

Regulatory Role of Lymphoid Chemokine CCL19 and CCL21 in the Control of Allergic Rhinitis¹

Kaoru Takamura,*[†] Satoshi Fukuyama,* Takahiro Nagatake,* Dong-Young Kim,*[‡]
Aya Kawamura,* Hideyuki Kawauchi,[†] and Hiroshi Kiyono^{2*}

The lymphoid chemokines CCL19 and CCL21 are known to be crucial both for lymphoid cell trafficking and for the structural organization of lymphoid tissues such as nasopharynx-associated lymphoid tissue (NALT). However, their role in allergic responses remains unclear, and so our current study aims to shed light on the role of CCL19/CCL21 in the development of allergic rhinitis. After nasal challenge with OVA, OVA-sensitized *plt* (paucity of lymph node T cells) mice, which are deficient in CCL19/CCL21, showed more severe allergic symptoms than did identically treated wild-type mice. OVA-specific IgE production, eosinophil infiltration, and Th2 responses were enhanced in the upper airway of *plt* mice. Moreover, in *plt* mice, the number of CD4⁺CD25⁺ regulatory T cells declined in the secondary lymphoid tissues, whereas the number of Th2-inducer-type CD8 α ⁺CD11b⁺ myeloid dendritic cells (m-DCs) increased in cervical lymph nodes and NALT. Nasal administration of the plasmid-encoding DNA of CCL19 resulted in the reduction of m-DCs in the secondary lymphoid tissues and the suppression of allergic responses in *plt* mice. These results suggest that CCL19/CCL21 act as regulatory chemokines for the control of airway allergic disease and so may offer a new strategy for the control of allergic disease. *The Journal of Immunology*, 2007, 179: 5897–5906.

The CCR7 ligands CCL19 and CCL21 are lymphoid chemokines involved in the chemotaxis of lymphoid cells such as leukocytes and dendritic cells (DC)³ (1, 2). Indeed, these chemokines play important roles in the formation of appropriate cellular microcompartmentalization and homeostasis in lymphoid tissues (3–5). CCL19 is produced primarily by stromal cells in the thymus and by the T cell area of secondary lymphoid tissues (1, 6). CCL21 is encoded by two genes, *Scya21a* (CCL21-Ser) and *Scya21b* (CCL21-Leu) (7). CCL21-Ser is produced by stromal cells in the T cell area and by the high endothelial venules of the secondary lymphoid tissues, CCL21-Leu by the lymphatic endothelium alone (1). Paucity of lymph node T cells (*plt*) mice have a genomic deletion that includes the CCL19/

CCL21-Ser gene, leading to defective homing of naive T cells to the secondary lymphoid tissues and thereby to insufficient architectural development of nasopharynx-associated lymphoid tissue (NALT) and of other organized lymphoid tissues such as spleen, cervical lymph nodes (CLN), and Peyer's patches (2, 5, 7, 8). Among these CCL19/CCL21-associated lymphoid tissues, NALT is considered one of the most important mucosal inductive sites for the initiation and regulation of both mucosal and systemic immune responses to inhaled Ags (9). NALT has been shown to be rich in Th0-type CD4⁺ T cells, which are capable of differentiating into Th1 or Th2 cells based on the nature of the nasally administered Ag (10). For example, nasal immunization of the fimbrial protein of anaerobe together with cholera toxin as mucosal adjuvant resulted in the induction of Ag-specific Th2-mediated IgA responses (11). Nasal administration of soluble protein together with a mucosal delivery molecule or immunomodulator (e.g., *Escherichia coli* heat-labile toxin B subunit, cholera toxin) can suppress pathogenic responses (12, 13). Further, nasal administration of Ag can induce Ag-specific regulatory T cells (Tregs) for the establishment of immunological homeostatic conditions (14). Respiratory-associated lymphoid tissues including NALT have thus been shown to play a pivotal role in the regulation of both active and quiescent mucosal immune responses in the respiratory tract as well as in systemic immune responses. However, their contribution to the induction and regulation of allergic responses in the upper airway remains to be elucidated.

