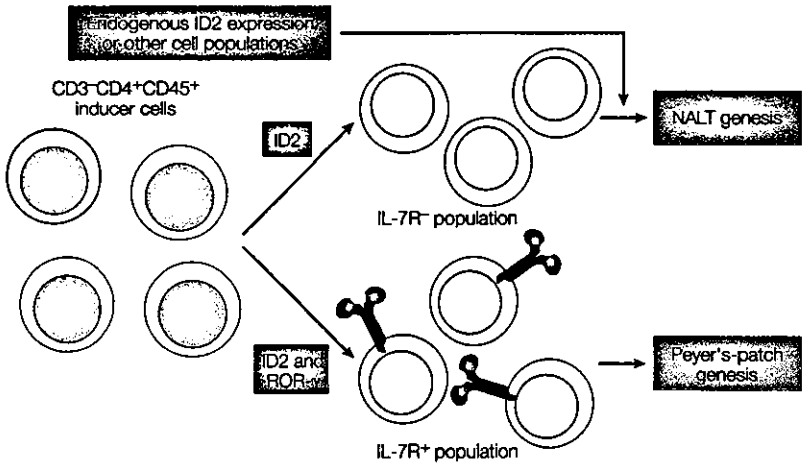

Figure 4 | **Model for the induction of organogenesis of NALT and Peyer's patches by two subsets of CD3⁻CD4⁺CD45⁺ inducer cells.** CD3⁻CD4⁺CD45⁺ cells differentiate from fetal-liver progenitors. We propose that both ROR-γ (retinoic-acid-receptor-related orphan receptor-γ) and ID2 (inhibitor of DNA binding 2) are essential for the generation of interleukin-7 receptor (IL-7R)-expressing CD3⁻CD4⁺CD45⁺ cells for the induction of Peyer's-patch organogenesis. By contrast, the generation of the IL-7R⁻CD3⁻CD4⁺CD45⁺ inducer cells that are involved in nasopharynx-associated lymphoid tissue (NALT) organogenesis is regulated by ID2 but not ROR-γ. However, this model remains to be tested experimentally, and other possibilities exist (see main text). NALT genesis in ID2-deficient mice can be initiated by the adoptive transfer of CD3⁻CD4⁺CD45⁺ cells from wild-type mice, but the maturation of NALT formation is incomplete. This indicates that other cell populations, or the endogenous expression of ID2 at the site of NALT, might be required for the full maturation of NALT.

WALDEYER'S RING
Human nasopharynx-associated lymphoid tissues, including the palatine tonsils and adenoids, which are considered to have an important role in the induction and modulation of mucosal immunity in the upper respiratory tract.

MIDDLE CONCHA
Bony plate that extends from the central section of the lateral wall of the nasal cavity.

T_H0 CELLS
Precursors of T helper 1 (T_H1) cells and T_H2 cells, which produce both interferon-γ and interleukin-4. This T-cell population has the capacity to become T_H1- and/or T_H2 cells.

CLASS-SWITCH RECOMBINATION
Molecular alteration of the constant-region gene of the immunoglobulin heavy chain (C_H) that leads to a switch in expression from the Cμ (or Cδ) region to one of the other C_H genes. This leads to a switch in the class of the immunoglobulin that is displayed on the cell-surface of the B cell (and that subsequently differentiating plasma cells produce) — from IgM (or IgD) to IgG, IgA or IgE — without altering the specificity of the immunoglobulin.

Immunological features of NALT

In rodents, NALT is found on both sides of the nasopharyngeal duct, dorsal to the cartilaginous soft palate, and it is considered analogous to WALDEYER'S RING in humans[34,35]. Also, in a recent study, a NALT-like structure of lymphocyte aggregates that form follicles was identified in human nasal mucosa, particularly in the MIDDLE CONCHA of children less than two years of age[36], indicating that an equivalent to mouse NALT can develop in humans. NALT consists of follicle-associated epithelium (FAE), HEVs, and T-cell- and B-cell-enriched areas. Antigen-sampling M cells are present in the epithelium of NALT, which is specialized for antigen uptake similar to the FAE of Peyer's patches[7,37]. Antigen-presenting cells, including dendritic cells (DCs) and macrophages, are also found in NALT[38]. So, NALT contains all of the lymphoid cells that are required for the induction and regulation of mucosal immune responses to antigens that are delivered to the nasal cavity. For example, the intranasal administration of reovirus resulted in the formation of germinal centres in NALT, leading to the clonal expansion of antigen-induced IgA⁺ B cells and the subsequent generation of reovirus-specific IgA in the respiratory and intestinal tracts[39]. Moreover, reovirus-specific CTLs were also induced in NALT with a high frequency. These findings show that NALT can be a potent inductive site for the mucosal immune system. In addition to the induction of positive immune responses, the nasal deposition of antigen has been shown to be effective for the induction of systemic unresponsiveness — a form of mucosally induced tolerance[40]. So, NALT has been shown to be involved in the generation of positive- and negative-regulatory signals for the induction of antigen-specific immunity and tolerance respectively. The cellular and molecular contributions of the immunocompetent cells present in NALT to the generation of tolerance to mucosally exposed antigens are unknown. Because little is known about the induction of nasally induced tolerance, we focus here on the role of NALT in the induction of protective immunity.

T_H0 environment. Characterization of the mRNA that encodes T_H1 and T_H2 cytokines in CD4⁺ T cells isolated from mouse NALT revealed a dominant cytokine profile of T_H0 CELLS, indicating that these T cells are capable of becoming T_H1 or T_H2 cells immediately after antigen exposure of the nasal tract[41–43]. CD4⁺ T cells isolated from NALT of naive wild-type mice are T_H0 cells[42], so they can become either T_H1 or T_H2 cells depending on the identity of the nasally administered antigen. Nasal delivery of protein antigens (such as bacterial cell-wall components or virus-associated antigens) together with cholera toxin as a mucosal adjuvant induces antigen-specific T_H2-type responses that promote the generation of antigen-specific IgA-producing B cells, both in the nasal passages and at distant mucosal effector sites, including the genito-urinary, respiratory and intestinal tracts[41,44,45]. By contrast, intranasal vaccination with antigen-expressing recombinant *Mycobacterium bovis* bacillus Calmette-Guérin (rBCG) results in T_H1-cell-mediated immunity[43].

IgA class switching. Peyer's patches have long been thought to be the sites for the initiation of CLASS-SWITCH RECOMBINATION (CSR) of μ- to α-gene expression in the gastrointestinal tract, because they contain all of the cellular and microarchitectural elements that are required for the generation of IgA-committed B cells, including germinal-centre-containing B-cell follicles, a FOLLICULAR DC network and an interfollicular T-cell area[1,46,47]. The germinal-centre region contains a high frequency of IgM⁺B220⁺ B cells that express activation-induced cytidine deaminase (AID), which is essential for μ- to α-gene conversion[48]. In an early study, it was shown that incubation of IgM⁺IgA⁻ B cells isolated from Peyer's patches in the presence of the cytokine transforming growth factor-β (TGF-β) resulted in the generation of IgM⁻IgA⁺ B cells[49–51]. These post-switch IgA-committed B cells then migrated to mucosal effector tissues (such as the intestinal lamina propria), a process mediated by a group of homing and chemokine receptors and their ligands (such as MADCAM1–$\alpha_4\beta_7$-integrin and CCL25–CCR9 interactions)[52,53] (discussed later). In the intestinal lamina propria, these cells became IgA⁺ plasma cells in the presence of the IgA-enhancing cytokines IL-5, IL-6 and IL-10 (REFS 50,54–58). So, it was generally accepted that organized lymphoid structures of MALT, such as Peyer's patches, function as the inductive sites for generating IgA-committed B cells through μ- to α-gene CSR, whereas the diffuse tissues of the intestinal lamina propria function as effector sites for the production of IgA[1,2] (FIG. 1). However, the finding that IgA class switching can occur in the intestinal lamina propria without involvement of

the Peyer's patches[59] cast these assumptions into doubt. Stromal-cell-derived TGF-β present in the intestinal lamina propria was shown to trigger IgM⁺B220⁺ cells to switch to IgA⁺ B cells[59]. Mice with a deficiency in the programmed-inflammation-associated cytokine LT-α do not form Peyer's patches, so the levels of IgA responses are reduced compared with those of wild-type mice[60]. However, reconstitution of *Lt-α*$^{-/-}$ mice with LT-expressing bone-marrow cells, or transplantation of an intestinal segment from recombination-activating gene (*Rag*)$^{-/-}$ mice to *Lt-α*$^{-/-}$ mice, resulted in the recovery of IgA responses[60]. These findings imply that at least some IgA-committed B cells can develop, even in the absence of Peyer's patches. *Lt-α*$^{-/-}$ mice have also been shown to be capable of inducing antigen-specific IgA responses to orally administered *Salmonella typhimurium*, despite the absence of organized lymphoid tissues associated with the mucosal compartment[61]. Although these findings provide supporting evidence that the environment of the diffuse lamina propria region is self-sufficient for the μ- to α-gene CSR that leads to the generation of IgA-committed B cells, it is also possible that unidentified, programmed-inflammation-independent miniature lymphoid aggregates, and recently characterized isolated lymphoid follicles[62], have a role in mounting IgA responses in cases of Peyer's-patch deficiency.

The finding that IgA-specific CSR can occur in diffuse mucosal effector tissues indicates that organized mucosal tissue is not essential for the generation of IgA-committed B cells in the digestive tract[59], although this new view is still controversial[63,64]. Because it has been shown that intestinal IgA is produced by two groups of cells, B1 cells and B2 CELLS[63,65,66], an interesting possibility is that IgA-specific CSR of B1 cells does not require organized lymphoid structures, whereas these structures are essential for IgA-isotype switching in B2 cells. In support of this view, we have shown that most B cells in gut-associated lymphoid tissues are B2 cells, whereas B1 cells are located preferentially in the intestinal lamina-propria region[66]. So, we think that the initial antigenic stimulation for the triggering of IgA-isotype switching in B2 cells might be provided by antigen sampling through M cells that are located in the dome epithelium (or FAE) of Peyer's patches. By contrast, the IgA-specific CSR process that occurs for B1 cells might be triggered by antigens sampled through newly identified villous M cells that are located adjacent to lamina-propria regions that do not contain observable lymphoid-like structures[67].

Because NALT is of similar importance for the initiation of IgA⁺ B-cell responses as Peyer's patches, we have investigated whether IgA-isotype switching can also occur in the diffuse tissue of the nasal passage. IgM⁺B220⁺ B cells, which are a prerequisite for CSR, were found in the organized inductive sites (NALT) but were mostly absent from the diffuse effector tissues (nasal passage) of the respiratory mucosal immune system[68]. Similarly, IgM⁺B220⁺ B cells were observed in the organized Peyer's patches of the intestinal tract but not in the intestinal lamina propria. So, in this study, IgM⁺B220⁺ B cells that are preconditioned to undergo IgA class switching are selectively located in the organized mucosa-associated inductive tissues of NALT and Peyer's patches[68]. This finding was confirmed by the molecular analysis of IgA CSR-associated mRNA specific for AID, Iα-Cμ CIRCULAR TRANSCRIPTS and Iμ-Cα TRANSCRIPTS. Because the expression of AID and the Iα-Cμ circular transcript are upregulated preferentially during μ- to α-gene conversion and then quickly down-regulated, these molecular events are considered to be a hallmark of B cells that are undergoing IgA class switching[69]. The expression of Iμ-Cα transcripts indicates the completion of IgA-specific CSR[59]. This analysis showed that the expression of AID-, Iα-Cμ circular transcript- and Iμ-Cα transcript-specific mRNA was restricted to the organized mucosal inductive tissues of NALT and Peyer's patches but was not found in the diffuse effector tissues of the nasal passage and intestinal lamina propria[68]. Furthermore, these organized mucosal lymphoid tissues are known to be associated with B2 cells[66]. So, these findings indicate that the IgA class switching, at least for B2 cells, requires the organized lymphoid structures of NALT and Peyer's patches in the aero-digestive tract. NALT was also recently shown to be an important site for the generation of memory B cells, which produce high-affinity IgA[70]. Taken together, these findings show that NALT contains all of the immunocompetent cells that are required for the induction and regulation of antigen-specific T_H1- or T_H2-cell-mediated responses and B-cell immune responses.

