Nasal Flt3 Ligand cDNA Elicits CD11c$^+$CD8$^+$ Dendritic Cells for Enhanced Mucosal Immunity[1]

Kosuke Kataoka,*† Jerry R. McGhee,* Ryoki Kobayashi,* Keiko Fujihashi,* Satoshi Shizukuishi,† and Kohtaro Fujihashi[2]*

Nasal immunization is an effective way to induce both mucosal and systemic immune responses. In this study, we assessed a cDNA vector for Flt3 ligand (FL) for its potential to enhance mucosal immunity or tolerance. Interestingly, tolerance was avoided and elevated levels of OVA-specific Ab responses were induced in nasal washes, fecal extracts, and saliva as well as in plasma when compared with mice given nasal OVA plus DNA plasmid without the FL gene. In addition, significant levels of OVA-specific CD4$^+$ T cell proliferative responses and OVA-induced IL-4 and IL-2 production were noted in spleen and cervical lymph nodes. Further, marked increases in FL protein occurred in the nasal lamina propria and submandibular glands and the frequencies of CD11c$^+$CD8$^+$ dendritic cells (DCs) significantly increased in the mucosal tissues. Moreover, these DCs expressed high levels of CD40, CD80, CD86, and MHC class II molecules. Nasal delivery of plasmid FL with OVA resulted in FL expression in both mucosal inductive and effector sites and resulted in expanded activated lymphoid DCs. Thus, nasal plasmid FL prevents mucosal tolerance and enhances active immunity when given by a mucosal route. ***The Journal of Immunology,* 2004, 172: 3612–3619.**

Flt3 ligand (FL),[3] a type 1 transmembrane protein, binds either feral liver kinase 2 (flk2) or fms-like tyrosine kinase 3 (flt3) receptor. FL mobilizes and stimulates myeloid and lymphoid progenitor cells (1), dendritic cells (DCs) (2), and NK cells (3). In addition, FL given with IL-7 costimulates the expansion of primitive B cell progenitors (4). Thus, FL has multiple roles in early hemopoiesis and B lymphopoiesis (4). In this regard, FL activates stem cells in bone marrow and promotes their differentiation into mature B cells (4). Furthermore, FL given with IL-15 expands NK cell progenitors (3). It has been shown that daily injection of FL into mice resulted in dramatic increases in DCs in the bone marrow, the peritoneal cavity, spleen, and thymus, and subsequently enhanced Ag-specific immune responses, comparable to those supported by cholera toxin (CT) when used as mucosal adjuvant (2, 5, 6). Other studies have now shown that the FL treatment also favors the induction of immune responses when given by mucosal (5), systemic (7), or cutaneous routes (8). In each of these studies, mice were injected daily with recombinant FL on consecutive days. In recent studies, plasmid DNA encoding FL has been given with plasmids encoding protein Ag (9) or linked to the Ag itself (10). These studies confirm the adjuvant activity of FL for both Ab- and cell-mediated immunity and suggest that frequent treatment with recombinant FL can also be accomplished by injection of FL cDNA itself.

Nasal Ag delivery is the most effective way to induce mucosal secretory-IgA (S-IgA) Ab responses and plasma-specific Abs (11–13). To elicit maximal levels of Ag-specific immune responses in both mucosal and systemic lymphoid tissue compartments, it is necessary to use an appropriate mucosal adjuvant (11, 14, 15). To date, both CT produced by *Vibrio cholerae* and the heat-labile enterotoxin from *Escherichia coli* are two of the most potent mucosal adjuvants for enhancement of Ag-specific Ab responses when coadministered with protein Ag by either the oral or nasal routes (16–18). It is known that CT acts as a mucosal adjuvant by inducing CD4$^+$ Th type 2 (Th2) cells and the cytokines IL-4, IL-5, IL-6, and IL-10, which are essential for induction of IgA, IgG1, and IgE Ab responses (19–23). In contrast, mucosal delivery of heat-labile enterotoxin results in mixed Th1 and Th2-type CD4$^+$ T cells with subsequent mucosal S-IgA Ab responses (24). In addition, it has been shown that mucosal application of cytokines or chemokines with protein Ag successfully induces both mucosal and systemic CD4$^+$ T cell and Ab responses. For example, nasal immunization with tetanus toxoid and IL-12 enhanced tetanus toxoid-specific Ab responses (25). Furthermore, both IL-1α and IL-1β are potent mucosal adjuvants (26). Finally, it has been shown that lymphotactin and RANTES possess mucosal adjuvant activity for enhanced Ag-specific Ab responses (27, 28).

It is well known that mucosal administration of protein Ag without appropriate adjuvant or delivery systems generally induces systemic unresponsiveness to the same Ag. This unresponsive state has been termed oral tolerance (29). In general, prolonged and frequent low doses of Ag administration or a single high dose of Ag delivery is required for the induction of mucosal tolerance. However, it has been shown that conjugation of Ag with the B subunit of CT (CT-B) facilitated induction of systemic unresponsiveness by reducing the levels and numbers of Ag doses required (30). Further, it was also reported that the expansion of DCs by parental injection of FL enhanced oral tolerance induction to OVA (31). Thus, oral tolerance was established in FL-treated mice using

*Departments of Oral Biology and Microbiology, Immunobiology Vaccine Center, University of Alabama at Birmingham, Birmingham, AL 35294; and †Department of Preventive Dentistry, Graduate School of Dentistry, Osaka University, Suita, Osaka, Japan

Received for publication September 29, 2003. Accepted for publication January 14, 2004.

The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked *advertisement* in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

[1] This work is supported by National Institutes of Health Grants and Contracts DE 12242, AI 43197, DC 04976, AI 65299, AI 18958, and DK 44240.

[2] Address correspondence and reprint requests to Dr. Kohtaro Fujihashi, Departments of Oral Biology and Microbiology, Immunobiology Vaccine Center, University of Alabama at Birmingham, Bevill Biomedical Research Building 761, 845 19th Street South, Birmingham, AL 35294-2170. E-mail address: kohtarof@uab.edu

[3] Abbreviations used in this paper: FL, Flt3 ligand; DC, dendritic cell; CT, cholera toxin; S-IgA, secretory-IgA; FE, fecal extract; CLN, cervical lymph node; NP, nasal passage; NALT, nasopharyngeal-associated lymphoreticular tissue; SMG, submandibular gland; LP, lamina propria; AFC, Ab-forming cell; NW, nasal wash; MHC II, MHC class II.

Copyright © 2004 by The American Association of Immunologists, Inc. 0022-1767/04/$02.00

very low doses of OVA that generally failed to induce tolerance in normal mice (31).

To date, no reports have determined the outcome of immune responses when FL or FL cDNA is given with Ag by mucosal routes. Our study was designed to examine whether a nasal plasmid encoding FL given with OVA would simultaneously enhance mucosal immunity or alternately, induce mucosal tolerance. We show here that an expression vector containing the FL gene given with the protein OVA nasally resulted in FL protein expression in the nasal tract which enhanced OVA-specific Ab responses instead of tolerance induction through a dramatic expansion of activated, mature DCs.

Materials and Methods

Mice

Female C57BL/6 mice (6- to 8-wk-old) were purchased from the Frederick Cancer Research Facility (Frederick, MD). These mice were transferred to microisolators and maintained in horizontal laminar flow cabinets, and provided sterile food and water as part of a specific pathogen-free facility. The health of the mice was monitored by both serology for bacterial and viral pathogens and immunohistology. All of the mice used in these experiments were free of bacterial and viral pathogens.

DNA plasmid

The plasmid pORF9-mFLt3L (pFL) consists of the pORF9-mcs vector (pORF) plus the full-length murine FL cDNA gene (InvivoGen, San Diego, CA). The pORF is an expression vector containing the hybrid elongation factor (EF)1-α/human T cell leukemia virus promotor and the ampicillin resistance gene (858 bp) which allowed selection of bacteria carrying the plasmid. This plasmid DNA was purified using the GeneElute Endotoxin-Free Plasmid kit (Sigma-Aldrich, St. Louis, MO). The *Limulus* amebocyte lysate assay (BioWhittaker, Walkersville, MD) resulted in <0.1 endotoxin unit of LPS per 1 μg of plasmid.

