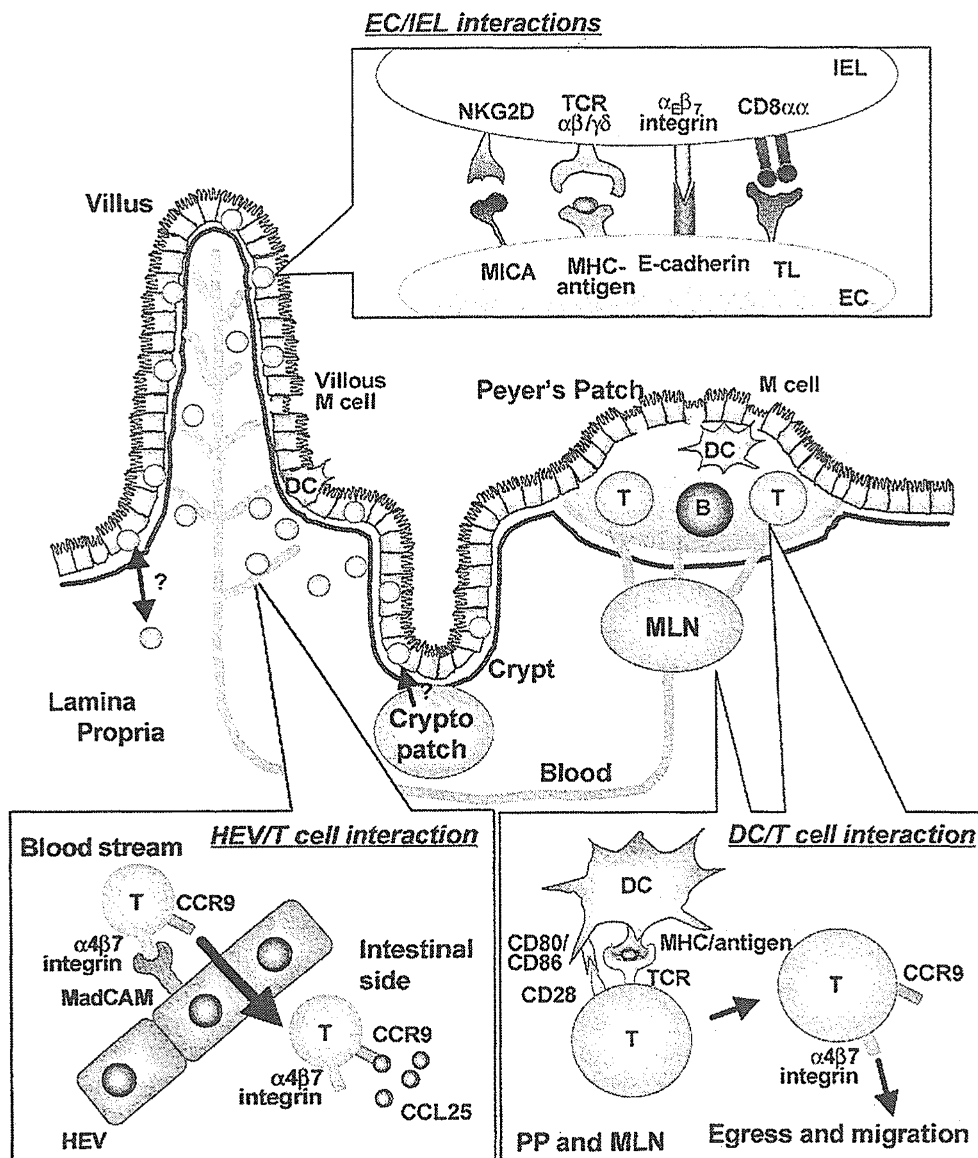

Figure 2. Induction pathway of intestinal T cells. Dendritic cells (DCs) in Peyer's patches (PPs) take up antigens transported through M cells and present them to T cells in an MHC-dependent manner. The activated T cells express $\alpha4\beta7$ integrin and CCR9, allowing them to migrate to mucosal effective sites, such as the lamina propria. $\alpha4\beta7$ interacts with intestinal high endothelial venules (HEV) expressing mucosal vascular addressin cell adhesion molecule 1 (MAdCAM-1), and CCR9 allows specific migration to the intestinal epithelial cell (EC)-chemokine, CCL25. These migrating lamina propria T cells exhibit cytotoxic activity primarily in an MHC-restricted manner. Another population of intestinal T cells is formed by intraepithelial lymphocytes (IELs), which are located between intestinal ECs. One population of IELs originates in the lamina propria, while the others are derived from cryptopatches. They express several molecules interacting with intestinal EC (e.g., TCR, NKG2D and CD8$\alpha\alpha$) and so act as a bridge between innate and acquired immunity. CCR9, CC- type chemokine receptor; MHC, major histocompatibility complex; TCR, T cell receptor; MLN, mesenteric lymph node.