Differences between NALT- and Peyer's-patch-initiated immune responses. NALT and Peyer's patches are thought to have similar immunological characteristics and biological functions, as well as to contain the same types of resident immunocompetent cell. So, similar to oral immunization, nasal immunization can stimulate antigen-specific T_H1- or T_H2-cell-mediated responses and IgA responses in distant mucosal effector tissues[1,2,41,43–45]. However, in general, NALT-targeted immunization effectively induces antigen-specific immunity in the respiratory and reproductive tissues, whereas Peyer's-patch-targeted immunization promotes the generation of protective immunity in the gastrointestinal-tract tissues[1,2]. Further support for a compartmentalized CMIS was provided recently when it was shown that nasal immunization induces the expression of high levels of CCR10 and $\alpha_4\beta_1$-integrin by IgA-committed B cells, allowing them to efficiently traffic to the respiratory and genito-urinary tracts, which express the corresponding ligands, CCL28 and VCAM1 (REFS 71,72). By contrast, orally induced IgA-committed B cells express CCR9 and CCR10, as well as $\alpha_4\beta_7$- and $\alpha_4\beta_1$-integrins, so they migrate to sites such as the small intestine, which express CCL25 and/or CCL28 together with MADCAM1 and/or VCAM1 (REF. 73).

So, despite NALT and Peyer's patches both belonging to the mucosal immune system, the subtle differences that we have discussed indicate that the tissue genesis and biological functions of NALT and Peyer's patches might differ because of their anatomically and

FOLLICULAR DC
(FDC). Cell with a dendritic morphology that is present in lymph nodes. These cells display on their surface intact antigens that are held in immune complexes, and B cells present in the lymph node can interact with these antigens. FDCs are of non-haematopoietic origin and are not related to dendritic cells.

B2 CELLS
IgM^{low}IgD^{hi}MAC1^{-}B220^{hi}CD23^{+} cells that originate from bone marrow and are distributed to mucosal and systemic immune compartments for the continuous secretion of antibodies with high affinity and fine specificity.

Iα-Cμ CIRCULAR TRANSCRIPTS
Circular DNA molecules that are present in activated B cells and consist of Iα (intervening region-α) and Cμ genes. They are a hallmark of B cells that are in the process of IgA class switching.

Iμ-Cα TRANSCRIPTS
Germline transcripts that can be detected in IgA-committed B cells after class switching.

Table 2 | **Novel mucosal adjuvants and delivery systems for the development of nasal vaccines**

Antigen	Adjuvant and delivery vehicle	T_H cells	Secretory IgA	Serum IgG	Protective immunity	References
Influenza HA	mCTA–nLTB	$T_H2 > T_H1$	+	+	+	93
PspA	mCT	$T_H2 > T_H1$	+	+	+	90
V3J1	rBCG	$T_H1 > T_H0$	–	+	+	43
HIV gp160	HVJ liposome	$T_H1 = T_H2$	+	+	+	76

gp160, glycoprotein 160; HA, haemagglutinin; HVJ, haemagglutinating virus of Japan; nLTB, B subunit of native form of heat-labile enterotoxin; mCT, mutant cholera toxin; mCTA, A subunit of MCT; PspA, pneumococcal surface adhesin A; rBCG, recombinant *Mycobacterium bovis* bacillus Calmette-Guérin; T_H, T helper cell; V3J1, peptide containing neutralizing epitope of HIV.

environmentally distinct locations. Targeting the CMIS would therefore seem to be a logical choice for the development of a second generation mucosal (nasal or oral) vaccine to induce antigen-specific immune responses — such as a combination of T_H1-cell or T_H2-cell responses, CTL responses, and IgA and IgG responses — in the common and/or selective regions of the mucosal compartments, as well as at systemic sites, through the use of the NALT- and Peyer's-patch-initiated mucosal immune responses (FIG. 1).

NALT-based vaccine development

As we have described, NALT is one of the key components of the organized lymphoid tissue, and it contains all of the immunocompetent cells that are required for the induction of antigen-specific immune responses. It is therefore likely to have a central role in the development of a 'nasal vaccine'. Nasal vaccination has proven to be an effective regimen for the stimulation of the respiratory immune system[1,2,41,45]. Furthermore, this route of mucosal immunization can elicit both humoral and cell-mediated antigen-specific immune responses[1,2,41,43–45]. Another attractive feature of nasal immunization is that it requires a much smaller dose of antigen than does oral vaccination for the induction of antigen-specific mucosal and systemic immune responses, because the antigens are not exposed to degradation by digestive enzymes. Because intranasal administration of vaccine antigen alone has failed to fully stimulate NALT, researchers are attempting to develop an effective NALT-targeted vaccine-antigen delivery system and to develop a safe and effective immune-enhancing molecule (or adjuvant) for intranasal administration with the vaccine antigen.

NALT-targeted vaccine delivery. Antigens are known to be more immunogenic in particulate form than in soluble form, but they are vulnerable to antigen-degrading enzymes and acids that are associated with the mucosal environment. To overcome these obstacles, much effort has been focused on the creation of novel non-toxic and non-immunogenic vaccine vehicles that can effectively deliver even the soluble form of antigen to the organized mucosal inductive tissue. Such vehicles need to protect vaccine components from degradation, enhance their uptake from mucosal surfaces and perhaps function as an adjuvant. Among the various candidates for mucosal antigen delivery, Sendai-virus-associated fusion protein seems particularly suited to function as a molecule that guides antigen to the mucosal epithelium, because the Sendai virus itself uses this fusion protein for the invasion of respiratory epithelial cells[74]. A novel hybrid antigen-delivery vehicle has also been devised using this envelope fusion glycoprotein of Sendai virus (or using the haemagglutinating virus of Japan, HVJ); the fusion protein is displayed on the surface of liposomes (either fusogenic liposomes or HVJ liposomes) that contain the antigen of interest. When this delivery vehicle was used, intranasally administered antigen that was conjugated to green-fluorescent protein successfully reached the antigen-sampling M cells that are located in the epithelium of NALT[75]. Fusogenic liposomes were also found to effectively deliver antigen to epithelial cells and macrophages in both NALT and the nasal passages[75]. Furthermore, it was shown that an intranasally administered HVJ liposome containing the HIV glycoprotein 160 antigen (gp160; also known as env) was a powerful tool for inducing gp160-specific serum IgG, and gp160-specific mucosal IgA was also detected in nasal wash, saliva, faecal extract and vaginal wash[76] (TABLE 2). These findings show that the novel hybrid antigen-delivery vehicle of fusogenic liposomes (or HVJ liposomes) effectively transports vaccine antigen to NALT for the initiation of antigen-specific IgA responses at distant mucosal effector sites. Furthermore, this immunization method can also induce antigen-specific immune responses (such as production of IgG) in the systemic compartment (TABLE 2).

Because antigen-sampling M cells are scattered throughout the NALT epithelium[77], it seems logical to develop an M-cell-targeted nasal vaccine. One promising approach has been to use a molecule that is involved in the normal course of invasion of an infectious agent. Reovirus, an enteric pathogen, is known to invade its host through M cells that are located in the epithelium of Peyer's patches[78]. The 45-kDa viral haemagglutinin σ1 protein of reovirus has a crucial role in its attachment to and entry into M cells[79]. The virus has been shown to recognize mouse M cells that are present in the airways[80], and the recombinant form of the σ1 protein can bind to M cells that are associated with NALT epithelium[80]. On the basis of these findings, attempts have been made to develop an M-cell-targeted DNA vaccine using the σ1 protein as a guiding molecule[81]. When conjugated to a eukaryotic expression vector that encodes luciferase (known as pCMVLuc) and administered intranasally, the σ1 protein can specifically bind to the apical surface of M cells that are situated in the follicular epithelium

of NALT; it then leads to the generation of luciferase-specific serum IgG and mucosal IgA responses[80]. A nasal vaccine assembled using the σ1 protein and gp160 resulted in gp160-specific CTL responses in various mucosa-associated and systemic immune compartments, including reproductive tissue and spleen respectively[81]. These findings further emphasize the efficacy of NALT-targeted immunization for the induction of humoral and/or cell-mediated antigen-specific immune responses in mucosal and systemic immune compartments.

Because it only infrequently causes serious complications, BCG, a commonly used vaccine for the control of tuberculosis, is considered to be a low-risk vaccine. The recombinant form of BCG is a useful vaccine-antigen delivery vehicle, because it has strong adjuvant activity that can induce both humoral and cell-mediated immune responses[82]. Indeed, systemic administration of rBCG that expresses HIV antigen has been shown to effectively induce cell-mediated immunity[43,83]. Our own studies have shown that intransal administration of rBCG that expresses V3J1, a neutralizing epitope of HIV, can induce V3-peptide-specific IgG that has neutralizing activity for more than 0.5–1 years in both normal and immunodeficient (interferon-γ-deficient or *Il-4*$^{-/-}$) mice[43] (TABLE 2). Furthermore, V3J1–rBCG-induced serum IgG has also been shown to effectively neutralize a homologous strain of HIV[43]. Accordingly, rBCG shows promise as an effective nasal-immunization vehicle for the induction of prolonged antigen-specific antibody responses.

Creation of safe toxin-based adjuvants. Both cholera toxin that is produced by *Vibrio cholerae* and the heat-labile enterotoxin of *Escherichia coli* function as adjuvants to enhance mucosal and serum antibody responses to co-administered protein antigens delivered by oral or nasal routes[2,84]. Unfortunately, despite their efficacy as mucosal adjuvants, the native forms of cholera toxin (nCT) and heat-labile enterotoxin (nLT) cause severe diarrhoea and so are unsuitable for use in humans. To overcome these hurdles, researchers have substituted a single amino acid to generate non-toxic mutant forms of cholera toxin (mCT) and heat-labile enterotoxin (mLT)[85–88]; these retain the adjuvanticity of the native forms but do not induce the ribosylation of ADP that is associated with toxic activity. Our efforts to devise a safe first generation toxin-based adjuvant have focused on mCT S61F (in which phenylalanine replaces serine at position 61) and mCT E112K (in which lysine replaces glutamic acid at position 112); these mutations were created by making a single amino-acid substitution in the active centre of the ADP-ribosyltransferase in the A subunit of cholera toxin[89]. The two mutant forms of cholera toxin have been shown to be safe by *in vitro* analyses of ADP-ribosylation activity and cyclic AMP formation, as well as by *in vivo* examination for diarrhoea-like symptoms. When pneumococcal surface-protein A (PspA) — a new candidate vaccine antigen for preventing infection with *Streptococcus pneumoniae* — was intranasally administered with mCT, antigen-specific mucosal IgA and systemic IgG responses were elicited[90]. Mice intranasally immunized with PspA and mCT were also protected against a lethal challenge with *S. pneumoniae*[90]. Interestingly, when the tetanus-toxoid vaccine (which is currently administered by injection) was intranasally administered with one of these two mCTs, it generated protective immunity against challenge with the toxin[91]. An independent study has also shown that mCT E112K is the safest and most effective of the currently available toxin-based mutant adjuvants[92]. Taken together, these findings support the idea that mCT is a strong candidate for an effective mucosal adjuvant to generate protective immunity by the nasal route of administration. Indeed, these findings indicate that the current preference for injection-type vaccines should be reconsidered, and in future, greater use should be made of spray-type vaccines that include mCT and other safe toxin-based adjuvants (BOX 1).