Nasal immunization and sample collection

Mice were immunized three times at weekly intervals nasally with 6 μl/nostril PBS containing 50 μg of pFL and 100 μg of OVA (fraction V; Sigma-Aldrich). As controls, mice were immunized nasally with 50 μg of pORF (empty plasmid) and 100 μg of OVA under anesthesia. Plasma, saliva, and fecal extracts (FEs) were collected on days 0 and 21. Stimulated saliva and FE samples were obtained as described previously (18, 32, 33). Mice were sacrificed 7 days after the last immunization (day 21) and nasal washes were obtained by instillation of 1 ml of PBS on three occasions into the posterior opening of the nasopharynx with a 30-gauge hypodermic needle (34, 35).

OVA-specific Ab assays

OVA-specific Ab levels in plasma and mucosal secretions were determined by ELISA as previously described (18, 33, 36, 37). Briefly, 96-well Falcon microtest assay plates (BD Biosciences, Oxnard, CA) were coated with 1 mg/ml OVA in PBS. After blocking with 1% BSA in PBS, 2-fold serial dilutions of samples were added to each well. Following incubation overnight at 4°C, HRP-labeled goat anti-mouse μ, γ, or α H chain-specific Abs (Southern Biotechnology Associates, Birmingham, AL) were added to wells. For IgG Ab subclass analysis, biotinylated mAbs specific for IgG1, IgG2a, IgG2b, and IgG3 (BD PharMingen, San Diego, CA) and peroxidase-conjugated goat anti-biotin Ab (Vector Laboratories, Burlingame, CA) were used for detection. The color reaction was developed for 15 min at room temperature with 100 μl of 1.1 mM 2,2′-azino bis (3-ethylbenzthiazoline-6-sulfonic acid) in 0.1 M citrate phosphate buffer (pH 4.2) containing 0.01% H_2O_2. Endpoint titers were expressed as the reciprocal $\log_2$ of the last dilution that gave an OD_{415} of 0.1 greater than background.

Enumeration of Ab-forming cells (AFCs)

Mononuclear cells from the spleen and cervical lymph nodes (CLNs) were isolated aseptically by a mechanical dissociation method using gentle teasing through stainless steel screens as described previously (18, 33, 37, 38). For isolation of mononuclear cells from nasal passages (NP) and nasopharyngeal-associated lymphoreticular tissues (NALT), a modified dissociation method was used based upon a previously described protocol (39–41). Mononuclear cells from submandibular glands (SMG), Peyer's patches, and lamina propria (LP) were isolated by a combination of an enzymatic dissociation procedure with collagenase type IV (0.5 mg/ml; Sigma-Aldrich) followed by discontinuous Percoll (Amersham Biosciences, Uppsala, Sweden) gradient centrifugation (32). Mononuclear cells obtained from mucosal and systemic lymphoid tissues were subjected to ELISPOT assay to detect numbers of OVA-specific AFCs (33, 37, 39, 41). Briefly, 96-well nitrocellulose plates (Millititer HA; Millipore, Bedford, MA) were coated with 1 mg/ml for analysis of anti-OVA-specific AFCs. The numbers of OVA-specific AFCs were quantified with the aid of a stereomicroscope as described elsewhere (18, 32, 38).

OVA-specific $CD4^+$ T cell responses

$CD4^+$ T cells from spleen and CLNs were purified by use of an automated magnetic activated cell sorter (AutoMACS) system (Miltenyi Biotec, Auburn, CA) as described previously (18, 37). Briefly, a nylon wool column enriched T cell fraction was incubated with biotinylated anti-CD4 mAb (GK 1.5) (BD PharMingen) followed by streptavidin-conjugated microbeads and sorted to purity with the AutoMACS. This purified T cell fraction was $>97\%$ $CD4^+$ and the cells were $>99\%$ viable. This purified $CD4^+$ T cell fraction was resuspended in RPMI 1640 (Cellgro; Mediatech, Washington, DC) supplemented with HEPES buffer (10 mM), L-glutamine (2 mM), nonessential amino acid solution (10 ml/L), sodium pyruvate (10 mM), penicillin (100 U/ml), streptomycin (100 μg/ml), gentamicin (80 μg/ml), and 10% FCS (complete RPMI 1640) (4×10^6 cells/ml), and cultured in the presence of 1 mg/ml OVA of cultures of T cell-depleted, irradiated (3000 rad) splenic APCs taken from nonimmunized, normal mice. To assess OVA-specific T cell proliferative responses, an aliquot of 0.5 μCi of tritiated [^{3}H]TdR (Amersham Biosciences, Arlington Heights, IL) was added during the final 18 h of incubation, and the amount of [^{3}H]TdR incorporation was determined by scintillation counting. The supernatants of identically treated T cell cultures not incubated with [^{3}H]TdR were then subjected to a cytokine-specific ELISA as described below.

Cytokine-specific ELISA

The levels of cytokines in $CD4^+$ T cell culture supernatants from spleen or CLNs were determined by cytokine-specific ELISA as described previously (36, 42–44). The culture supernatants were collected on day two for IFN-γ, IL-2, IL-4, IL-5, IL-6, and IL-10 analysis. The immunoplates (Nalge Nunc International, Rochester, NY) were coated with anti-cytokine capturing mAb. After blocking with 3% BSA in PBS, serial 2-fold diluted samples and standards were added, and incubated overnight at 4°C. The plates were washed and respective detection mAb was added. After incubation overnight at 4°C, HRP-labeled goat anti-biotin Ab (Vector Laboratories) was added and incubated for 4 h at room temperature. The color reaction was developed at room temperature with 100 μl of 1.1 mM 2, 2′-azino bis (3-ethylbenz-thiazoline-6-sulfonic acid) in 0.1 M citrate phosphate buffer (pH 4.2) containing 0.01% H_2O_2. The detection limits for each cytokine were: 106.3 pg/ml for IFN-γ, 15.6 pg/ml for IL-2, 4.66 pg/ml for IL-4, 156 pg/ml for IL-5, 156 pg/ml for IL-6, and 391 pg/ml for IL-10.

Quantitative analysis of cytokine-specific mRNA

For evaluation of cytokine-specific mRNA levels in OVA-stimulated $CD4^+$ T cells, real-time PCR was used with a LightCycler (Roche Applied Science, Indianapolis, IN) (45, 46). The $CD4^+$ T cells were harvested after 2 days of incubation and total RNA was isolated by the acid guanidium thiocyanate-phenol-chloroform extraction procedure. Aliquots of extracted RNA (25 μg/ml) were subjected to RT reaction using Superscript II Reverse Transcriptase (Invitrogen/Life Technologies, Carlsbad, CA). Samples were treated with 1 μl of 10 μg/ml RNase H (Invitrogen/Life Technologies) and incubated at 37°C for 30 min. The levels of synthesized cDNA were measured by GeneQuant RNA/DNA calculator (Amersham Biosciences). The sample cDNA and the external standards were amplified with cytokine-specific primers and SYBR Green I by using the LightCycler (Roche Applied Science). The specificity of PCR products was confirmed by a melting curve as well as by agarose gel electrophoresis. The concentration of sample cDNA was determined using linear, diluted external standards obtained by an identical PCR protocol with the LightCycler.

Flow cytometry analysis

Aliquots of mononuclear cells (0.2–1.0 $\times 10^6$ cells) were isolated from tissues of mice immunized with OVA plus pFL or mice given OVA plus pORF (empty plasmid). Cells were stained with FITC-conjugated anti-mouse CD11b, CD8, or B220 mAbs, PE-labeled anti-mouse I-A^b, CD11c, CD40, CD80, or CD86 mAbs (BD PharMingen) and biotinylated anti-mouse CD11c mAbs (BD PharMingen) followed by CyChrome-streptavidin. The samples were then subjected to FACS analysis (FACSCalibur; BD Biosciences).