tion [73]. S-IgA prevents mucosal infection with viruses and neutralized microbial toxins [74–76]. Additionally, the J-chain- and pIgR-mediated transport machinery through ECs is an effective physical system not only for the delivery of dimeric/polymeric IgA from the basolateral surface to the lumen, but also for the creation of a one-way transport pathway that blocks antigens from penetrating the body [77]. Furthermore, the mucosal IgA plays a pivotal role in impairing pathogen penetration by neutralizing pathogens during transcytosis by ECs, especially within the apical recycling endosome [78, 79].

The mucosal immune system acts as more than just the first line of defense against pathogenic microorganisms: the mucosal epithelium, especially the intestinal tract, serves as the means by which nonpathogenic commensal bacteria cross-talk with the immune system to foster the development and maintenance of the mucosal IgA production pathway [31]. For example, the unusually small and flattened PPs of germ-free mice, which also showed a paucity of IgA-producing B cells, matured normally once commensal bacteria were introduced, and an increase in the number of IgA plasma cells was seen as well [80]. It has also been reported that disruption of the AID gene resulted in ILF hyperplasia and a high degree of germinal-center formation, as discussed above [59]. These observations were associated with an increase in anaerobic flora and with the antibiotic treatment meant to destroy them, indicating that ILF development was also regulated by the interaction with commensal bacteria [59]. Further, most recent studies suggest that secretions of intestinal IgA are a key factor in the regulation of commensal microflora [81]. Thus, an altered bacterial flora characterized by an aberrant increase in segmented filamentous bacteria was observed in the intestinal tract of IgA-deficient mice.

Since both commensal and pathogenic bacteria express conserved molecular features of microbes (so-called pathogen-associated molecular patterns; PAMPs) necessary for stimulation of innate immunity and eventually, of acquired immunity, one obvious question would be why commensal bacteria do not induce inflammatory responses [82]. Several recent investigations offer plausible explanations for this intriguing interaction between intestinal commensal microflora and the host immune system. First, induction of S-IgA responses against commensal bacteria is derived from T cell-independent B1 cells, while the S-IgA response against pathogen-derived epitopes required antigen-specific T cell help presumably belonging to B2 cells [53]. As mentioned above, the T cell-independent IgA antibodies originating from B1 cells possessed reactivity to conserved bacterial products, which resulted in the undiscriminating blockade of commensal bacteria attachment to mucosal surfaces. It was further demonstrated that intestinal macrophages rapidly kill commensal bacteria, while intestinal DCs retain small numbers of live commensal organisms and migrate only into MLNs, and do not stray beyond them. This function ensures a commensal bacteria-specific IgA response that is specifically produced at gut mucosa, but not at systemic compartments [83]. In contrast, pathogenic *Salmonella enterica* serovar Typhimurium are detected in both DCs and macrophages from the MLNs as well as the spleen, which allows bacteria to persist longer and induce more pathogenic effects at both the local and the systemic compartments [83].

Second, it was reported that avirulent *Salmonella* were capable of disrupting inflammatory cytokine synthesis from intestinal ECs by inhibiting ubiquitin-mediated degradation of IκB, leading to the blocking of nuclear factor kappa B (NF-κB)-mediated transactivation of the inflammatory gene [84]. The third possible mechanism of inhibiting inflammatory response at mucosal sites is the generation of tolerance to subsequent stimulation from bacterial products. Otte et al. reported that repeated contact with bacterial components (e.g., lipopolysaccharide) induced downregulation of Toll-like receptors (TLRs) on the surface of ECs, and inhibition of intracellular signaling though TLRs by upregulation of Tollip [85]. These data suggest mechanisms by which inflammatory responses induced by commensal bacteria are inhibited to create and maintain an immunological silence at the intestinal mucosa. However, the exact means by which the mucosal immune system cleverly distinguishes commensal from pathogenic bacteria remains to be clarified.