To further enhance the efficacy of the mCT mucosal adjuvant, a second-generation, chimeric-type adjuvant was constructed from the A subunit of mCT (mCTA) and the B subunit of nLT (nLTB); therefore, the adjuvant has the immunobiological properties of both cholera toxin and heat-labile enterotoxin[93]. Nasal immunization with influenza-virus haemagglutinin plus the newly created chimeric mucosal adjuvant mCTA–nLTB resulted in significant haemagglutinin-specific serum IgG and IgA responses[93] (TABLE 2). In addition, mice that were intranasally immunized with haemagglutinin and mCTA–nLTB showed high levels of haemagglutinin-specific IgA in nasal and lung washes and were protected from viral challenge[93]. These findings show that nasal vaccines containing mCT or mCTA–nLTB are effective for the induction of protective immunity. The goal of mucosal-vaccine development cannot be realized without the creation of such novel and safe mucosal adjuvants.

Box 1 | Advantages and disadvantages of nasal vaccination

Advantages

- Is the most effective route to elicit optimal protective immunity in both mucosal and systemic immune compartments.
- Can effectively induce antigen-specific immunity in the reproductive tract, as well as in the upper respiratory tract.
- Can generate cross-protective immunity in the gut through the common mucosal immune system.
- Can avoid degradation of vaccine antigen caused by digestive enzymes, so requires a smaller dose of antigen than oral immunization.
- Does not require injection, so is less painful.
- Does not require trained medical personnel for delivery.

Disadvantages

- Possible deposition of antigen in the central nervous system through the olfactory bulbs and olfactory nerves; this requires further investigation.
- Requires adjuvant safety to be clinically determined; clinical studies indicate that Bell's palsy is caused by influenza nasal vaccine that contains the native form of *Escherichia coli* heat-labile enterotoxin as a mucosal adjuvant.

BELL'S PALSY
Facial paralysis that is thought to be triggered by viral infection. The facial nerve is oedematous in patients suffering from this disease.

Recent progress in clinical application of nasal vaccination. Between the late 1950s and the early 1960s, the efficacy of immunization with an intranasally administered vaccine against infection with influenza virus was shown in a large clinical trial in Osaka, Japan, in which a nasal-spray vaccine containing live attenuated influenza virus was administered to more than 10,000 volunteers[94,95]. In recent years, two types of intranasally administered influenza vaccine, an inactivated form and a live attenuated form, were introduced in Switzerland and the United States respectively. Indeed, as early as 1997, an inactivated form of nasal vaccine containing a small amount of nLT as a mucosal adjuvant was introduced in Switzerland. However, this influenza vaccine was withdrawn from the market because of the development of BELL'S PALSY by some recipients after nasal vaccination. A causal relationship between the intranasally administered inactivated-influenza vaccine used in Switzerland and the incidence of Bell's palsy was formally established in a recent case–control study[96]. At this stage, the causes and pathogenesis of Bell's palsy remain unclear; however, because nLT has been shown to have pro-inflammatory properties and possible neurological toxicity[97], the co-formulated nLT that is present in the inactivated-influenza vaccine is suspected to be the causative agent[98]. These findings highlight that the development of a safe mucosal adjuvant is crucial if progress is to be made towards a safe and effective mucosal vaccine.

In the past year, on the basis of promising clinical trials showing the induction of protective immunity, an intranasally administered cold-adapted influenza vaccine, known as 'FluMist', has been made available to healthy Americans of ages 5 to 49 (REFS 99,100). It should be noted that the concept of cold-adapted influenza virus was reported in 1967 (REF. 101), so more than 35 years were required for this discovery to be translated into an intransal vaccine against infection with influenza virus. More recently, the concept of nasal immunization was adopted for the development of a vaccine against severe acute respiratory syndrome (SARS). An experimental nasally administered vaccine against SARS that consists of a recombinant attenuated parainfluenza virus expressing the envelope spike protein of the SARS coronavirus was able to induce protective immunity in African green monkeys, including SARS-coronavirus-specific neutralizing antibodies[102]. Although further experiments are essential before this finding can be applied to developing a vaccine against SARS for use in humans, the study emphasizes the usefulness of mucosal immunization for the immunoprophylaxis of infectious diseases.

Concluding remarks

The mucosal immune system is now recognized to be an important first line of defence against invading pathogens. NALT and Peyer's patches are important inductive sites for the initiation of antigen-specific mucosal IgA and serum IgG responses, as well as CTL immune responses, at both mucosal and systemic sites; in this way, both NALT and Peyer's patches function to maximize the two-tiered immunological barrier of the host. The respiratory mucosal immune system has several immunological characteristics that are distinct from those of the Peyer's-patch-centred intestinal mucosal immune system. Although the tissue-genesis programme for other secondary lymphoid tissues, including Peyer's patches, begins during embryonic life, the NALT-organogenesis programme is initiated only after birth. Lymphoid organogenesis of Peyer's patches requires cytokine-mediated programmed inflammation (through the LT-βR) and signalling through the IL-7R, whereas the initiation of NALT development seems to be independent of the IL-7R, LT-$\alpha_1\beta_2$ and the LT-βR. Although the inducer cells for both NALT and Peyer's patches have a common phenotype, that is, $CD3^-CD4^+CD45^+$, NALT-inducer cells seem to be regulated by ID2 alone, whereas Peyer's-patch-inducer cells depend on both ID2 and ROR-γ. Because the organogenesis programme of NALT is different from that of other secondary lymphoid tissues, such as Peyer's patches, efforts should now be aimed at elucidating the distinct molecular characteristics of the NALT-genesis programme and the functional consequences of this. Clearly, it is important to have a thorough understanding of the unique molecular and cellular properties of the NALT-centred mucosal immune system for the development of a successful nasal vaccine.

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Using an *in vivo* model, this paper directly showed that ID2-regulated $CD3^-CD4^+CD45^+$ inducer cells are involved in the postnatal initiation of NALT genesis. In addition, together with reference 31, it described that NALT organogenesis is independent of IL-7R- and LT-βR-mediated signalling.

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Competing interests statement

The authors declare no competing financial interests.

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Pathological Role of Large Intestinal IL-12p40 for the Induction of Th2-Type Allergic Diarrhea

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IL-12 consists of two disulfide-linked subunits, p40 and p35, that form functionally active heterodimers for the induction of Th1 cells. In contrast to IL-12 heterodimers, p40 monomers and homodimers possess inhibitory effects on Th1 cells leading to the creation of a Th2 environment. Although it has been shown that IL-12p40 acts as antagonist of IL-12p70 *in vitro*, no evidence is currently available whether IL-12p40 is functional *in vivo*. We now report that IL-12p40 plays an important pathological role in an intestinal allergic disease. A high expression of IL-12p40 protein was demonstrated in epithelial cells, dendritic cells, and macrophages in large but not small intestine of allergic diarrhea-induced mice. Interestingly, neutralization with anti-IL-12p40 mAbs reduced the incidence and delayed the onset of disease development. Lower levels of ovalbumin (OVA)-specific IgE Abs in serum were detected in anti-IL-12p40 mAb-treated mice than in control Ab-treated mice. The secretion of Th2 cytokines and eotaxin by the mononuclear cells isolated from the large intestine of anti-IL-12p40 mAb-treated mice was significantly decreased. Finally, the removal of the IL-12p40 gene resulted in complete inhibition of disease development. These results show that over-expression of IL-12p40 is an important contributing factor for the generation of the dominant Th2-type environment in the large intestine of mice with allergic diarrhea. *(Am J Pathol 2004, 164:1327–1335)*

In general, intestinal allergic reactions are provoked by the activation of allergen-specific Th2-type cells, excessive eosinophil and mast cell recruitment, and IgE Ab production.[1,2] We previously reported that systematically primed BALB/c mice developed severe diarrhea after repeated oral challenge with ovalbumin (OVA).[1] Diarrhea-induced mice revealed a Th2-type allergic response characterized by high levels of Ag-specific IgE Abs in serum, increased numbers of IgG1, IgA and IgE Abs in the large intestine, and high numbers of mast cells and eosinophils in the large intestine. Furthermore, large intestinal $CD4^+$ T cells isolated from mice with allergic diarrhea secreted IL-4, IL-5, and IL-13, but not IFN-γ. On the other hand, a murine model of eosinophilic gastrointestinal hypersensitivity induced by challenge with oral allergen, in the form of enteric-coated beads, resulted in marked allergen-induced IL-4 and IL-5 production and eosinophil accumulation in the small intestine. Although several interesting intestinal allergic models were recently reported,[2,3] the exact underlying molecular and cellular mechanisms remain to be elucidated.

In allergic asthma, allergen-specific T cells have been shown to also acquire the Th2 phenotype and to avoid from the Th1-type pathway.[4,5] A recent study has demonstrated that Th1/Th2 imbalance induced allergic disease at the level of transcription factors. Interestingly, a high expression of GATA-3 and/or a lack of T-bet signaling markedly influenced the development of allergic asthma.[6,7] In addition, the expression of not only Th2-type cytokine but also Th1-type cytokine (ie, IFN-γ or IL-12) played a critical role in murine dermatitis and asthma models.[8,9] It has also been demonstrated that natural killer (NK) cells, like Th2 cells, play an important role in the development of allergen-induced asthma.[9]

It has been suggested that antigen-presenting cells (APCs) play a crucial role in the skewing of Th1 and Th2 differentiation.[10,11] IL-12 is a heterodimeric cytokine composed of p40 and p35 which strongly promotes the differentiation of naive $CD4^+$ T cells to the Th1 phenotype and suppresses the synthesis of Th2-type cytokines.[12] IL-12 is produced primarily by APCs and the production is regulated by IL-10 and IFN-γ.[13,14] In addition, biological effects of IL-12 are counter-balanced by IL-12p40 itself, which binds to the receptor complex without induc-

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ing intracellular signals.[15] Thus, murine IL-12p40 inhibits IL-12-mediated responses by means of the competitive binding to IL-12 receptor with an affinity similar to that of IL-12p70.[16,17] Further, IL-12p40 can behave as an IL-12p70 antagonist *in vivo*, delaying the allograft rejection of cardiac myoblast.[18] IL-12p40 transgenic mice also showed increased susceptibility to the malaria infection.[19]

With regard to allergic responses, a potential contribution of IL-12 has been suggested for the development of allergic asthma.[9,20] Mixed Th1- and Th2-associated cytokines, including IFN-γ, IL-2, IL-5, GM-CSF, and IL-12, were secreted by smooth muscle cells located in the sensitized airways of atopic asthma-induced mice.[21] A previous study demonstrated that IL-12p40 mRNA expression was detected in the lung tissue of mice with asthma.[21] However, the role of IL-12 in intestinal allergic disease has not yet been carefully examined. To investigate the potential roles of IL-12 in intestinal hypersensitivity, we have assessed the expression pattern of IL-12p40 or IL-12p35 in the intestinal tract of diarrhea-induced mice and examined the therapeutic effects of modulating IL-12 involvement in allergic diarrhea.