Allergic rhinitis (AR) is a Th2-mediated disorder characterized by Ag-specific IgE production, infiltration of inflammatory cells including eosinophils into the nasal mucosa, and several nasal symptoms such as sneezing, nasal congestion, itching, and rhinorrhea (15). Exposure to allergens following allergic presensitized conditions leads to the cross-linking of allergen-specific mast-cell surface-bound IgE and basophils via the Fc ϵ R, to the degranulation of these cells and to the release of histamine and other allergy-associated chemical mediators responsible for the early phases of allergic responses. When released by mast cells and other cells,

*Division of Mucosal Immunology, Department of Microbiology and Immunology, Institute of Medical Science, The University of Tokyo, Tokyo, Japan; [†]Department of Otorhinolaryngology, The Shimane University School of Medicine, Izumo, Japan; and [‡]Department of Otorhinolaryngology, Seoul National University College of Medicine, Seoul, Korea

Received for publication February 23, 2007. Accepted for publication August 14, 2007.

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 was supported by the Core Research for Evolutional Science and Technology (CREST) Program, the Japan Science and Technology Corporation, a Grant-in-Aid from the Ministry of Education, Science, Sports and Culture, and the Ministry of Health. S.F. and T.N. were supported by research fellowships from the Japan Society for the Promotion of Science for Young Scientists. D.-Y.K. was supported by research fellowships from the Japan Society for the Promotion of Science for Foreign Researchers.

² Address correspondence and reprint requests to Dr. Hiroshi Kiyono, Division of Mucosal Immunology, Department of Microbiology and Immunology, The Institute of Medical Science, The University of Tokyo, 4-6-1 Shirokanedai, Minato-ku, Tokyo, Japan. E-mail address: kiyono@ims.u-tokyo.ac.jp

³ Abbreviations used in this paper: DC, dendritic cell; *plt*, paucity of lymph node T cells; NALT, nasopharynx-associated lymphoid tissue; CLN, cervical lymph nodes; Treg, regulatory T cell; AR, allergic rhinitis; m-DC, myeloid DC; NP, nasal passage; l-DC, lymphoid DC; WT, wild type.

chemokines such as CCL5 (RANTES), CCL11 (eotaxin), CCL17 (thymus and activation-regulated chemokine), and CCL22 (macrophage-derived chemokine) trigger recruitment of inflammatory cells such as eosinophils and Th2 cells, thereby contributing to the induction of late-phase allergic responses (16). Inasmuch as the chemokine family has been shown to play a critical role in most physiological and pathological immune scenarios, we thought it logical next to determine whether the NALT-associated lymphoid chemokines CCL19/CCL21 are involved in the development of allergic responses in the upper respiratory compartment.

DCs are the front-line sentinels for Ag detection in both organized lymphoid tissues such as Peyer's patches and NALT and the diffused connective tissues of the lamina propria region in mucosal compartments. Mucosal DCs have been shown to play an important role in the induction of both physiological and pathological Th1/Th2 polarization in protective immunity, inflammation, and allergy (17, 18). Mucosal DCs are also involved in the induction of Tregs for the creation of immunologically quiescent conditions in the harsh environment of the aero-digestive mucosa (19, 20). Tregs are a distinct population of CD4⁺ T cells constitutively expressing IL-2 receptor α -chains (CD25) (21). Tregs play a central role in the regulation of autoimmune, infectious, and allergic diseases by cell-to-cell contact-dependent inhibition and by the secretion of anti-inflammatory cytokines such as IL-10 and TGF- β (22). Th2 responses have been shown to be down-regulated by naturally occurring CD4⁺CD25⁺ Tregs expressing forkhead/winged-helix family transcription factor P3 (Foxp3) and by inducible populations of Ag-specific IL-10-secreting Tregs (23, 24).