Cytotoxic functions of mucosal T cells as a cellular barrier

The mucosal immune system does not rely solely on S-IgA-mediated humoral immunity to provide an effective first line of defense. Since the mucosal immune system is continuously facing harsh environmental stress, and because a rupture of this first defense line can lead to serious disease, the system must be equipped with multiple layers of protective immunity. The experiments using IgA$^{-/-}$ mice pointed out that compensatory mechanisms other than S-IgA might be responsible for protection from viral or bacterial infection [86, 87]. In this respect, there is substantial evidence that mucosal T cells harbor cytolytic activity and are thus capable of killing cells infected with virus or bacteria [88–90]. Like IgA-producing B cells, large numbers of mucosal T cells, including both CD4^{+} and CD8^{+} T cells, are situated in the intestinal lamina propria for the delivery of protective functions, including cytotoxicity (fig. 2). Moreover, a unique mucosal T cell population exists in the intestinal epithelium. Next we focus on the cytotoxic effects of intestinal T cells as a major provider of cell-mediated immunity at the mucosal surface.

Intestinal lamina propria T cells with cytotoxic function

Intestinal lamina propria T cells are largely composed of $\alpha\beta$TCR lymphocytes expressing either $CD4^+$ or $CD8\alpha\beta^+$. In addition, $CD4^+$ and $CD8^+$ T cells distribute in different areas of intestinal tissue sections, as observed by immunostaining. $CD4^+$ T cells are largely located in the lamina propria, while $CD8^+$ T cells reside along the epithelium [91]. Although we have no explanation for this histological segregation, the most obvious interpretation would be that mucosal $CD8^+$ T cells with cytotoxic activity are situated close to the entry sites for pathogenic invaders to ensure the immediate elimination of the pathogens and infected ECs. Most of these mucosal T cells are thought to derive from the CMIS-dependent induction pathway. Recent studies have demonstrated that PP- and MLN-derived DCs determine the gut tropism of lamina propria lymphocytes (LPLs) by the induction of high levels of $\alpha_4\beta_7$ integrin and CCR9 expression, resulting in selective migration to the small intestine (fig. 2) [92–94]. Thus, oral antigen-educated mucosal T cells originating from PP migrate to distant effector sites by obtaining the mucosal trafficking molecules (e.g., $\alpha_4\beta_7$ and CCR9) via the CMIS.

At the periphery, $CD8^+$ T cells recognize the antigens derived from the cytosolic antigen as a complex with MHC class I molecules [95, 96]. Heterodimeric CD8 ($CD8\alpha\beta$) T cells are involved in the subsequent killing of target or virus-infected cells [97, 98]. Thus, the α chain of the CD8 molecule associates with MHC class I molecules, and the β chain acts as a TCR co-receptor for the recognition of cytotoxic T cell epitope antigens. Consistent with the expression of $CD8\alpha\beta$ on LPL T cells, these $CD8^+$ LPLs present cytotoxic activities against MHC class I-restricted antigens originating from various kinds of intracellular antigens [99, 100]. Similar to peripheral $CD8^+$ T cells, $CD8\alpha\beta$ LPLs express the pore-forming protein perforin and cytolytic granules containing granzyme proteases to exhibit cytotoxic activity against pathogenic cells [101].

Intraepithelial T cells, an anonymous cell population, are important as a first line of defense