Materials and Methods

Mice

BALB/c mice were purchased from Japan Clea Company (Tokyo, Japan). Breeding pairs of IL-12p40-deficient [IL-12p40 knockout (KO)] mice were purchased from The Jackson Laboratories (Bar Harbor, ME), and colonies were established and maintained in the experimental animal facility at the University of Tokyo and the University of Alabama at Birmingham. All mice were 6 to 7 weeks of age at the beginning of individual experiments.

Antibodies

Recombinant murine IL-12 p40 and p70 were obtained from BD PharMingen (San Diego, CA). Biotin-anti-CD11b (M1/70, rat IgG2b) and biotin-anti-CD11c (HL3, hamster IgG) were also purchased from BD PharMingen. Biotin-SP-conjugated, affinity-purified anti-rat IgG (H+L) mouse $F(ab')_2$ and anti-hamster IgG(H+L) goat $F(ab')_2$ were obtained from Jackson ImmunoResearch Laboratories, Inc. (West Grove, PA). Anti-IL-12p40 (C17.8.20, rat IgG2a) was a generous gift from Dr. G. Trinchieri, Wistar Institute, Philadelphia, PA[22] and was purified from ascites on a protein G column (Pharmacia Biotech, Uppsala, Sweden). Purified rat IgG was purchased from Sigma Chemical, Inc. (St. Louis, MO).

Induction of Allergic Diarrhea

For the induction of allergic diarrhea, our well-established protocol was used as described previously.[1] Briefly, on the first day of the experiment (day 0), mice were primed by subcutaneous (SC) injection of 1 mg of OVA in Complete Freund Adjuvant (CFA) (Difco Laboratories, Detroit, MI). One week after the systemic priming (day 7), mice were repeatedly challenged with 50 mg of OVA by oral administration (PO) three times per week for several weeks.[1] These mice were sacrificed and analyzed within 1 and 2 hours after a total of 10 times of oral administration with OVA. In a timed kinetics study, mice were sacrificed at indicated intervals, ie, 0, 30 minutes, 1 hour, and 2 hours following the last oral administration of OVA. As controls, mice were repeatedly given oral OVA in phosphate-buffered saline (PBS) without systemic priming or were injected SC with 1 mg OVA in CFA without repeated oral challenge.

Treatment of Mice with Anti-IL-12p40

In vivo Ab treatment was performed as described previously.[23] Anti-IL-12p40 (C17.8.20, rat IgG2a) was a generous gift from Dr. G. Trinchieri, Schering-Plough Research Institute, Dardilly, France.[22] BALB/c mice were intraperitoneally administered with 0.5 mg to 2.0 mg of purified anti-IL-12p40 (C17.8) or control rat IgG (Sigma Chemicals, Inc.) per week for the duration of the experiment. Ab treatment was started 1 week before or at the time systemic priming with OVA in CFA. Among different concentrations tested, the protocol of 1 mg/mouse of per week was the most optimal condition. Further, when the mAb treatment schedule was compared between the start at 1 week before or at the same time as OVA systemic priming, an identical effect was noted in this study. Thus, the mAb anti-IL-12 (1 mg/mouse) was started on 1 week before the OVA systemic priming.

ELISA for OVA-Specific IgE Abs in Serum

To assess OVA-specific IgE Ab levels in serum, a sandwich ELISA system was adopted.[1] End-point titers of OVA-specific IgE Abs were expressed as the reciprocal $\log_2$ of the last dilution that showed a level of 0.1 higher absorbance than that of sera of non-immune mice as background.

Isolation of Mononuclear Cells and Cytokine-Specific ELISA Assay

To isolate mononuclear cells from small and large intestines, we used a enzymatic dissociation method.[24] Briefly, mononuclear cells were dissociated by collagenase from small and large intestines after the removal of Peyer's patches and were then subjected to a discontinuous Percoll gradient.[25] Mononuclear cells from small and large intestines were then co-cultured in the presence of 1 mg OVA. After 3 days of culture, the supernatant was collected and assayed for cytokines by using an ELISA Kit specific for IL-4 (Endogen, Woburn, MA), IL-5 (Amersham Pharmacia Biotech, Piscataway, NJ), IL-13 (R&D Systems, Minneapolis, MN) and eotaxin (Techne Corporation, Minneapolis, MN).

Table 1. Primers and Probes Used for Qualitative and Quantitative RT-PCR

		Conventional PCR*	
HPRT	primer	F	GTT GGA TAC AGG CCA GAC TTT GTT G
		R	GAG GGT AGG CTG GCC TAT AGG CT
IL-12p40	primer	F	ATG GCC ATG TGG GAG CTG GAG
		R	TTT GGT GCT TCA CAC TTC AGG
IL-12p35	primer	F	ACC CAG TTG GCC AGG GTC
		R	CAA GGC ACA GGG TCA TCA TC
		Real-time PCR†	
HPRT	primer	F	AAC TTT GCT TTC CCT GGT
		R	AGT CAA GGG CAT ATC CAA CA
	probe	FITC	CAG TAC AGC CCC AAA ATG GTT AAG GTTGC
		LCRed640	AGC TTG CTG GTG AAA AGG ACG TCT CG
IL-12p40	primer	F	AGA GGA GGG GTG TAA CCA G
		R	GGG AAC ACA TGC CCA CTT G
	probe	FITC	ACC GAA GTC CAA TGC AAA GGC GG
		LCRed640	AAT GTC TGC GTG CAA GCT CAG G
IL-12p35	primer	F	CCT GTG CCT TGG TAG CAT CT
		R	AGA CTG CAT CAG CTC ATC G
	probe	FITC	ACC AGA CAG AGT TCC AGG CCA TCA
		LCRed640	TGA TGG CCT GGA ACT CTG TCT GGT

*, Primers were designed based on the published sequence.[13]
†, Primers and probes were designed and produced by Nihon Gene Research Laboratories (Sendai, Japan).

Immunoprecipitation and Western Blot Analyses

For the detection of different forms of IL-12, intestinal tissue extracts were prepared as previously described with minor modifications.[26] Small and large intestines were removed, minced in cold PBS with protease inhibitor, homogenized, and incubated to allow cytokine release from the tissue. After centrifugation intestinal tissue extracts were subjected to the measurement of protein concentration and then pre-cleared with protein G Sepharose beads (Pharmacia Biotech, Uppsala, Sweden), subsequently incubated with anti-IL-12p40, mixed with protein G Sepharose beads. The beads were washed, subjected to SDS-PAGE under non-reducing condition. After electrophoresis, proteins were transferred to a polyvinylidene difluoride microporous membrane (PVDF Immobilon; Millipore, Bedford, MA) and the membrane was reacted with biotinylated anti-IL-12 (C17.8) followed by incubation with biotin-streptavidin complex (ABC-AP Kit; Vector Laboratories, Inc.). Visualization of the signal was performed by NBT/BCIP Substrate Kit (BioRad, Hercules, CA).

Analysis for IL-12 mRNA Accumulation

The expression of IL-12p40 or p35 in small and large intestines of mice was examined using conventional RT-PCR as previously described[13] and quantitative real-time PCR method using a Lightcycler (Roche Diagnostics, GmbH Mannheim, Germany), with some modifications.[27,28] The sequences of primers and probes for real-time PCR were designed by Nihon Gene Research Laboratories (Sendai, Japan) (Table 1). Total RNA was extracted by using TRIzol reagent (Invitrogen Life Technologies, Carlsbad, CA) and 2 μg of extracted RNA was subjected to RT reaction using Superscript II Reverse Transcriptase (Life Technologies).[14] Hypoxanthine phosphoribosyl transferase (HPRT) cDNA was used to standardize the total RNA content. The cDNA from each experimental sample was then subjected to the Lightcycler FastStart DNA Master Hybridization Probes Kit (Roche Diagnostics). The external standards of HPRT, IL-12p40, and IL-12p35 DNA prepared by PCR between 20 pg to 0.02 fentograms were used for the quantification of specific cDNA in each sample. The ratio of the p40 and p35 increase in experimental mice was calculated and compared with non-treated mice as follows; the ratio = (p40 or p35 mRNA amounts from experimental mice/HPRT mRNA amounts from experimental mice)/(p40 or p35 mRNA amounts from non-treated mice/HPRT mRNA amounts from non-treated mice).

Immunohistochemical Analysis

Following extensive washing, small and large intestines were fixed in 4% paraformaldehyde-PBS and treated with sucrose-gradient, frozen in OCT-embedding medium as previously described with minor modifications.[29] For IL-12p40 immunostaining, cryosections were subjected to antigen retrieval using 10 mmol/L citric buffer pH 6.0 for 5 minutes at 98°C. Slides were then blocked with normal mouse IgG and incubated with rat anti-IL-12p40 or control rat IgG for 16 hours at 4°C. The section were then treated with biotinylated goat anti-rat IgG $F(ab')_2$ (Jackson ImmunoResearch Laboratories, Inc.), ABC-AP Kit, and red chromogen (Vector Red; Vector Laboratories, Inc.). For IL-12p35 immunostaining, we used goat anti-IL-12p35 (Santa Cruz, Inc., Santa Cruz, CA) or control goat IgG. The sections were then treated with biotinylated donkey anti-goat IgG $F(ab')_2$ and ABC-AP Kit. In the case of surface marker staining, serial sections were incubated with anti-CD11b (M1/70, BD PharMingen) or anti-CD11c (HL3, BD PharMingen), biotinylated second anti-