Immunohistochemistry of NALT

NALT were taken from mice immunized nasally with OVA plus pFL or OVA plus pORF (empty plasmid) and were snap-frozen at −160°C. Cryostat sections of 4 μm were then fixed with 4% formaldehyde before staining using conjugated Abs. Sections were stained with PE-conjugated biotin coupled anti-CD40, -CD80, -CD86, and anti-CD11c mAbs. Biotin-labeled anti-CD11c mAb was followed by HRP-conjugated streptavidin-Alexa Fluor 488 (Molecular Probes, Eugene, OR). Sections were examined with a fluorescence microscope (BX50/BXFLA; Olympus, Tokyo, Japan) equipped with a digital image capture system (Olympus).

Detection of FL cDNA

To determine the tissues which take up the plasmid, DNA was extracted from the NPs, NALT, and SMG and amplified with ampicillin resistance gene-specific primers by PCR. Tissues were digested by proteinase K (100 μg/ml), and the DNA was extracted twice with phenol equilibrated with Tris buffer (pH 8.0) (47). For detection of the ampicillin resistance gene, extracted DNA was amplified by oligonucleotide primers specific for the ampicillin resistance gene (sense, 5′-CCAATGCTTAATCAGTGAGGC-3′; antisense, 5′-ATGAGTATTCAACATTTCCGTGTCG-3′). The PCR products were separated by electrophoresis in 2% agarose gels and visualized by UV light illumination following ethidium bromide (0.5 mg/ml) staining.

Analysis of FL protein expression

To determine the levels of FL protein present in nasal washes (NW), saliva, and plasma, samples were collected from mice given nasal Ag plus plasmid. These samples were then analyzed with a Quantikine M mouse Flt3 Ligand ELISA kit (R&D Systems, Minneapolis, MN).

Statistical analysis

The results are expressed as the mean ± SEM, and mouse groups were compared with control mice using an unpaired Mann-Whitney *U* test with Statview software (Abacus Concepts, Berkley, CA) designed for Macintosh computers. Values of p of <0.05 or <0.01 were considered significant.

Results

Nasal pFL administration enhances Ag-specific mucosal and plasma Ab responses

We initially examined whether nasal administration of FL plasmid (pFL) would enhance OVA-specific immune responses or alternatively would result in tolerance in both mucosal and systemic lymphoid tissues. Mice given nasal OVA plus pFL exhibited OVA-specific IgA Ab responses in NW, FEs, and saliva (Fig. 1*a*). In contrast, OVA-specific S-IgA Ab levels of mice immunized with either OVA alone or OVA with empty plasmid gene (pORF) did not produce S-IgA anti-OVA Ab responses (Fig. 1*a*). To further support these findings, mice given the nasal pFL plus OVA displayed high numbers of OVA-specific IgA AFCs in NPs and SMG. In addition, a high frequency of OVA-specific IgA AFCs were seen in small intestinal LP (Fig. 1*b*). Interestingly, our results showed that OVA-specific IgA AFCs were also effectively induced in mucosal inductive tissues themselves, i.e., in NALT and Peyer's patches following nasal immunization with pFL and OVA (Fig. 1*c*). These findings clearly show that nasal immunization with OVA and pFL is an effective way to induce Ag-specific IgA Abs in mucosal secretions. Further, this result clearly shows that nasal pFL does not induce mucosal tolerance. Elevated levels of OVA-specific plasma IgG Ab responses were seen in mice given nasal OVA plus pFL when compared with mice immunized with OVA plus empty plasmid (pORF) (Fig. 2*a*). The plasma from mice nasally immunized with OVA alone did not contain detectable Ab levels (data not shown). Both OVA-specific IgG1 and IgG2b Ab levels were markedly elevated when compared with control mouse groups (Fig. 2*b*). Relatively low IgG2a and no IgG3 Ab responses were seen (Fig. 2*b*). These findings suggest that a nasal vaccine containing FL plasmid induces a Th2-type cytokine-mediated OVA-specific immune response. To support this, the numbers of OVA-specific IgM, IgG, and IgA AFCs in spleen and CLNs were significantly increased when compared with control mice (Fig. 2*c*). Taken together, these results indicate that nasal immunization with pFL as mucosal adjuvant effectively induces OVA-specific Ab responses in both mucosal and systemic immune compartments. Further, pFL did not induce mucosal tolerance.

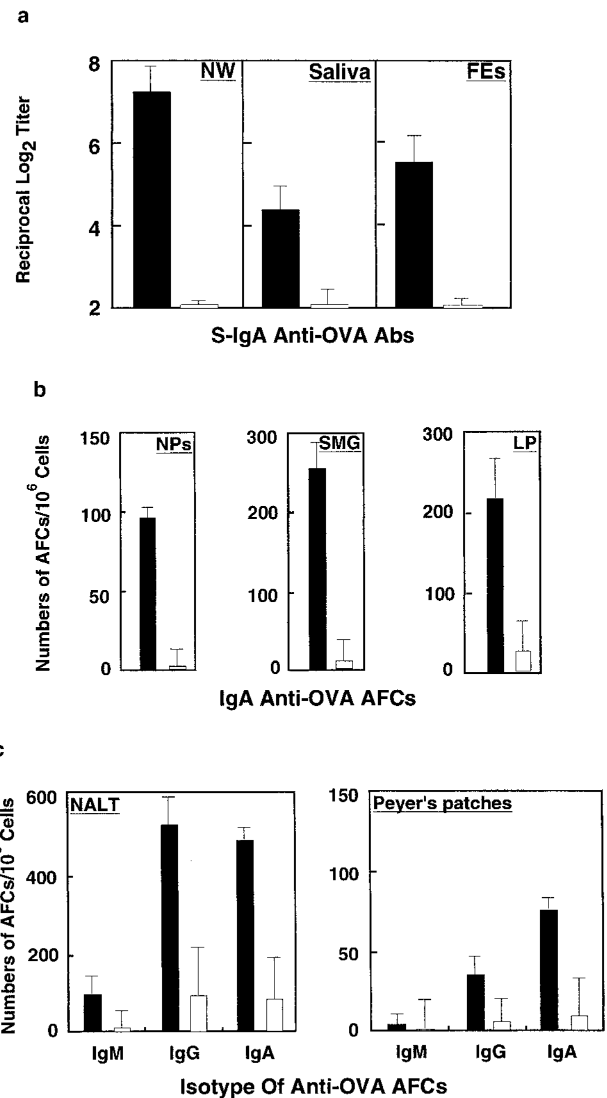

FIGURE 1. OVA-specific Ab responses in mucosal sites. *a*, Comparison of OVA-specific IgA Ab responses in NW, FEs, and saliva of mice immunized with OVA plus pFL (■) or OVA with the empty plasmid (pORF) (□). Each mouse group was nasally immunized weekly for three consecutive weeks with 100 μg of OVA plus 50 μg of pFL or pORF as mucosal adjuvants. Seven days after the last immunization, the levels of S-IgA anti-OVA Abs in NW, FEs, and saliva were determined by OVA-specific ELISA. The values shown are the mean ± SEM taken from 25 mice in each experimental group. *b* and *c*, Analysis of OVA-specific IgA AFCs in mucosal effector tissues and OVA-specific AFCs mucosal inductive sites. Each mouse group was nasally immunized weekly for three consecutive weeks with OVA plus pFL (■) and with OVA plus pORF (□). Seven days after the last immunization, mononuclear cells isolated from NPs, SMG, the small intestinal LP, NALT, and Peyer's patches were subjected to OVA-specific ELISPOT assay to determine the numbers of IgM, IgG, and IgA AFCs. Mice immunized nasally with OVA alone as a control group did not exhibit any anti-OVA AFCs. The values shown are the mean ± SEM of 25 mice in each experimental group.