An additional unique feature of the mucosal immune system is the presence of T cells in the intestinal epithelium known as IELs (fig. 2). IELs are located at every four to nine ECs and are mainly composed of heterogeneous groups of T cells based on the usage of TCRs as well as CD4 and CD8 [102]. LPL $CD8^+$ T cells are exclusively $\alpha\beta$TCR-positive cells with heterodimeric $CD8\alpha\beta$ (70% of $CD8^+$ LPLs are $\alpha\beta$TCR-positive, and 15% are $\gamma\delta$TCR-positive). In contrast, few $\alpha\beta$TCR $CD8\alpha\beta$ *T* cells are found in IELs (about 10%), and most $CD8^+$ IELs are either $\gamma\delta$TCR- or $\alpha\beta$TCR-positive cells with homodimeric $CD8\alpha\alpha$ (about 50%) [103]. Similar to $CD8^+$ T cells at the periphery and lamina propria, $CD8\alpha\beta$ IELs develop in the thymus and migrate specifically into the mucosal compartments by the selective expression of CCR9 and $\alpha_4\beta_7$ integrin [104]. However, CCR9-deficient mice exhibited a modest decrease of IELs, indicating that other chemokines might be involved in IEL migration [105, 106]. Thus, several studies suggest the contribution of other chemokines and chemokine receptor pathways as mediators of gut tropism [107–109]. Intriguingly, TCR clonotypes the $CD8\alpha\beta$ $\alpha\beta$TCR IELs were almost identical to those of the $CD8\alpha\beta$ LPLs and thoracic duct $CD8^+$ T cells [110], implying that $CD8\alpha\beta$ IELs were primed to antigen in PPs and migrated into the intestinal region via the CMIS-dependent pathway. The finding that DCs in PPs and MLNs induce $\alpha_4\beta_7$ integrin and CCR9 expression on IELs as well as LPLs lends support to this theory (fig. 2) [93, 94]. Under the influence of TGF-β, a cytokine produced by ECs and numerous activated lymphocytes and macrophages, inhibition of $\alpha4$ expression occurs simultaneously with the induction of αE, leading to the expression of $\alpha E\beta 7$, a hallmark of IELs for the cell-to-cell interaction with E-cadherin [111]. The presence of a two-way communication of T cells between the epithelial region and the lamina propria was also predicted [112], but the exact governing of it remains to be elucidated. At the least, these findings suggested that IELs provide an additional layer of defense over and above IgA-committed B cell-mediated humoral immunity.

In contrast to so-called thymus-dependent $CD8\alpha\beta$ IELs, at least some populations of $CD8\alpha\alpha$ IELs, such as $\gamma\delta$TCR T cells, are thought to be thymus-independent and thus develope in gut-associated cryptopatches (CPs) [113]. CP lymphocytes do not originate from the thymus, because nude mice contain CPs of identical size, structure, number and cell phenotype with normal mice. In contrast, CPs are absent in mice that have a defective cytokine-receptor γ chain gene and that also lack $CD8\alpha\alpha$ $\alpha\beta$TCR and $\gamma\delta$TCR IEL fraction, but contain thymus-dependent $CD4^+$ and $CD8^+$ $\alpha\beta$TCR IELs [114, 115]. The main population of CP cells displayed a c-kit, IL-7R and CD44-positive, but lineage markers (CD3, B220, Mac-1, Gr-1 and TER-119)-negative lympho-homopoietic stem cell phenotype [116]. Consistent with the IL-7R expression on CP lymphocytes, gut epithelium-derived IL-7 has been shown to be important in the induction of $CD8\alpha\alpha$ IEL T cells and CP maturation, since IL-$7^{-/-}$ mice do not have $\gamma\delta$TCR IELs and CPs. The introduction of IL-7 into IL-7-deficient mice results in the recovery of $\gamma\delta$TCR IELs and CPs [117, 118]. In vivo studies demonstrated that CPs had an ability to generate both $\alpha\beta$TCR and $\gamma\delta$TCR IELs without the influence of the thymus [115, 116]. However, other studies questioned the thymus-independent nature of IELs and implied that $CD8\alpha\alpha$ $\alpha\beta$TCR and $\gamma\delta$TCR IELs developed at the thymus [119, 120]. The most recent study has demonstrated that all of

the intestinal T cells expressing $\alpha\beta$TCR, regardless of co-expression of heterodimeric or homodimeric CD8, are progeny of $CD4^+CD8^+$ thymocytes [121]. Although the issue of thymus-independent development of IELs remains controversial, CPs are still considered key members of the gut-associated lymphoid tissue network and at least serve as one immunological nest for the development of some populations of intestinal T cells. Some researchers have reported the expression of CCR6 by CP lymphocytes and have noted that the expression of E-cadherin on ECs could be a tethering molecule for IELs, helping them migrate to and reside in the intestinal epithelium [111, 122]. However, the molecular sequence mechanism for IEL egress from CP and migration into the EC compartment is still obscure.