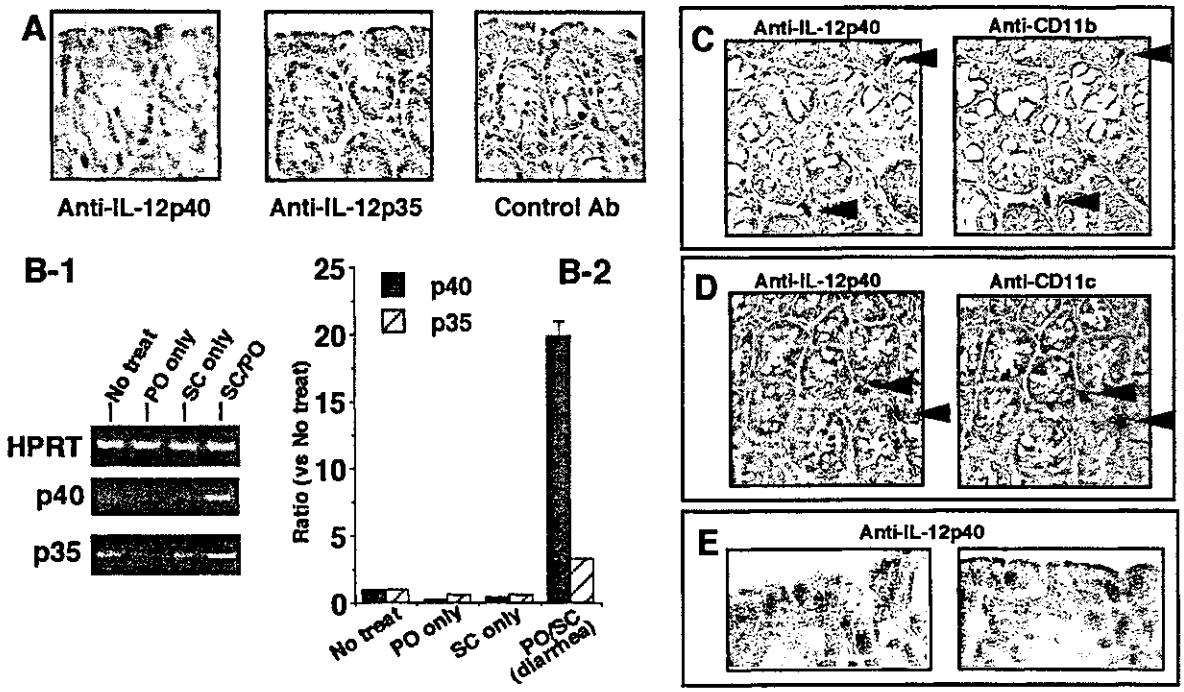

Figure 1. Selective production of IL-12p40 by the large intestine of diarrhea-induced mice. In **A**, large intestinal tissues from diarrhea-induced mice were immunostained with anti-IL-12p40 mAb, anti-IL-12p35 mAb, or control IgG. Control non-disease mice section gave no signal above background (data not shown). In **B-1**, IL-12p40-specific mRNA was expressed selectively in the large intestine of mice with allergic diarrhea. In **B-2**, quantitive real-time PCR analysis of IL-12p40- and p35-specific mRNA expression was performed. The ratio was obtained as the level of IL-12p40 or p35 expression in non-treated mice as a scale of one. The detailed information for the expression of this ratio is described in the Materials and Methods section. In **C-E**, IL-12p40 was detected in MØ and DC and epithelial cells in the large intestine. The serial sections of the large intestine from diarrhea-induced mice were stained with anti-IL-12p40 mAb and anti-CD11b mAb (**C**), with anti-IL-12p40 mAb and anti-CD11c mAb (**D**). The **arrows** point to double-positive cells. Large intestinal epithelial cells were stained with anti-IL-12p40 mAb (**E**).

body and ABC-AP. The color reaction was developed using Vector Red Substrate Kit I.

Statistical Analysis

Statistical analyses were performed by the two sample non-parametric Welch test with a significance level of 0.01 (**) for body weight and Ig levels, respectively. Mouse disease rates were determined using the Wilcoxon rank-sum test with a significance level of 0.01 (**). Values for cytokine-synthesis in the samples between anti-IL-12 p40-treated and control antibody-treated mice were analyzed by using Student's *t*-test at *P* values of <0.01(**).

Results

Detection of IL-12p40 Protein in the Large Intestine of Allergic Diarrhea Mice

To examine whether IL-12p40 was expressed in the large intestine of OVA-induced diarrhea mice, we analyzed IL-12 expression using a variety of available detection methods. First, we performed immunohistochemical analysis to directly demonstrate the enhanced IL-12p40 expression in the large intestine of mice with allergic diarrhea. As shown in Figure 1A, IL-12p40, but not IL-12p35, was expressed in the large intestine of diarrhea-induced mice. To further confirm enhanced expression of IL-12p40 in the large intestine of mice with diarrhea, we next performed IL-12-specific RT-PCR analysis. Interestingly, IL-12p40 mRNA was only detected in the large intestine of diarrhea-induced mice, not in control mice without the disease [eg, SC only or per oral challenge (PO) only; Figure 1B]. In contrast, IL-12p35 mRNA expression was detected in both groups of mice (Figure 1B-1). When IL-12-specific mRNA quantitative real-time PCR analysis was performed, high levels of IL-12p40-specific mRNA were noted in the large intestine of OVA-induced allergic diarrhea mice (Figure 1B-2). In contrast, the level of p35 did not vary among the four different groups including experimental diseased (SC/PO) and control non-diseased mice (non-treated, SC only, and PO only). Taken together, these results clearly indicate that IL-12p40, but not p35, was selectively enhanced at the levels of both mRNA and protein in the large intestine of allergic diarrhea mice.

Inasmuch as the induction of IL-12p40 selectively occurred in the large intestine of OVA-induced allergic diarrhea mice, it was important to determine which cell types produced IL-12p40 in the large intestine. Immunohistochemical analysis demonstrated that IL-12p40-producing cells were co-stained with anti-CD11b mAb [ie, macrophages (MØ)]. Further, $CD11c^+$ cells [ie, dendritic cells (DC)] were also positively stained for IL-12p40 (Figure 1, C and D). Further, some epithelial cells were also positive for IL-12p40 expression (Figure 1E). Taken together, these findings show that large intestinal macrophages, dendritic cells, and epithelial cells are responsi-

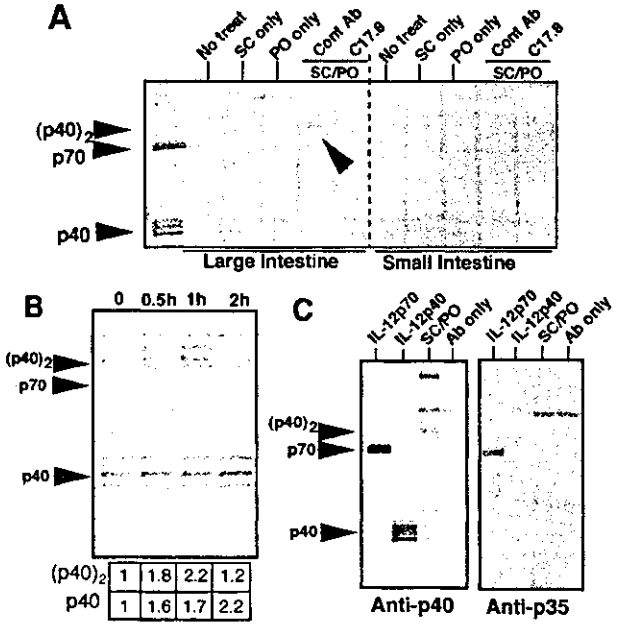

Figure 2. Induction of IL-12p40 homodimer in the large but not small intestine of diarrhea-induced mice. Large and small intestinal tissue extracts were subjected to immunopreciptation and Western blotting analysis using anti-IL-12p40 (C17.8) mAb under non-reducing conditions (**A**). The captions above the figure indicate the experimental mouse group receiving different *in vivo* treatments. Thus, the samples were obtained from SC/PO mice treated with C17.8 or control antibodies. Further, the samples were isolated from mice treated with PO only, SC only, or non-treated mice. The **arrow** points to IL-12p40 homodimer expression in the large intestine of diarrhea-induced mice. The data represent four independent experiments. In **B**, at the indicated times after oral administration of OVA, large intestinal tissue extracts isolated from diarrhea-induced mice were assayed for IL-12p40 by the same method as in **A**. In **C**, the large intestinal tissue extracts of diarrhea-induced mice were subjected to Western blotting with anti-IL-12p35 Ab as well as anti-IL-12p40. IL-12p70 protein was used as a positive control for the IL-12p35 detection system. As negative control, immunoprecipitation was performed without the tissue specimens (Ab only). The data represent three different experiments.

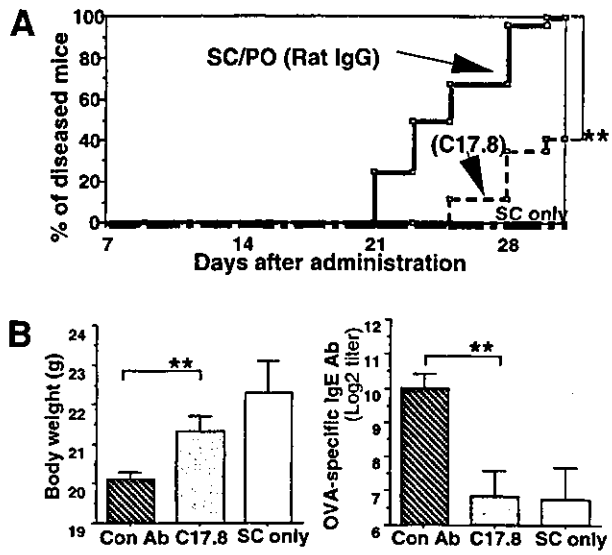

Figure 3. Inhibition of allergic diarrhea disease by the treatment with anti-IL-12p40 mAb. In **A**, anti-IL-12p40 mAb (C17.8) treatment (**thin dashed line**) delayed the development of allergic diarrhea when compared with the rat IgG-treated group (**solid line**). Statistical differences were determined by Wilcoxon rank-sum test and are indicated by **, $P < 0.01$. Mice with SC only were used as controls (**thick dashed line**). In **B**, **left**, body weight was recovered in allergic diarrhea mice treated with anti-IL-12p40 mAb (C17.8). In **B**, **right**, OVA-specific IgE Abs were reduced in the serum of allergic diarrhea mice treated with anti-IL-12p40 mAb (C17.8). The data are expressed as the mean of ± SE and are representative of five independent experiments. Statistical differences between anti-IL-12p40 mAb and control rat IgG-treated mice are indicated as **, $P < 0.01$.

ble for the production of IL-12p40 at the disease site of OVA-induced allergic diarrhea.

The Western blotting method was adopted for the examination of IL-12 p40 expression in the small and large intestinal tissue extracts from OVA-induced allergic diarrhea mice within 1 to 2 hours after the last oral challenge. In the large intestine of diarrhea-induced mice, the 80kD form of IL-12 predominated clearly demonstrating the presence of IL-12p40 homodimer but not 70kD IL-12 heterodimer, in contrast to the environment observed in the large intestine of control mice or the small intestine of mice with/without diarrhea (Figure 2A). The multiple bands of p40 and p80 are the result of glycosylation heterogenity.[15] We thus analyzed three bands of p40 and three bands of p80 as specific bands. In the case of spleen, IL-12p40 was detected in control, healthy mice. The levels of IL-12p40 did not change after development of allergic diarrhea (data not shown). To examine the kinetics of the response, we next assessed the time course of IL-12p40 expression in the large intestine of the diarrhea-induced mice. The expression of IL-12p40 or p80 in the large intestine peaked between 1 and 2 hours after the last oral challenge, at the same time that severe symptoms of OVA-induced allergic diarrhea were observed (Figure 2B). These data suggest that there is an intimate relationship between the development of diarrhea and the expression of IL-12p40 in the large intestine.

To further confirm the expression of IL-12p80 or p40 instead of the p70 form, the protein extracts from the large intestine of the diarrhea-induced mice were immunoprecipitated with anti-IL-12p40 mAb and then Western blotting was performed using anti-IL-12p35 mAb. No molecular bands corresponding to IL-12p70 proteins were detected in the large intestine of diarrhea-induced mice, while predominant IL-12p40 protein was detected (Figure 2C). The large molecular weight band above the p70 and p80 bands was non-specific and was caused by the nature of antibody used in the immunoprecipitation, since the large molecular weight band was also seen following immunoprecipitation in the absence of tissue specimens (Ab only in Figure 2C). These results indicate that the secretion of IL-12p40, but not IL-12p70, in the large intestine is critically important in the development of OVA-induced allergic diarrhea.