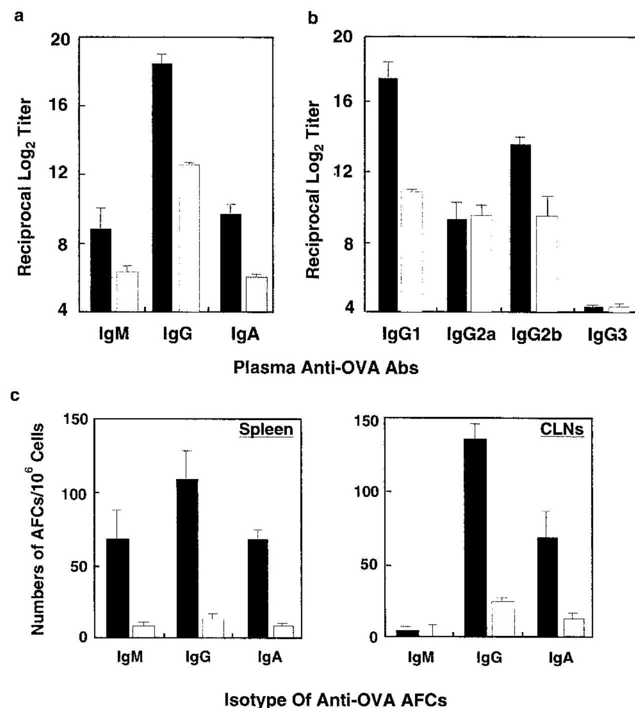

FIGURE 2. Comparison of OVA-specific Ab responses in systemic lymphoid tissues of mice immunized with OVA plus pFL (■) or OVA given with pORF (□). *a* and *b*, Each mouse group was nasally immunized weekly for three consecutive weeks. Seven days after the last immunization, IgM, IgG and IgA, or IgG subclass anti-OVA Ab responses in plasma were determined by OVA-specific ELISA. Mice immunized with OVA alone as a control group did not exhibit detectable plasma anti-OVA Abs. The values shown are the mean ± SEM of 25 mice in each experimental group. *c*, Analysis of OVA-specific AFCs in spleen and CLNs of mice given nasal OVA plus pFL (■) or OVA plus pORF (□) as mucosal adjuvants. Each mouse group was nasally immunized weekly for three consecutive weeks with 100 μg of OVA and 50 μg of pFL or pORF. Seven days after the last immunization, mononuclear cells were isolated from spleen and CLNs and were then subjected to OVA-specific ELISPOT assay to determine the numbers of IgM, IgG, and IgA AFCs. The values shown are the mean ± SEM of 25 mice in each experimental group.

Nasal pFL as mucosal adjuvant enhances OVA-specific $CD4^+$ T cell proliferative and cytokine responses

We next assessed OVA-specific $CD4^+$ T cell responses induced by the expression vector containing the FL gene as nasal adjuvant. Both splenic and CLN $CD4^+$ T cells from mice given nasal pFL plus OVA showed higher proliferative responses than did mice immunized with pORF plus OVA (Table I). We next examined levels of Ag-specific Th1- and Th2-type cytokine produced by OVA-specific $CD4^+$ T cells from spleen and CLNs of mice given nasal pFL as mucosal adjuvant. OVA-stimulated $CD4^+$ T cells isolated from spleen or CLNs of mice given nasal OVA plus pFL exhibited significantly higher levels of IL-2 and IL-4 production when compared with mice given OVA plus pORF (empty plasmid). In contrast, no significant increases in other Th1- or Th2-type (IL-5, IL-6, and IL-10) cytokines were seen (Table I). These results were further confirmed by quantitative real-time PCR. Thus, OVA-stimulated $CD4^+$ T cells from the spleen and CLNs of mice given nasal pFL contained significantly increased levels of IL-4-specific mRNA when compared with mice immunized nasally with OVA plus pORF (Table I). In addition, higher levels of IL-2-specific mRNA were detected in $CD4^+$ T cells from spleen and CLNs of mice given pFL as nasal adjuvant when compared with mice given pORF (Table I). In contrast, IFN-γ-specific mRNA levels were essentially identical between mice given pFL and empty plasmid (Table I). These results show that both IL-2 and IL-4 are essential cytokines for the induction of the adjuvant effects of pFL.

Nasal administration of pFL expands DCs in mucosal tissues

We next investigated the frequency of $CD11c^+$ DCs in various mucosal inductive and effector tissues. Our results showed major increases in numbers of $CD11c^+$ DCs in NALT, NPs, SMG (Table II), and LP (data not shown) of mice given nasal FL when compared with mice given pORF. It is important to note that the numbers of $CD11c^+$ DCs in the mucosal effector sites, such as NPs, NALT, and SMG were remarkably enhanced (Table II; Fig. 3, *a* and *b*). These results indicate that increased numbers of $CD11c^+$ DCs were due to nasal application of the expression plasmid encoding the FL gene. To characterize the phenotype of these expanded DCs, we assessed cell surface expression by $CD11c^+$ DCs in mucosal tissues of CD8, CD11b, B220, MHC class II (MHC II), CD40, CD80, and CD86. Interestingly, the majority of DCs in NPs

Table I. *OVA-induced $CD4^+$ Th1- and Th2-type cytokine profiles from mice given nasal OVA plus pFL or OVA and pORF*[a]

			Th1- and Th2-Type Cytokine[c,d] (pg/ml)			Levels of Cytokine-Specific cDNA[d,e] (Attomole/1 ng total cDNA)		
Tissue Used	Nasal OVA Plus	Stimulation Index[b]	IFN-γ	IL-2	IL-4	IFN-γ	IL-2	IL-4
Spleen	pFL	5.4 (±2.1)	780 (±110)	347* (±50)	31.3* (±1.1)	9.3 (±2.2)	72* (±23)	21* (±3.5)
	pORF	1.2 (±1.0)	320 (±146)	27 (±10)	17.2 (±1.8)	7.4 (±1.7)	26 (±8.2)	5.2 (±2.7)
CLNs	pFL	6.6 (±0.9)	940 (±180)	101 (±21)	56.0* (±13.6)	1.3 (±1.5)	19 (±3.6)	8.0* (±2.1)
	pORF	1.0 (±0.8)	680 (±150)	58 (±14)	18.8 (±9.9)	1.0 (±0.4)	7.8 (±2.1)	3.2 (±1.1)

[a] The $CD4^+$ T cells (4×10^6 cells/ml) from spleen and CLNs from each mouse group were cultured with 1 mg/ml OVA in the presence of T cell-depleted and irradiated splenic feeder cells (8×10^6 cells/ml).

[b] Analysis of OVA-specific $CD4^+$ T cell proliferative responses induced following nasal immunization with OVA plus pFL. The stimulation index was determined as cpm of wells with OVA/wells without OVA (controls). The levels of [^{3}H]TdR incorporation for each control well were between 500 and 1000 cpm. The results represent the individual values from three separate experiments of five mice per experimental group.

[c] Culture supernatants were harvested after 48 h of incubation and analyzed by the respective cytokine-specific ELISA.

[d] The values shown are the mean ± SEM of 25 mice in each experimental group.

[e] The $CD4^+$ T cells were harvested after 48 h of incubation. Total RNA was extracted from these cells and subjected to quantitative RT-PCR analysis.

* $p < 0.05$ compared with control mice.