Another key difference between CD8$\alpha\beta$ IEL T cells and CD8$\alpha\alpha$ IEL T cells was revealed using MHC class I-deficient mice. The experiments demonstrated that CD8$\alpha\beta$ $\alpha\beta$TCR IELs were dependent on MHC class I, while CD8$\alpha\alpha$ IELs were not [123, 124]. Thus, CD8$\alpha\beta$ $\alpha\beta$TCR IELs showed cytotoxic activity against nonself cytoplasmic antigens in an MHC class I-restricted manner, whereas CD8$\alpha\alpha$ IELs exerted extremely low cytotoxic activity against antigens associated with MHC class I [89]. These observations raise the obvious question about the nature of antigens and presentation molecules interacting with CD8$\alpha\alpha$ IELs. Mice lacking the MHC-regulating molecules β2-microglobulin (β2m) and transporter associated with antigen processing (TAP) shed new light on the process of antigen presentation and recognition. The number of CD8$\alpha\alpha$ IELs were markedly reduced in β2m-deficient mice compared with TAP-deficient mice, implying that nonclassical MHC molecules might contribute to antigen presentation to subpopulations of IELs [125, 126]. In support of this hypothesis, intestinal ECs express several nonclassical MHC molecules, including thymus leukemia antigen (TL), Qa-1, Qa-2, CD1 and MHC class I-related molecules (MICA and MICB) (fig. 2) [127]. Some populations of these nonclassical MHC molecules (TL and MICA) are capable of interacting with their ligand without antigen, but the other populations present lipid antigen (e.g., CD1). As expected, Qa-$2^{-/-}$ mice contained a few CD8$\alpha\alpha$ IELs, and the mice were susceptible to parasitic infections [128, 129]. The other molecules interacting with $\gamma\delta$TCR IELs are MICA, capable of activating $V\gamma1V\delta1^+$ IELs (fig. 2) [130]. Additionally, MICA interacts with an 'activating type' of natural killer (NK) receptor, NKG2D [130, 131], and CD8$\alpha\alpha$ $\gamma\delta$TCR IELs display both T and NK cell markers and cytotoxic feasibility [132]. Since MICA is not expressed constitutively on normal ECs but is induced by bacterial or viral infection [133, 134], it has been thought that CD8$\alpha\alpha$ $\gamma\delta$TCR IELs recognize infected ECs via MICA, hampering systemic dissemination of virus or bacteria. These responses mediated by nonclassical MHC molecules were induced promptly after infection; hence, it has been proposed that $\gamma\delta$TCR IELs provided a bridge between rapid innate responses and slower acquired immune responses [135]. In support of this idea, a recent study using a *Listeria* infection model demonstrated that MHC class I-restricted memory T cells regulate H2-M3 (one of the nonclassical MHC molecules)-restricted memory T cells by limiting antigen presentation by DCs, thereby preventing the contribution of H2-M3-restricted protective mechanisms at late stages of infection [136].

In addition to the interaction between nonclassical MHC and TCR or NKG2D, CD8$\alpha\alpha$ itself interacts with TL, a β2m-dependent nonclassical MHC class I molecule [137]. TL is constitutively expressed by the ECs of the small intestine and, like the other nonclassical MHC molecules, does not present peptide antigens [138, 139]. Functional studies have demonstrated that the interaction of TL with CD8$\alpha\alpha$ on IELs promotes the production of cytokines but does not induce their proliferation and cytotoxic response [137]. These unique functions seem to lead to IEL-mediated protection without destruction of the EC layer. Regardless of the origin of IELs (thymic versus extra-thymic development), these gut-oriented T cells seem to be key players in establishing a surface barrier-associated immunological flow of innate and acquired immunity.

Concluding remarks

This review has been aimed at elucidating the functional aspects of the molecular and cellular regulation of mucosal B- and T-cell-mediated S-IgA and cell-mediated immunity as a first line of defense against invading pathogens. The mucosa-associated immunocompetent cells, including mucosal ECs, DCs, macrophages, Th1, Th2, CTL and IgA-committed B cells, harmoniously interact in both innate and acquired immunity at mucosal sites, thereby playing an important role in the early and late phases of pathogenic microorganism invasion, respectively. These facts have led to considerable efforts at developing a mucosal vaccine using mucosal adjuvant and/or mucosal delivery systems that could effectively upregulate the induction of protective immunity at the initial entry of pathogens via the aerodigestive and reproductive tracts [140, 141]. In addition to protecting against microorganism invasion at mucosa, the mucosal immune system is capable of inducing and regulating a mucosal homeostasis between host and outside environments. Thus, disruption of the system leads to the development of mucosal immune diseases such as inflammatory bowel disease, asthma and food allergies [142]. A comprehensive molecular and cellular understanding of the mucosal immune system will facilitate novel