Anti-IL-12p40 Treatment Reduced the Symptoms of Allergic Diarrhea

Inasmuch as the preferential localization of IL-12p40 was observed in mice with allergic diarrhea, we next performed a neutralization experiment using anti-IL-12p40 mAb (C17.8). We observed a significant delay in the onset of diarrhea and reduced the frequency of diarrhea to 40% by treatment with anti-IL-12p40 mAb (Figure 3A). Obvious body weight loss was seen in control Ig-treated

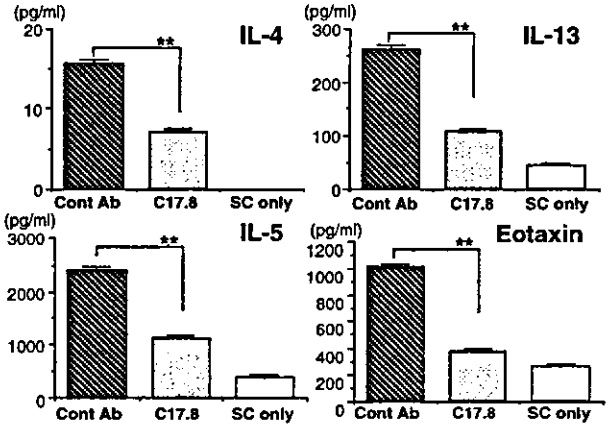

Figure 4. *In vivo* treatment with anti-IL-12p40 (C17.8) reduced the predominant antigen-specific Th2 type responses by large intestinal mononuclear cells isolated from diarrhea-induced mice. The mononuclear cells isolated from the large intestine (1.5×10^5 cells/well) were cultured with OVA (1 mg/ml) for 3 days. Culture supernatants were harvested and then assayed for IL-4, IL-13, IL-5, and eotaxin by ELISA assay. These data are expressed as the mean ± SE and are representative of three independent experiments. The statistical differences between anti-IL-12p40 mAb and control antibody treated mice are indicated as **, $P < 0.01$.

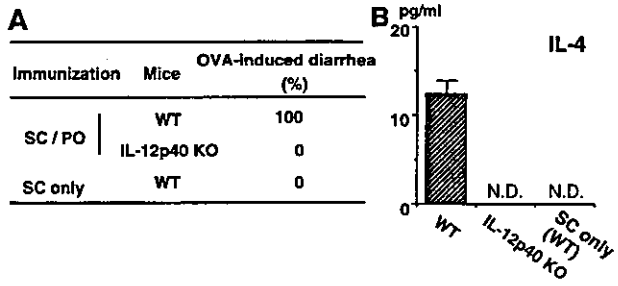

Figure 5. Suppression of allergic diarrhea development in IL-12p40 KO mice. In **A**, the incidence of allergic diarrhea was reduced in the IL-12p40 KO mice when compared with wild-type mice immunized subcutaneously and then given OVA repeatedly by the oral route (SC/PO). In **B**, the large intestinal LP mononuclear cells from IL-12p40 KO mice did not produce IL-4. Mononuclear cells isolated from the large intestine were restimulated with OVA for the assessment of IL-4 synthesis as described in Figure 4A. The data are expressed as the mean ± SE and represent three different experiments.

diarrhea mice, while treatment with anti-IL-12p40 mAb resulted in partial recovery from body weight loss (Figure 3B, left). In addition, high levels of OVA-specific IgE Abs were detected in the serum of diarrhea-induced mice treated with control Ab, whereas the mice treated with anti-IL-12p40 mAb showed low levels of OVA-specific IgE Abs (Figure 3B, right). These results indicate that treatment with anti-IL-12p40 mAb alters the environment from a disease-inducing one to one fastening recovery in OVA-induced allergic diarrhea.

Suppression of Intestinal Th2-Type Cytokine by Anti-IL-12p40 Treatment

To confirm decreased Th2-type responses in the large intestine after anti-IL-12p40 mAb treatment, we next examined antigen-induced cytokine production by the large intestinal mononuclear cells. Interestingly, the anti-IL-12p40 treatment resulted in decreased levels of OVA-induced Th2 cytokine synthesis including those of IL-4, IL-5, and IL-13 (Figure 4). Production levels of the Th2 cytokines were comparable to those of control mice without allergic diarrhea (SC only). In contrast to the alterations observed in OVA-induced Th2 cytokine synthesis, there was no difference in the level of IFN-γ production between the mice treated with anti-IL-12p40 mAb and control IgG (data not shown). We further confirmed that IL-4 producing cells were $CD4^+$ Th2 cells by intracellular staining (data not shown).

Finally, the level of eotaxin, a well-known chemokine for eosinophil recruitment in allergic disease,[30] was also examined, since our previous study demonstrated that the frequency of eosinophils was increased in the large intestine of allergic diarrhea mice.[1] Likewise, the level of eotaxin could also be presumed to be increased in the large intestine of allergic diarrhea mice (Figure 4). Interestingly however, the level of eotaxin synthesis was significantly decreased by the treatment with anti-IL-12p40 mAb (Figure 4). These results indicate that anti-IL-12p40 mAb inhibited the immunopathological Th2 cytokine environment of the large intestine in allergic diarrhea mice. Thus, an interesting scenario could be the presence of high levels of IL-12p40 monomer and/or homodimers instead of IL-12p70 in the disease site of OVA-induced allergic diarrhea mice. Therefore, treatment with anti-IL-12p40 mAb might result in the inhibition of Th2-type responses in the large intestine of allergic disease mice.

IL-12p40-Deficient Mice Do Not Develop Allergic Diarrhea

To directly confirm the pathological role of IL-12p40 in the development of allergic diarrhea, IL-12p40 knockout (KO) mice were used. IL-12p40 KO mice did not develop the allergic diarrhea completely (Figure 5A). OVA-induced IL-4 production by large intestinal LP mononuclear cells was not detected in IL-12p40 mice (Figure 5B). The levels of other Th2-type cytokines (IL-5 and IL-10) were also reduced in IL-12p40 KO mice (data not shown). Taken together, these results clearly show that IL-12p40 plays an important role in the development of this large intestinal allergic disease.

Discussion

Our present findings provide new, strong evidence for an immunopathological role for locally produced IL-12p40 in the development of OVA-induced allergic diarrhea. Here we demonstrate the high expression of IL-12p40, without IL-12p35, in the large intestine but not in the small intestine of mice with allergic diarrhea. These IL-12p40 were locally produced by large intestinal MØ, DC, and epithelial cells. Based on our knowledge, this is the first demonstration of the presence of IL-12p40 in the selected part of the intestinal tract (eg, large intestine) in mice with allergic diarrhea. Although IL-12 is thought to drive the Th1-dominant environment,[31] our present findings provide additional supportive evidence that IL-12p40 contributes to the generation of a Th2-dominant environ-

ment.[18,19] It should be noted that our results directly demonstrate the *in vivo* immunopathological contribution of locally produced mucosal IL-12p40 to the development of OVA-induced diarrhea. Thus, the anti-IL-12p40 treatment reduced the incidence of OVA-induced allergic diarrhea. An attractive explanation would be that large intestinal MØs and DCs as well as epithelial cells contribute to the development of pathological Th2-dominant responses by the production of IL-12p40 in OVA-induced allergic diarrhea. Thus, the administration of anti-IL-12p40 resulted in the inhibition of the locally produced, mucosal IL-12p40-created, pathological Th2 condition, leading to the reduction of disease development.

Our present and previous results clearly show that large intestinal antigen-specific Th cells produce high levels of Th2 cytokine in OVA-induced allergic diarrhea.[1] The presence of monomeric or dimeric forms of IL-12p40, behaving as an antagonist to IL-12p70, is an additional contributing factor for the creation of a dominant pathological Th2 environment. Thus, the severe symptoms of allergic diarrhea were reduced by treatment with anti-IL-12p40 mAb, since the production of Th2 cytokines was significantly decreased in the large intestine. Overall, IL-12p40-supported, Th2-type cytokine synthesis plays a critical and pathological role in the induction of allergic reactions in large intestinal tissues. Although we do not have any specific explanation for the generation of IL-12p40 at the disease site, one possibility could be antigen overload in the intestinal tract. Our previous study demonstrated that oral administration of high doses of OVA induced Th2-mediated allergic diarrhea in systematically pre-sensitized BALB/c mice.[1] In contrast, low doses of oral OVA failed to induce allergic diarrhea. It was also shown that high doses of OVA peptide increased the numbers of naive $CD4^+$ T cells with Th2-like phenotype, which in turn produced dramatically large amounts of IL-4.[32] Therefore, high doses of oral antigen may create an immunological environment favoring Th2 cell development. To support this view, it has also been shown that high doses of oral antigen preferentially inhibit IFN-γ-producing Th1-type cells.[1] Further, the dose of antigen can determine whether Th1- or Th2-type cells are generated by antigen-presenting cells including DC.[33] Taken together, these findings allow us to postulate that an overload of oral antigen may direct mucosal antigen-presenting cells, including DC and MØ, and epithelial cells, to produce monomeric or dimeric forms of IL-12p40 instead of IL-12p70.

IL-12 has been considered as an inhibitory factor for allergic responses induced by preferential Th2-cytokine production. Indeed, endogenous rIL-12 decreased IgE levels and Th2 cytokine production induced by allergic reaction.[34] In contrast, IL-12 has also been shown to be involved in the pathological phase of mucosa-associated allergic diseases of the respiratory tract. In the murine asthma model, IL-12 contributed to the recruitment of eosinophils into the respiratory tract via the induction of VCAM-1 on local vascular epithelial cells.[35] Thus, the deletion of the IL-12 gene (p40) resulted in a substantial reduction in the airway recruitment of eosinophils and in the expression of VCAM-1 when compared with wild-type mice exhibiting an asthma-like reaction induced by systemic sensitization followed by nasal OVA.[36] In addition, selective overexpression of IL-12p40 was noted in airway epithelial cells and bronchoalveolar lavage fluids of patients with asthma.[20] Our present findings also demonstrate that the locally produced p40 form of IL-12 was associated with the development of OVA-induced allergic diarrhea. Thus, IL-12p40 was preferentially expressed only in the large intestine of allergic diarrhea mice. In addition to these results generated through the characterization of an asthma model, our present finding suggests a critical role for IL-12, especially that of p40-associated molecules, for the development of allergic diseases including asthma and food allergy.

The treatment with anti-IL-12p40 mAbs effectively reduced the incidence as well as the severity of allergic diarrhea, an effect most likely due to an alteration in the dominant immunopathological Th2-type response to a Th1-type environment. To support this view, locally overexpressed IL-12p40 may compete with the well-known Th1 promoter IL-12p70 and IL-23 (p40/p19).[37–39] To this end, it has been shown that endogenous IL-12p40 can overcome the Th1-promoting activity of IL-12p70 and/or IL-23.[18] In this regard, our recent and separate study showed that IL-23p19-specific mRNA expression was not detected in either diseased or healthy BALB/c mouse groups (data not shown). The results suggest that IL-23 dose not play an important role in the development of our diarrhea model. Therefore, treatment with anti-IL-12p40 antibody likely eliminated the antagonistic effect of IL-12p40 at the local site, perhaps leading to the creation of an IL-12p70 environment for the initiation of down-regulation of Th2 responses. An alternative explanation would be that anti-IL-12p40 mAb used in this experiment may possess a higher affinity for the monomeric or dimeric form of IL-12 than for the IL-12p70 heterodimer. Although our emphasis has been on the inhibitory effects of anti-IL-12p40 mAb for the prevention of allergic diarrhea, one must accept the fact that complete prevention of disease development was never achieved through use of mAbs. A possible explanation for this finding could be that anti-IL-12p40 mAb inhibited Th1 induction of IL-12p70 in addition to IL-12p40. Thus, this alteration of a Th2 dominant environment and shift to one of a Th1-type may partially occur in the large intestine of mice with allergic diarrhea. To support this possibility, the mAb used in these experiments has been shown to neutralize IL-12p70 in addition to IL-12p40.[13,14] In addition, the experiments using IL-12p40 KO mice suggest that the absence of IL-12p40 results in a complete failure to develop allergic diarrhea. It clearly shows that IL-12p40 play a critical role in the development of this disease. However, one alternative and simple expectation would be that an IL-12p40 deficiency may lead to the creation of Th2 environment due to the lack of Th1 inducing IL-12p70. Thus, it may lead to the more susceptible condition for the development of Th2-mediated diarrhea. Although we do not have any specific data to negate the latter possibility, one possible explanation would be that the deficiency of IL-12p70 formation in IL-12p40 KO mice lead to the lack of ability to active antigen presenting cells. IL-12p70 deficiency may

result in the absence of induction antigen-specific T cell response including the pathological Th2-type cells. It has been shown that IL-12 or IL-12-induced IFNγ can directly activate antigen presenting cells.[40] To address the issue, a series of interesting experiment would be the adaptive transfer of large intestinal MØ, DC, and epithelial cells into IL-12p40 and/or p35 KO mice. These experiments are, of course, planned for our future study.