Table II. *Comparison of the frequency of $CD11c^+$ DCs[a] and CD8, CD11b, B220, and costimulatory molecule expression by $CD11c^+$ DCs in mucosal tissues of mice given nasal OVA plus pFL or OVA and pORF[b]*

Tissue Source	Nasal Adjuvant	% Total Lymphocytes	% $CD11c^+$ DCs						
		CD11c[a]	CD11b[b,c]	CD8[b,c]	B220[b,c]	CD40[b,c]	CD80[b,d]	CD86[b,d]	MHC II[b,d]
NPs	pFL	11.0 ± 4.3**	21.1 ± 5.9	40.2 ± 12.3*	0.5 ± 0.3	35.9 ± 9.5*	46.3 ± 5.2*	49.9 ± 10.2*	78.7 ± 7.3*
	pORF	3.1 ± 1.0	18.2 ± 4.8	17.1 ± 6.9	0.4 ± 0.2	13.4 ± 7.7	13.5 ± 6.3	20.4 ± 6.6	52.8 ± 4.5
NALT	pFL	5.6 ± 1.8*	29.5 ± 6.3	45.1 ± 9.4*	4.6 ± 1.2	56.4 ± 10.4*	45.9 ± 11.2*	36.4 ± 10.9	80.2 ± 5.6
	pORF	1.9 ± 0.8	26.9 ± 6.5	19.6 ± 5.8	4.5 ± 1.8	28.7 ± 5.5	25.5 ± 9.8	24.5 ± 7.3	76.7 ± 3.6
SMG	pFL	18.5 ± 5.6**	13.9 ± 3.3	13.9 ± 5.3	2.0 ± 0.5	41.4 ± 6.5*	41.9 ± 8.0*	47.3 ± 12.8*	63.1 ± 5.9
	pORF	5.4 ± 1.9	10.7 ± 2.5	4.7 ± 1.8	1.4 ± 0.6	10.7 ± 5.3	11.5 ± 6.4	13.1 ± 4.3	46.4 ± 9.9

[a] Mononuclear cells from the SMG, the NPs, and NALT of mice immunized with OVA plus pFL or OVA and pORF were stained with PE-conjugated anti-CD11c mAb and subjected to flow cytometry analysis by FACSCalibur.
[b] Mononuclear cells were stained with a combination of anti-CD11c and the respective mAb and subjected to flow cytometry analysis by FACSCalibur.
[c] Mononuclear cells were stained with FITC-conjugated anti-CD40, -CD11b, -CD8, or B220 and PE-labeled anti-CD11c.
[d] Mononuclear cells were stained with PE-labeled anti-CD80, -CD86, or I-A[b] and biotinylated anti-CD11c mAbs, followed by FITC-streptavidin.
* $p < 0.05$, ** $p < 0.01$ compared with immunized mice with OVA plus pORF.

and NALT express CD8 but not CD11b or B220 molecules. Further, high levels of MHC II, CD40, CD80, and CD86 were expressed by $CD11c^+$, $CD8^+$, $CD11b^-$, and $B220^-$ DCs (Table II; Fig. 3, *c–j*). Taken together, these results indicate that nasal administration of pFL preferentially expands mature DCs and also induces their activation in both mucosal inductive and effector tissues.

FL plasmid and protein is expressed in the nasal tract

To assess for expression of FL protein, DNA was isolated from various tissues and amplified with ampicillin resistance gene-specific primers. The highest expression of pFL was in NALT followed by NPs. In contrast, naive mice did not express the plasmid-specific gene (Fig. 4*a*). We next examined whether the levels of FL protein were increased after nasal administration of pFL. The levels of FL protein in plasma, FEs, and NWs of mice immunized given nasal pFL plus OVA were significantly elevated when compared with mice immunized with pORF plus OVA or mice given OVA only (Fig. 4*b*). These results indicate that nasal application of plasmid induced higher expression of FL protein in both mucosal effector and systemic lymphoid tissues.

Discussion

In our studies, we have shown that nasal delivery of the protein OVA with 50 μg of FL plasmid results in high titers of OVA-specific plasma IgG and IgA and mucosal S-IgA Ab responses. Further, elevated numbers of OVA-specific IgG and IgA AFCs were also detected in both mucosal and systemic tissues. Although it has been shown that both FL protein and encoded plasmid exhibit adjuvant activity (7–10), our study is the first to show that the plasmid vector encoding FL cDNA has potent mucosal adjuvanticity instead of tolerance induction when coadministered nasally with protein Ag. Induction of Ag-specific immune responses was associated with elevated numbers of activated DCs in NALT. In this regard, we demonstrated that pFL was preferentially taken up by NALT, and FL protein levels were increased in NWs of mice given nasal OVA plus pFL. Further, our study showed that $CD4^+$ T cells and IL-2 and IL-4 synthesis mediated the pFL nasal adjuvant activity.

The focus of the present study was to investigate whether nasal delivery of plasmid encoding FL would enhance mucosal immunity or alternatively would induce tolerance. It was previously reported that pretreatment with FL facilitated the induction of systemic unresponsiveness through expansion of immature DCs when protein Ag was subsequently administered via the oral route (31). In contrast, our results provide direct evidence that both mucosal and systemic immunity are enhanced when pFL is given simultaneously with Ag. The opposite outcomes between these two studies may be explained by the manner of FL treatment used. For example, we used mucosal FL plasmid administration simultaneously with OVA. Thus, significant increases in mature DCs were limited to the local nasal mucosa and SMG (Table II). In contrast, in the oral tolerance studies, consecutive FL protein delivery was performed and marked increases in DCs were seen in lymphoid tissues before oral delivery of OVA. Further, the phenotype of DCs induced by nasal pFL was totally distinct from DCs induced by consecutive injection of FL protein. Thus, increased numbers of $CD11c^+CD8^+$ DCs in NALT, NPs, and SMG with up-regulated expression of MHC II, CD40, CD80, and CD86 molecules were noted in mice given pFL when compared with DCs from mice given nasal OVA with pORF (Table II). In contrast, FL protein injection failed to induce up-regulated expression of these costimulatory molecules, although numbers of DCs in various tissues were significantly increased. Based upon these findings, it is possible that the presence of Ag with FL is an essential condition for the effective generation of activated DCs in immune inductive tissues which subsequently lead to Ag-specific immunity instead of mucosal tolerance. Indeed, DCs expanded by FL injection were capable of being activated and of expression of costimulatory molecules after LPS stimulation (5, 31). To further support this view, it has been shown that i.m. injection of a DNA vaccine encoded together with FL plasmid and Ag induced enhanced Ag-specific immune responses (10). Further, nasal application of pFL alone failed to induce costimulatory factor expression even though the DC population was markedly increased (data not shown).

It is well known that $CD4^+$ T cells and their derived Th1 and Th2 cytokine responses are essential for the induction of Ag-specific S-IgA Ab responses. In this regard, we have compared Th1- and Th2-type cytokine production by $CD4^+$ T cells from mice given either nasal pFL or pORF as mucosal adjuvant. Our results clearly showed that IL-4 production was significantly enhanced in both spleen and CLNs of mice given pFL. Further, increased levels of IL-2 synthesis by OVA-stimulated $CD4^+$ T cells were also noted in both spleen and CLNs of mice immunized with OVA plus pFL. Although IL-2 is considered to be a Th1-type cytokine, the enhanced IL-2 production by $CD4^+$ T cells from mice given pFL may account for IL-4 synthesis but not for Th1-type cytokine responses. To support this view, our results showed limited amounts of IFN-γ production (Table I). In this regard, OVA-specific IgG2a Ab which is a typical subclass supported by Th1-type responses

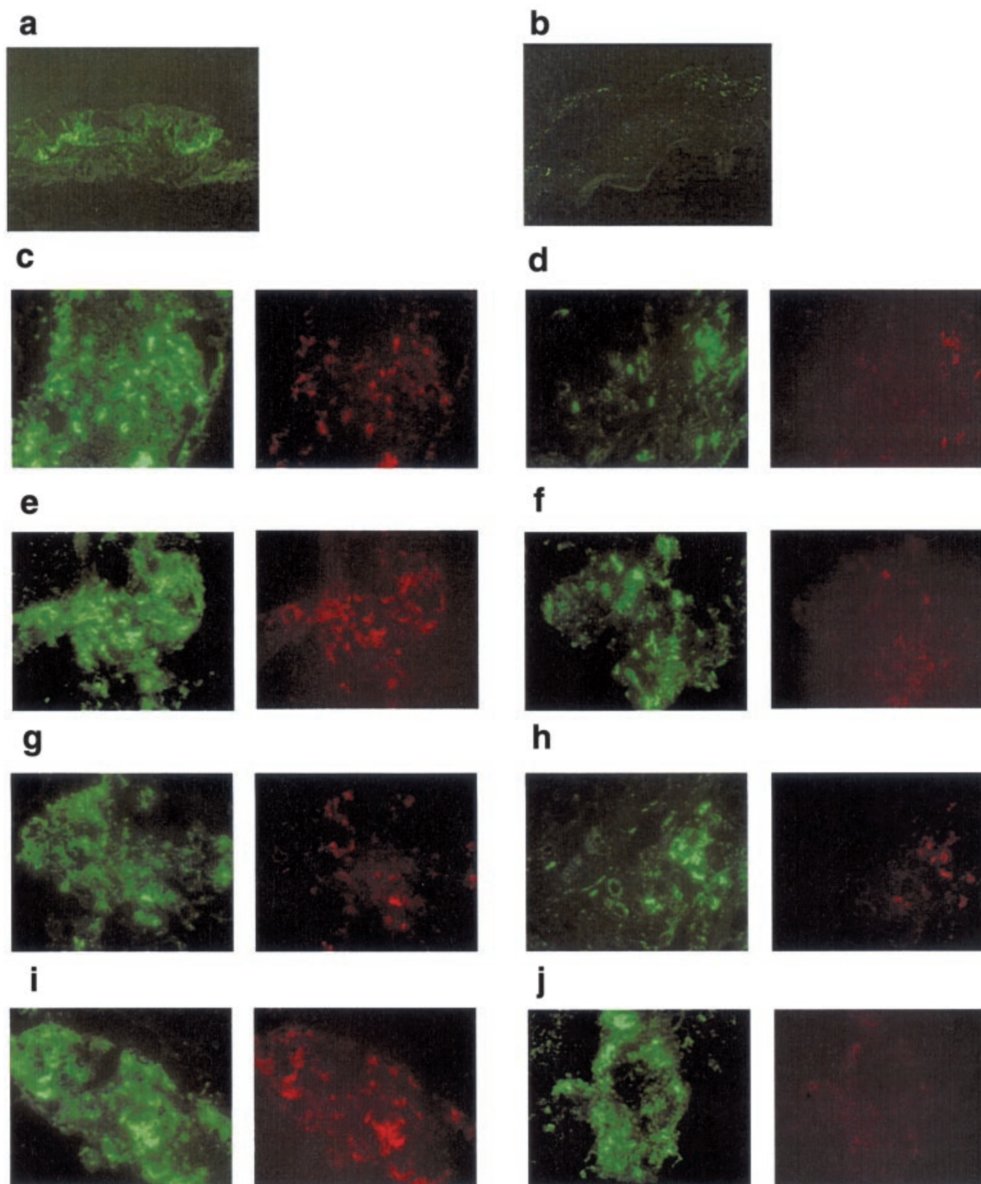

FIGURE 3. Immunofluorescent staining of DCs in NALT of mice treated with OVA plus pFL (*a*, *c*, *e*, *g*, and *i*) or OVA and pORF (*b*, *d*, *f*, *h*, and *j*). *a* and *b*, Frozen sections of NALT were stained with biotin conjugated anti-CD11c mAb followed by Alexa Fluor 488. Original magnification, × 40. *c–j*, NALT were stained with PE-conjugated anti-CD40 (*c* and *d*), -CD80 (*e* and *f*), -CD86 (*g* and *h*), or -I-A^b (*i* and *j*) together with biotin-conjugated anti-CD11c mAb followed by Alexa Fluor 488. Original magnification, × 400.

were quite low in mice given nasal pFL as mucosal adjuvant. It has been shown that nasal application of the unmethylated CpG motif possessed CD4$^+$ Th1-type T cell-mediated adjuvant activity when coadministered with influenza Ag (48). Thus, it would be logical to suggest that pFL adjuvanticity possibly resulted from unmethylated CpG motif effects because the FL encoded plasmid was used as mucosal adjuvant. However, our results revealed only low OVA-specific IgG2a Ab responses and essentially no OVA-induced IFN-γ synthesis by CD4$^+$ T cells which would be associated with CpG adjuvant activity. A minor increment of OVA-specific IgG Ab responses induced by control cDNA plasmid is due to the common results of DNA immunization. In this regard, others reported similar results that control plasmid possessed minor adjuvant activity (49, 50). The most important point of our study is that pFL successfully induced OVA-specific IgA Ab responses in mucosal secretions and plasma. In contrast, control plasmid failed to induce detectable levels of IgA Abs in mucosal secretions or plasma. Thus, the control immunization formula did not contain actual adjuvant activity. The adjuvant activity totally depends upon pFL. Taken together, we postulate that pFL mucosal adjuvant activity may be mediated by IL-4 producing Th2-type CD4$^+$ T cells. In this regard, we are currently testing pFL adjuvant activity in IL-4-deficient mice.

To date, an array of nasal adjuvants have been used successfully to elicit Ag-specific immunity in both mucosal and systemic sites; however, the precise mechanisms for the induction of Ag-specific immune responses still remains to be elucidated. For example, enterotoxins, their mutants, and chimeras have shown significant adjuvanticity when coadministered nasally with protein Ag (18, 34). Although evidence has been presented that oral native CT induces up-regulation of CD86 on Peyer's patch B cells and macrophages and expression of CD86 was required for adjuvanticity (23, 51), the primary and main target tissues or cells of nasal native CT for the initiation of adjuvant activity are currently unknown.

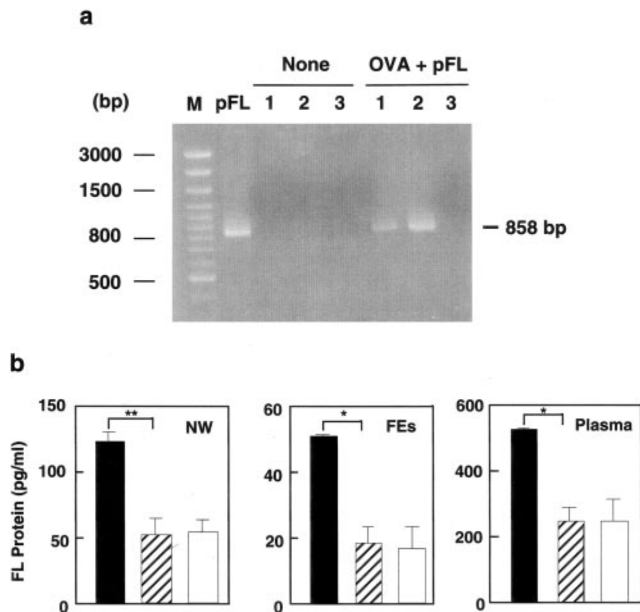

FIGURE 4. *a*, Detection of pFL uptake by use of ampicillin resistance gene (858 bp). Mice were nasally immunized weekly for three consecutive weeks with 100 μg of OVA plus 50 μg of pFL. Seven days after the last immunization, the DNA was extracted from 1) NPs, 2) NALT, and 3) the SMG and was then amplified with the ampicillin-resistance gene-specific primers by PCR. The pFL (0.1 μg) was used as a positive control. Numbers (base pair) to the left indicate migration positions of the DNA marker (left lane; M). The results are from one experiment and are representative of five separate experiments. *b*, Comparison of FL protein levels in NW, FEs, and plasma of mice nasally immunized with OVA plus pFL (■) or with OVA and pORF (□), or mice given OVA only (▨). Seven days after the last immunization, NW, FEs, and plasma were subjected to FL-specific ELISA. The values shown are the mean ± SEM of 15 mice in each experimental group. **, $p < 0.01$; *, $p < 0.05$ when compared with control mice.