Recently, it has been suggested that IL-12 is also one of the key cytokines for the regulation of the intestinal immune response.[41] Mouse IL-12p40 is produced as monomer and homodimer five to ninety times as frequently as IL-12p70 *in vivo* and *in vitro*,[40,42] implying the existence of additional immunological roles for IL-12p40. An interesting possibility would be that excess production of the monomeric and/or the homodimeric form of IL-12p40 could be a key contributing factor to the maintenance of immunological homeostasis at the mucosal compartment. Interestingly, our present findings demonstrate that over-expression of IL-12p40 occurred only in the large but not the small intestine following oral exposure to high doses of protein antigen. At the present time, we cannot offer any specific explanation for this distinct localization of IL-12p40. However, an interesting possibility would be that the expression of negative regulators for IL-12, including sCD40L and IL-10R,[14,43] could differ between the small and large intestine. To support this possibility, epithelial cells have been shown to express CD40 and IL-10R.[34,44] Since the large intestinal tract is continuously exposed to overloaded microflora, the level of co-stimulatory molecule expression such as CD40 by large intestinal epithelial cells could be lower to avoid unnecessary inflammatory responses. Thus, the large intestinal tract may form an immunological environment favoring the generation of IL-12p40. This interesting possibility is currently being tested in our laboratory.

In summary, our results demonstrated that locally produced IL-12p40 contribute to the Th2 cell generation of pathological polarization in the large intestine of OVA-induced allergic diarrhea. This study provides the first evidence for the association of over-expressed IL-12p40 from intestinal epithelial cells, DC and MØ, in the development of allergic diarrhea. Thus, the application of anti-IL-12p40 mAb resulted in the reduction of disease incidence and severity. Further, the disease development was completely eliminated in the deletion of IL-12p40 gene. Taken together, our studies provide an opportunity to consider that anti-IL-12p40 mAbs may be an alternative therapeutical regimen for the control of allergic intestinal disease.

Acknowledgments

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Non-toxic Stx derivatives from *Escherichia coli* possess adjuvant activity for mucosal immunity

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Abstract

Both B subunit of Shiga toxin 1 (Stx1-B), which mediates the binding of toxin to the membrane, and mutant Stx1 (mStx1), which is a non-toxic double-mutated Stx1 harboring double amino acid substitutions in the A subunit, possess potent mucosal adjuvant activity. Nasal immunization of mice with ovalbumin (OVA) plus the Stx1-B or mStx1 induced OVA-specific serum IgG and mucosal IgA responses. IgG subclass analysis revealed that mStx1 and Stx1-B as mucosal adjuvants supported Ag-specific IgG1 followed by IgG2b Abs. The co-administration of either mStx1 or Stx1-B with OVA enhanced the production of IL-4, IL-5, IL-6 and IL-10 with low IFN-γ, by OVA-specific $CD4^+$ T cells. To better elucidate the mechanisms underlying mStx1's and Stx1-B's adjuvant activity, we next sought to examine whether or not dendritic cells (DC) residing in the nasopharyngeal-associated lymphoreticular tissue (NALT) were activated by nasal administration of Stx1-B or mStx1. We found that mice nasally administered with Stx1-B or mStx1 showed an up-regulation in the expression of CD80, CD86 and especially CD40 on NALT DCs. Taken together, these results suggest that non-toxic Stx derivatives could be effective mucosal adjuvants for the induction of Th2-type, $CD4^+$ T cell mediated, antigen-specific mucosal IgA and systemic IgG Ab responses, and that they likely owe their adjuvant activity to the up-regulation of co-stimulatory molecules including CD80, CD86 and CD40 on NALT DCs.

Keywords: Stx; Adjuvant

1. Introduction

Adjuvants are key to the development of effective mucosal vaccine because they can compensate for the often poorly immunogenic nature of orally and nasally administered vaccine antigens by inducing vaccine antigen-specific humoral and/or cellular immune responses. Much of the protection available at mucosal surfaces such as the respiratory, gastrointestinal and urogenital tracts is provided by the production of secretory IgA (S-IgA) antibodies (Abs) which are effectively produced only when vaccine is administered by a mucosal route [1]. In an effort to develop new strategies to curb global infection, researchers in the field are currently trying to develop novel adjuvants which can be nasally or orally co-administered with vaccine antigen to maximize the induction of protective S-IgA antibodies.

Thus far, several bacterial enterotoxins including cholera toxin (CT) of *Vibrio cholerae* and heat labile enterotoxin (LT) of enterotoxigenic *Escherichia coli* have been identified as possessing strong immunoenhancing activity against co-administered protein antigen when given by the oral or nasal routes [2–5]. By eliciting antigen (Ag)-specific Th2-type $CD4^+$ T cell responses with high levels of IL-4 and IL-5 production, mucosally co-administerated CT enhances the generation of Ag-specific systemic IgG1, IgE and mucosal S-IgA responses [6]. In contrast, LT induces a mix of IFN-γ-producing $CD4^+$ Th1-type and IL-4-, IL-5-, IL-6-, and IL-10-secreting Th2-type cells for subsequent induction of serum IgG1, IgG2a, and mucosal S-IgA Ab

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responses [7]. Other bacterial toxins such as pertussis toxin and PT-9K/129G, the genetically detoxified derivative of pertussis toxin have also been shown to possess mucosal adjuvant activities [8,9]. Pertussis toxin potentiates both Th1 and Th2 responses to antigen co-injected via the systemic route [10]. The mucosal presentation of a chimeric molecule composed of the gp 120V3 loop region of the MN strain of HIV-1 and a non-toxic form of *Pseudomonas* exotoxin resulted in strong mucosal and systemic immune responses to an integrated HIV-Ag [11]. However, Shiga toxin (Stx) was found to possess immunogenicity but not adjuvant activity when given via the oral route [12].

Stx, which is generated by Stx-producing *E. coli* (STEC), is one of the major virulence factors for STEC infectious diseases. Stx is a holotoxin composed of an A subunit measuring approximately 32-kDa in non-covalent association with a pentameric ring of an identical B subunit, each with a molecular mass of 7.7-kDa [13]. The A subunit is the enzymatic component of the toxin and acts as a highly specific *N*-glycosidase enzyme hydrolyzing the bond between ribose and a single adenine residue found on a prominent loop structure in the 28S rRNA component of eukaryotic ribosomes [14,15]. The B subunits mediate the binding of toxin to the neutral glycolipids of cell membranes, globotriaosylceramide and globotetraosylceramide [16]. Stx is classified into two groups: Stx1, the amino acid sequence of which is identical to that of Shiga toxin: Stx2, which is immunologically distinct from Stx1 [17].

In an effort to develop a candidate for a vaccine against infectious diseases caused by enterohemorrhagic *E. coli*, we have previously used site-directed mutagenesis to generate E167Q & R170L (mStx1), a double mutant of Stx1, harboring double amino acid substitutions in its RNA *N*-glycosidase active center [18]. Due to these mutations, mStx1 lacks RNA *N*-glycosidase activity, cytotoxicity and mouse lethality [18]. In the present study, we have addressed whether or not mStx1 and Stx1-B elicit mucosal adjuvant activity when co-administered nasally with protein antigen. Furthermore, we have assessed the capability of nasally administered mStx1 and Stx1-B to activate dendritic cells (DC) in nasopharyngeal-associated lymphoreticular tissue (NALT). Our results suggest that both mStx1 and Stx1-B are effective mucosal adjuvants for the induction of Ag-specific Ab responses in both mucosal and systemic compartments. Moreover, when applied nasally, they are also capable of up-regulating co-stimulatory molecules including CD80, CD86 and CD40 on NALT DCs.

2. Materials and methods

2.1. Mice

C57BL/6 mice purchased from SLC (Shizuoka, Japan) or Cler Japan, Inc. (Tokyo, Japan) were maintained and bred in the experimental animal facility at Osaka University under pathogen-free conditions in microisolator cages. All mice were provided sterile food and water ad libitum. C57BL/6 mice were used in this study at 8–12 weeks of age.

2.2. Bacterial toxins

The mutant of Stx1 (mStx1), native (n)Stx1 and nStx2 were purified from *E. coli* MC 1061 strain M 23, strain 87-27 and strain Tp 8, respectively [18,19]. As described previously, purification steps included ion-exchange, chromatofocusing and HPLC as described previously [19]. The B subunit of Stx1 (Stx1-B) was derived from *Bacillus brevis* pNU212-VT1B and was purified by the use of ion-exchange and gel filtration [20].

The amount of endotoxin was measured in the toxin preparation with an Endospec-SP test (Seikagaku Co., Tokyo, Japan). The nStx1, nStx2, mStx1 and Stx1-B used in this study contained 7.03, 9.52, 34.0 and 3.05 pg of *lipopolysaccharide* (LPS) per 10 μg of protein, respectively. The range of these LPS contents (e.g. 3-35 pg/10 μg protein) has been shown to have no biological effect on the immune system [21,22].

2.3. Immunization protocol and sample collection

A standard nasal immunization protocol was used in this study [23]. Mice were nasally immunized on days 0, 7 and 14 with a 10 μl aliquot (5 μl per nostril) containing 100 μg of ovalbumin (OVA; Sigma, St. Louis, MO) alone or combined with various doses of mStx1, Stx1-B, nStx1 or nStx2 as mucosal adjuvants [23]. Saliva was obtained from mice following i.p. injection with 100 μl of 1 mg/ml pilocarpine (Sigma). Nasal washes were collected by gently flushing the nasal passage with 100 μl of sterile PBS [23].

2.4. Analysis of antibody responses

Ag-specific Ab titers in serum, saliva, and nasal washes were determined by ELISA as described previously [6,24]. Briefly, plates were coated with OVA (1 mg/ml) and blocked with 1% BSA in PBS. After the plates were washed, serial dilutions of serum, saliva, or nasal washes were added in duplicate. Following incubation, the plates were again washed and peroxidase-labeled goat anti-mouse μ, γ or α heavy chain-specific Abs [Southern Biotechnology Associates (SBA), Birmingham, AL] were added to appropriate wells. Finally, 3,3′,5,5′-tetramethylbenzidine (TMB) with H_2O_2 was added for color development.