Contrary to this, our findings clearly showed the target tissues and cells where the initiation of FL adjuvant function occurred. In this regard, our results showed that the highest expression of the plasmid-specific ampicillin resistant gene was in NALT followed by the NPs. The FL protein levels were significantly increased in NWs when compared with those from mice given OVA only or OVA plus pORF. Similarly, FL levels in plasma were also elevated. Because the spleen as well as other lymph nodes did not express the plasmid-specific gene, we postulate that high levels of FL in plasma were primarily due to transudation from the nasal mucosa. These results show that FL-encoded plasmid was mainly taken up by NALT and subsequently the FL protein was produced locally in these tissues, which resulted in the subsequent expansion and activation of DCs.

In summary, the present study has shown that plasmid encoding FL cDNA was an effective and safe mucosal adjuvant. The mechanisms of pFL adjuvanticity were clearly due to the function of FL. Thus, the numbers of DCs in nasal inductive tissues, e.g., NALT were significantly increased. Because Ag was simultaneously administered together with pFL, the expanded DCs were activated and expressed MHC II and costimulatory molecules (CD40, CD80, and CD86). Subsequently, these activated DCs induced mainly Th2-type IL-4-producing $CD4^+$ T cells for the induction of mucosal S-IgA and systemic IgG Ab responses. Use of pFL as mucosal adjuvant opens a new avenue for the development of effective and safe mucosal vaccines which prevent not only ordinary infectious diseases but also the category A agents for potential use by bioterrorists.

Acknowledgments

We thank Annette M. Pitts for excellent technical assistance in the immunohistochemistry assay, and Sheila D. Turner for the final preparation of the manuscript.

References

1. Brasel, K., H. J. McKenna, K. Charrier, P. J. Morrissey, D. E. Williams, and S. D. Lyman. 1997. Flt3 ligand synergizes with granulocyte-macrophage colony-stimulating factor or granulocyte colony-stimulating factor to mobilize hematopoietic progenitor cells into the peripheral blood of mice. *Blood 90:3781*.
2. Marakovsky, E., K. Brasel, M. Teepe, E. R. Roux, S. D. Lyman, K. Shortman, and H. J. McKenna. 1996. Dramatic increase in the numbers of functionally mature dendritic cells in Flt3 Ligand-treated mice: multiple dendritic cell subpopulations identified. *J. Exp. Med. 184:1953*.
3. Yu, H., T. A. Fehniger, P. Fuchshuber, K. S. Thiel, E. Vivier, W. E. Carson, and M. A. Caligiuri. 1998. Flt3 ligand promotes the generation of a distinct $CD34^+$ human natural killer cell progenitor that responds to interleukin-15. *Blood 92:3647*.
4. Hunte, B. E., S. Hudak, D. Campbell, Y. Xu, and D. Rennick. 1995. *flk2/flt3* ligand is a potent cofactor for the growth of primitive B cell progenitors. *J. Immunol. 156:489*.
5. Williamson, E., G. M. Westrich, and J. L. Viney. 1999. Modulating dendritic cells to optimize mucosal immunization protocols. *J. Immunol. 163:3668*.
6. Pulendran, B., J. L. Smith, G. Caspary, K. Brasel, D. Petit, E. Maraskovsky, and C. R. Maliszewski. 1999. Distinct dendritic cell subsets differentially regulate the class of immune response in vivo. *Proc. Natl. Acad. Sci. USA 96:1036*.
7. Pisarev, V. M., P. Parajuli, R. L. Mosley, J. Sublet, L. Kelsey, P. S. Sarin, D. H. Zimmerman, M. D. Winship, and J. E. Talmadge. 2000. Flt3 ligand enhances the immunogenicity of a *gag*-based HIV-1 vaccine. *Int. J. Immunopharmacol. 22:865*.
8. Baca-Estrada, M. E., C. Ewen, D. Mahony, L. A. Babiuk, D. Wilkie, and M. Foldvari. 2002. The haemopoietic growth factor, Flt3L, alters the immune response induced by transcutaneous immunization. *Immunology 107:69*.
9. Moore, A. C., W.-P. Kong, B. K. Chakrabarti, and G. J. Nabel. 2002. Effects of antigen and genetic adjuvants on immune responses to human immunodeficiency virus DNA vaccine in mice. *J. Virol. 76:243*.
10. Hung, C.-F., K.-F. Hsu, W.-F. Cheng, C.-Y. Chai, L. He, M. Ling, and T.-C. Wu. 2001. Enhancement of DNA vaccine potency by linkage of antigen gene to a gene encoding the extracellular domain of Fms-like tyrosine kinase 3-ligand. *Cancer Res. 61:1080*.
11. Ogra, P. L. 1996. Mucosal immunoprophyraxis: an introductory overview. In *Mucosal Vaccine.* H. Kiyono, P. L. Ogra, and J. R. McGhee, eds. Academic Press, San Diego, p. 3.
12. Mestecky, J., and J. R. McGhee. 1989. New strategies for oral immunization. *Curr. Top. Microbiol. Immunol. 146:1*.
13. McGhee, J. R., J. Mestecky, M. T. Dertzbaugh, J. H. Eldridge, M. Hirasawa, and H. Kiyono. 1992. The mucosal immune system: from fundamental concepts to vaccine development. *Vaccine 10:75*.
14. Vadolas, J., J. K. Davis, P. J. Wright, and R. A. Strugnell. 1995. Intranasal immunization with liposomes induces strong mucosal immune responses in mice. *Eur. J. Immunol. 25:969*.
15. Langermann, S., S. Palaszynsky, A. Sadziene, C. Stover, and S. Koenig. 1994. Systemic and mucosal immunity induced by BCG vector expressing outer-surface protein A of *Borrelia burgdorferi. Nature 372:552*.
16. Clements, J. D., N. M. Hartzog, and F. L. Lyon. 1988. Adjuvant activity of *Escherichia coli* heat-labile enterotoxin and effect on the induction of oral tolerance in mice to unrelated protein antigens. *Vaccine 6:269*.
17. Holmgren, J., N. Lycke, and C. Czerkinsky. 1993. Cholera toxin and cholera B subunit as oral-mucosal adjuvant and antigen vector systems. *Vaccine 11:1179*.
18. Yamamoto, S., H. Kiyono, M. Yamamoto, K. Imaoka, M. Yamamoto, K. Fujihashi, F. W. Van Ginkel, M. Noda, Y. Takeda, and J. R. McGhee. 1997. A nontoxic mutant of cholera toxin elicits Th2-type responses for enhanced mucosal immunity. *Proc. Natl. Acad. Sci. USA 94:5267*.
19. Marinaro, M., H. F. Staats, T. Hiroi, R. J. Jackson, M. Coste, P. N. Boyaka, N. Okahashi, M. Yamamoto, H. Kiyono, H. Bluethmann, K. Fujihashi, and J. R. McGhee. 1995. Mucosal adjuvant effect of cholera toxin in mice results from induction of T helper 2 (Th2) cells and IL-4. *J. Immunol. 155:4621*.
20. Xu-Amano, J., H. Kiyono, R. J. Jackson, H. F. Staats, K. Fujihashi, P. D. Burrows, C. O. Elson, S. Pillai, and J. R. McGhee. 1993. Helper T cell subsets for immunoglobulin A responses: oral immunization with tetanus toxoid by use of cholera toxin as adjuvant selectively induces Th2 cells in mucosa associated tissues. *J. Exp. Med. 178:1309*.
21. Xu-Amano, J., R. J. Jackson, K. Fujihashi, H. Kiyono, H. F. Staats, and J. R. McGhee. 1994. Helper Th1 and Th2 cell responses following mucosal or systemic immunization with cholera toxin. *Vaccine 12:903*.
22. VanCott, J. L., H. F. Staats, D. W. Pascual, M. Roberts, S. N. Chatfield, M. Yamamoto, M. Coste, P. B. Carter, H. Kiyono, and J. R. McGhee. 1996. Regulation of mucosal and systemic antibody responses by T helper cell subsets, macrophages, and derived cytokines following oral immunization with live recombinant *Salmonella. J. Immunol. 156:1504*.
23. Yamamoto, M., H. Kiyono, S. Yamamoto, E. Batanero, M.-N. Kweon, S. Otake, M. Azuma, Y. Takeda, and J. R. McGhee. 1999. Direct effects on antigen-presenting cells and T lymphocytes explain the adjuvanticity of nontoxic cholera toxin mutant. *J. Immunol. 162:7015*.