For IgG subclass analysis, biotinylated rat monoclonal anti-mouse γ1 (G1-7.3), γ2a (R19-15), γ2b (R12-3) or γ3 (R40-82) heavy chain-specific Abs (BD PharMingen, San Diego, CA) and streptavidin-conjugated peroxidase (Vector Laboratories, Inc., Burlingame, CA) were employed. For the analysis of total IgE antibodies, OptEIA ELISA for IgE was used (BD PharMingen). Endpoint titers were expressed

as the reciprocal $\log_2$ of the last dilution giving an optical density at 450 nm (OD_{450}) of $\geq$0.1 above negative control.

2.5. Detection of Ag-specific Ab-forming cells (AFCs) by the enzyme-linked immunospot (ELISPOT) assay

In the ELISPOT assay, numbers of Ag-specific AFCs from various tissues including salivary glands, nasal passages and spleens were determined by direct counting of spots as previously described in detail [4,24]. Ninty-six-well nitrocellulose-based plates (MultiScreen-HA, Millopore Co., Bedford, MA) were coated with 1 mg/ml of OVA diluted in PBS for enumeration of Ag-specific AFC. Wells were blocked with RPMI1640 medium containing 10% FCS, HEPES buffer (15 mM), L-glutamine (2 mM), penicillin (100 U/ml), and streptomycin (100 μg/ml) (complete medium). Cells at various dilutions were added and incubated for 6 h at 37 °C in 5% CO_2 in moist air. Antigen-specific AFCs were detected with peroxidase-labeled anti-mouse μ, γ, or α Ab (SBA) and then visualized by adding the chromogenic substrate, 3-amino-9-ethylcarbazole (Moss. Inc., Pasadena, MD). Spots were counted with the aid of a dissecting microscope (SZH Zoom Stereo Microscope System, Olympus, Lake Success, NY).

2.6. OVA-specific $CD4^+$ T cell responses

$CD4^+$ T cells were purified from cervical lymph node (CLN) and splenic cell suspensions by use of the magnet-activated cell sorter system (Miltenyi Biotec) [24]. Cells were added to a nylon wool column (Polysciences, Warrington, PA) and incubated at 37 °C for 1 h to remove adherent cells. The $CD4^+$ T cell subset was then obtained by positive sorting using a magnetic bead separation system consisting of anti-CD4 monoclonal (m)Ab (clone GK1.5)-conjugated microbeads (MACS; Miltenyi Biotec). Purified $CD4^+$ T cells (>98% purity) were cultured at a density of 4×10^6 cells/ml with OVA (1 mg/ml) with T cell-depleted, irradiated (3000 rads) splenic feeder cells (8×10^6 cells/ml) and rIL-2 (10 units/ml; PharMingen) in complete medium [23]. The $CD4^+$ T cell cultures were incubated for 3 days at 37 °C in 5% CO_2 in air. Culture supernatants were then harvested for quantitation of secreted IFN-γ, IL-4, IL-5, IL-6 and IL-10 by a commercial AN'LYZA immunoassay kit (R&D Systems, Minneapolis, MN). To measure the levels of Ag-specific T cell proliferation, 0.5 μCi of [^{3}H]thymidine (Amersham Pharmacia Biotech) was added to individual cultures 18 h before termination, and the uptake of [^{3}H]thymidine in counts per minute (cpm) was determined by scintillation counting [25].

2.7. FACS analysis

Cells were analyzed by FACS (FACS Calibur & CellQuest; Becton Dickenson Co. Inc., San Jose, CA) using the following antibodies from BD PharMingen: fluorescerin isothiocyanate (FITC)-conjugated anti-mouse CD11c (clone HL3), phycoerythrin (PE)-conjugated anti-mouse CD80 (clone 16-10A1), PE-conjugated anti-mouse CD86 (clone GL1), PE-conjugated anti-mouse I-A^b (clone AF6-120.1), and PE-conjugated CD40 (clone 3/23).

2.8. Isolation of NALT DC

NALT was isolated and then rinsed in complete medium [23] before being digested with collagenase D (400 Mandl units/ml; Roche, Indianapolis, IN), as previously described [26]. Briefly, NALT was incubated with collagenase D (400 Mandl units/ml) and DNase I (200 μg/ml; Roche) for 35 min at 37 °C in RPMI 1640 medium, and EDTA at a final concentration of 5 mM was added during the last 5 min of incubation. For the enrichment of DC, released cells were layered over a metrizamide gradient column (Accurate, Westbury, NY; 14.5 g of metrizamide added to 100 ml of complete medium) and centrifuged, and the low-density fraction was collected as DCs [27]. The enriched DC cells were counted and then stained with the appropriate monoclonal antibodies, as described above for FACS analysis.

2.9. Statistical analysis

The results are reported as mean ± one standard error (S.E.). Statistical significance ($P < 0.05$) was determined by Student's *t*-test and by the Mann–Whitney *U*-test of unpaired samples.

3. Results

3.1. Induction of Ag-specific systemic Ab responses by nasal administration of OVA and mStx1 or Stx1-B

We began by assessing whether nasal co-administration of newly developed non-toxic Stx1 derivatives such as mStx1 or Stx1-B would provide mucosal adjuvant activity for the induction of Ag-specific Ab responses (Fig. 1A). Mice were nasally immunized with an optimal dose of OVA in the presence or absence of different concentrations of the adjuvant candidates. Although all doses of mStx1 tested in this study (e.g. 0.1–20 μg) provided the adjuvant activity, administration of 0.5 μg of mStx1 resulted in the highest OVA-specific IgM and IgG Ab responses among several doses tested (data not shown). Of all the dosages of Stx1-B tested (e.g. 0.1–20 μg), the administration of 5 μg of Stx1-B produced the most impressive serum IgM and IgG anti-OVA Ab responses. Although a dosage of 0.5 μg of the native form of Stx1 (nStx1) resulted in some adjuvant activity, high doses (e.g. 2 μg) of nStx1 proved universally lethal to mice ($n = 5$) (data not shown). Due to its lethality, native Stx1 does not make a practical mucosal adjuvant candidate. That same lethality also makes nStx1 unsuitable for subcutaneous co-administration, as we found in an earlier study (unpub-

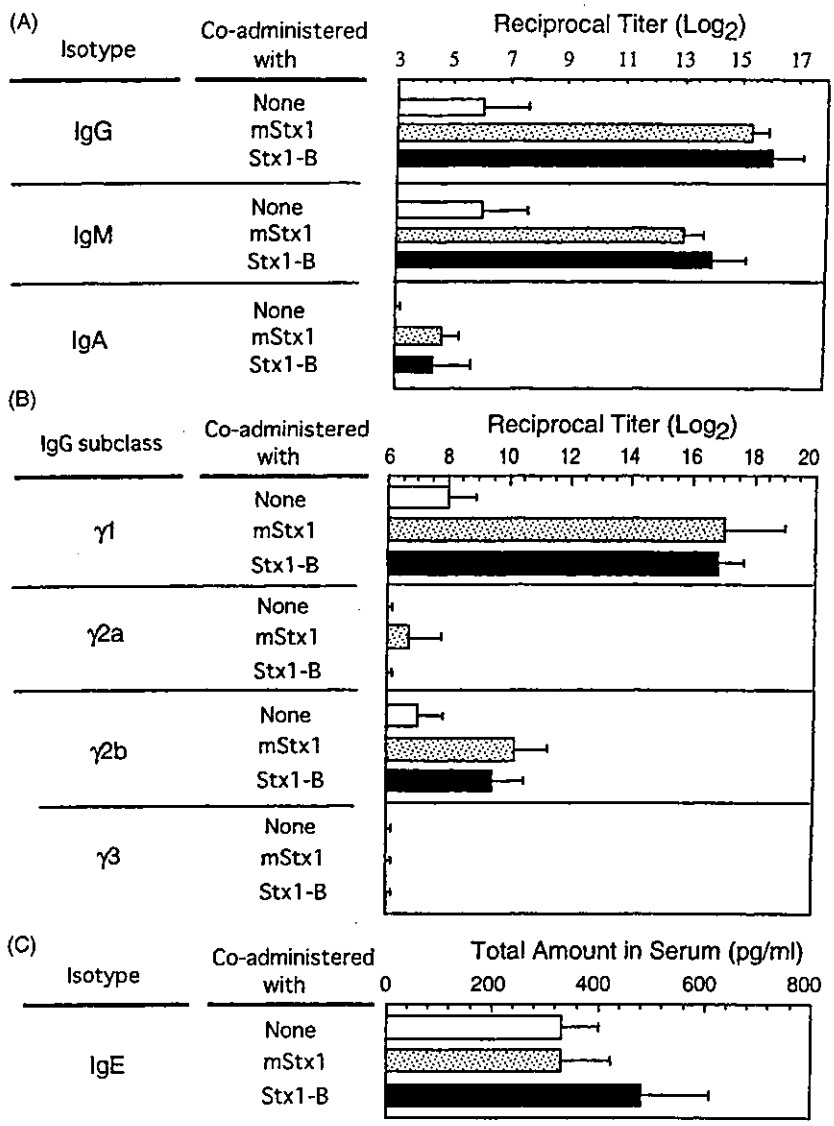

Fig. 1. Mucosal adjuvant activity of Stx1 derivatives for the induction of systemic antibody responses. OVA-specific serum IgM, IgG and IgA Ab responses (A), IgG subclass Ab responses (B) and total IgE responses (C) were examined and compared in serum of mice nasally immunized with OVA plus mStx1 or Stx1-B. Groups of C57BL/6 mice were nasally immunized with 100 μg of OVA plus 0.5 μg of Stx1 mutant (dotted bar) (E167R & R170L; mStx1), or 5 μg of Stx1-B (black bar) as mucosal adjuvant or with OVA alone (white bar) on days 0, 7 and 14. Serum samples were collected at day 21 and examined for OVA-specific IgM, IgG and IgA Abs, OVA-specific IgG subclass Ab responses and total IgE by ELISA. The results are expressed as the mean ± S.E.M. from five to six mice per group and from a total of three separate experiments.

lished data). In summary, we found mStx and Stx1-B to be the best candidates for possible mucosal adjuvants, since an optimal dose of 0.5 or 5 μg, respectively, induced serum IgM, IgG and relatively low IgA anti-OVA Ab responses (Fig. 1A). Consequently, the remainder of our experiments focused on the mucosal adjuvant activity of these two forms of non-toxic derivatives. As expected, antigen-specific Ab responses were low after nasal immunization with OVA alone (Fig. 1A). Analysis of OVA-specific IgG subclass responses revealed that co-administration of mStx1 or Stx1-B resulted in a major IgG subclass response with IgG1 subclass appearing, followed by IgG2b (Fig. 1B). The levels of total IgE were not statistically changed between mice immunized with OVA and Stx1 derivatives and those administered OVA alone (Fig. 1C). Further, nasal immunization of OVA and Stx1 derivatives did not mount for antigen-specific IgE antibodies (Stx1-B: 6.85 ± 0.05 and mStx1: 6.80 ± 0.02) when compared with OVA alone (<6.0) (data not shown).

When antigen-specific IgG antibody forming cells (AFC) in the spleen and cervical lymph node (CLN) of mice nasally immunized with OVA plus Stx1 derivatives were analyzed, significant numbers of OVA-specific IgG AFC were detected, confirming the results obtained by the characterization of OVA-specific serum Ab responses. In contrast, low numbers of OVA-specific IgG AFC were seen in spleen and CLN of mice given OVA alone (Fig. 2A).