24. Takahashi, I., M. Marinaro, H. Kiyono, R. J. Jackson, I. Nakagawa, K. Fujihashi, S. Hamada, J. D. Clements, K. L. Bost, and J. R. McGhee. 1996. Mechanisms for mucosal immunogenicity and adjuvancy of *Escherichia coli* labile enterotoxin. *J. Infect. Dis. 173:627*.
25. Boyaka, P. N., M. Marinaro, R. J. Jackson, S. Menon, H. Kiyono, E. Jirillo, and J. R. McGhee. 1999. IL-12 is an effective adjuvant for induction of mucosal immunity. *J. Immunol. 162:122*.
26. Staats, H. F., C. P. Bradney, W. M. Gwinn, S. S. Jackson, G. D. Sempowski, H.-X. Liao, N. L. Letvin, and B. F. Haynes. 2001. Cytokine requirements for induction of systemic and mucosal CTL after nasal immunization. *J. Immunol. 167:5386*.
27. Lillard, J. W. J., P. N. Boyaka, J. A. Hedrick, A. Zlotnik, and J. R. McGhee. 1999. Lymphotactin acts as an innate mucosal adjuvant. *J. Immunol. 162:1959*.
28. Lillard, J. W. J., P. N. Boyaka, D. D. Taub, and J. R. McGhee. 2001. RANTES potentiates antigen-specific mucosal immune responses. *J. Immunol. 166:162*.
29. McGhee, J. R., and H. Kiyono. 1999. The mucosal immune system. *In Fundamental Immunology*, 4th Ed. W. E. Paul, ed. Academic Press, San Diego, p. 909.
30. Kato, H., K. Fujihashi, R. Kato, Y. Yuki, and J. R. McGhee. 2001. Oral tolerance revisited: prior oral tolerization abrogates cholerae toxin-induced mucosal IgA responses. *J. Immunol. 166:3114*.
31. Viney, J. L., A. M. Mowat, L. M. O'Malley, E. Williamson, and N. A. Fanger. 1998. Expanding dendritic cells in vivo enhances the induction of oral tolerance. *J. Immunol. 160:5815*.
32. Fujihashi, K., J. R. McGhee, M.-N. Kweon, M. D. Cooper, S. Tonegawa, I. Takahashi, T. Hiroi, J. Mestecky, and H. Kiyono. 1996. T cell deficient mice have impaired mucosal IgA responses. *J. Exp. Med. 183:1929*.
33. Yamamoto, M., D. E. Briles, S. Yamamoto, M. Ohmura, H. Kiyono, and J. R. McGhee. 1998. A nontoxic adjuvant for mucosal immunity to pneumococcal surface protein A. *J. Immunol. 161:4115*.
34. Hagiwara, Y., J. R. McGhee, K. Fujihashi, R. Kobayashi, N. Yoshino, K. Kataoka, Y. Etani, M.-N. Kweon, S. Tamura, T. Kurata, et al. 2003. Protective mucosal immunity in aging is associated with functional $CD4^+$ T cells in nasopharyngeal-associated lymphoreticular tissues. *J. Immunol. 170:1754*.
35. Tamura, S., K. Miyata, K. Matsuo, H. Asanuma, H. Takahashi, K. Nakajima, Y. Suzuki, C. Aizawa, and T. Kurata. 1996. Acceleration of influenza virus clearance by Th1 cells in the nasal site of mice immunized intranasally with adjuvant-combined recombinant nucleoprotein. *J. Immunol. 156:3892*.
36. Yamamoto, S., Y. Takeda, M. Yamamoto, H. Kurazono, K. Imaoka, M. Yamamoto, K. Fujihashi, M. Noda, and H. Kiyono. 1997. Mutants in the ADP-ribosyltransferase cleft of cholera toxin lack diarrhoeagenicity but retain adjuvanticity. *J. Exp. Med. 185:1203*.
37. Koga, T., J. R. McGhee, H. Kato, R. Kato, H. Kiyono, and K. Fujihashi. 2000. Evidence for early aging in the mucosal immune system. *J. Immunol. 165:5352*.
38. Kweon, M.-N., M. Yamamoto, F. Watanabe, S. Tamura, F. W. van Ginkel, A. Miyauchi, H. Takagi, Y. Takeda, T. Hamabata, K. Fujihashi, et al. 2002. A non-toxic chimeric enterotoxin adjuvant induces protective immunity in both mucosal and systemic compartments with reduced IgE Ab. *J. Infect. Dis. 186:1261*.
39. Hiroi, T., K. Iwatani, H. Iijima, S. Kodama, M. Yanagita, and H. Kiyono. 1998. Nasal immune system: distinctive Th0 and Th1/Th2 type environments in murine nasal-associated lymphoid tissues and nasal passage, respectively. *Eur. J. Immunol. 28:3346*.
40. Asanuma, H., Y. Inaba, C. Arizawa, T. Kurata, and S. Tamura. 1995. Characterization of mouse nasal lymphocytes isolated by enzymatic extraction with collagenase. *J. Immunol. Methods 187:41*.
41. Wu, H. Y., E. B. Nikolova, K. W. Beagley, and M. W. Russell. 1996. Induction of antibody-secreting cells and T-helper and memory cells in murine nasal lymphoid tissue. *Immunology 88:493*.
42. Dickinson, B. L., and J. D. Clements. 1995. Dissociation of *Escherichia coli* heat-labile enterotoxin adjuvanticity from ADP-ribosyltransferase activity. *Infect. Immun. 63:1617*.
43. Power, D. C. 1992. Immunological principles and emerging strategies of vaccination for the elderly. *J. Am. Geriatr. Soc. 40:81*.
44. Schmucker, D. L., M. F. Heyworth, R. L. Owen, and C. K. Daniels. 1996. Impact of aging on gastrointestinal mucosal immunity. *Dig. Dis. Sci. 41:1183*.
45. Simpson, D. A., S. Feeney, C. Boyle, and A. W. Stitt. 2000. Retinal VEGF mRNA measured by SYBR green I fluorescence: A versatile approach to quantitative PCR. *Mol. Vision 6:178*.
46. Nakanishi, H., Y. Kodera, Y. Yamamura, S. Ito, T. Kato, T. Ezaki, and M. Tatematsu. 2000. Rapid quantitative detection of carcinoembryonic antigen-expressing free tumor cells in the peritoneal cavity of gastric-cancer patients with real-time RT-PCR on the LightCycler. *Int. J. Cancer 89:411*.
47. Blin, N., and D. W. Stafford. 1976. A general method for isolation of high molecular weight DNA from eukaryotes. *Nucleic Acids Res. 3:2303*.
48. Moldoveanu, Z., L. Love-Homan, W. Q. Huang, and A. M. Kreig. 1998. CpG DNA, a novel immune enhancer for systemic and mucosal immunization with influenza virus. *Vaccine 16:1216*.
49. Herve, M., L. Dupre, E. Ban, A.-M. Schacht, A. Capron, and G. Riveau. 2001. Features of the antibody response attributable to plasmid backbone adjuvanticity after DNA immunization. *Vaccine 19:4549*.
50. Donnelly, J. J., J. B. Ulmer, J. W. Shiver, and M. A. Liu. 1997. DNA vaccines. *Annu. Rev. Immunol. 15:617*.
51. Cong, Y., C. T. Weaver, and C. O. Elson. 1997. The mucosal adjuvanticity of cholera toxin involves enhancement of costimulatory activity by selective up-regulation of B7.2 expression. *J. Immunol. 159:5301*.