In this study, we examine whether the NALT-associated lymphoid chemokines CCL19/CCL21 help regulate T cell-mediated control of allergic responses in nasopharyngeal tissue. *plt* mice show aggravated allergic symptoms with aberrant Th2 responses, increased numbers of CD8 α ⁺CD11b⁺ myeloid DCs (m-DCs), and a reduction in CCR7-expressing Tregs in the NALT and CLN. The worsened allergic responses in *plt* mice could be reversed by nasal administration of plasmids encoding CCL19/CCL21-Ser DNA to reduce Th2-inducer-type m-DCs. Our results suggest that the lymphoid chemokines CCL19 and CCL21 play a role in the control of AR by reducing the numbers of m-DCs and thereby the likelihood of inhibiting a Th2 environment.

Materials and Methods

Mice

BALB/c mice were purchased from Japan SLC. *Plt* mice on a BALB/c background were provided by Dr. Terutaka Kakiuchi (Toho University School of Medicine). Mice transgenic for a TCR that recognizes the OVA_{323–339} peptide in the context of I-A^d (DO11.10 TCR- $\alpha\beta$ transgenic mice) on a BALB/c background were purchased from The Jackson Laboratory Animal Resources Center. These mice were maintained under specific pathogen-free conditions in the Laboratory Animal Research Center of The Institute of Medical Science (The University of Tokyo). All mice were 6–7 wk of age at the beginning of individual experiments.

Induction of AR

For the induction of AR, we employed a previously described protocol with some modifications in the quantity of Ags and the sensitization schedule (25). In brief, female BALB/c mice and *plt* mice were presensitized by means of an i.p. injection of 25 μ g of OVA (Grade V; Sigma-Aldrich) with 1 mg of aluminum hydroxide hydrate gel (Alum) (LSL Co.) in 200 μ l of PBS on days 0, 7, and 14. Thereafter, mice were challenged by nasal administration of either 500 μ g of OVA in 20 μ l of PBS (for AR group) or 20 μ l of PBS alone (for control group) for 14 consecutive days from day 21 to 34.

Assessment of allergic symptoms

On days 20 (after three rounds of i.p. sensitization) and on days 27 and 34 (after 7 and 14 nasal challenges, respectively), the instances of sneezing

and nasal rubbing in a 5-min period were counted by investigators in a blinded fashion after the last nasal challenge (25). At the same time, the behavior of the mice was recorded by video camera.

ELISA for the analysis of IgE Abs and histamine in serum

For the analysis of total and OVA-specific IgE levels in serum, a sandwich ELISA system was employed in accordance with the manufacturer's protocol (26). Ninety-six-well plates were coated with purified anti-mouse IgE mAb (clone R35–72; BD Pharmingen) and a purified mouse IgE isotype (27–74; BD Pharmingen) was used as a standard. HRP-conjugated anti-mouse IgE (23G3; Southern Biotechnology) (for total IgE) and HRP-labeled anti-biotin (Vector Laboratories) following biotin-labeled OVA (for OVA-specific IgE) were added to the plates as detection enzymes. The reaction was developed by 3,3',5,5'-tetramethylbenzidine (Moss) and terminated by the addition of 2 N H₂SO₄. OD was recorded by a luminometer (iEMS Reader; Labsystems) set at 450 nm. End-point titers of OVA-specific IgE were expressed as the reciprocal log₂ of the last dilution of a sample giving an OD value 0.1 higher than background. Serum was collected within 10 min after the last nasal challenge and its histamine levels analyzed using a histamine immunoassay kit (Immunotech) (27).

Histological analysis for eosinophil infiltration

After the analysis of nasal symptoms, mice were sacrificed and their heads fixed in 4% paraformaldehyde at 4°C for 16 h. Fixed tissues were then decalcified in EDTA solution at 4°C for 10 days and embedded in paraffin. Samples were sliced into 5 μ m coronal sections and the sections subjected to H&E staining (28). The number of eosinophils that had infiltrated into the nasal septal mucosa was counted using a high magnification ($\times 400$) microscope.

Isolation of mononuclear cells

Spleen, CLN, and thymus were removed, and single-cell suspensions were prepared by mechanical dissociation (10). Mononuclear cells of NALT and nasal passage (NP) were isolated as previously described with some modifications (10). In brief, the palatine plate containing NALT was removed and then NALT was dissected out. NP tissues without NALT were also extracted from the nasal cavity, and mononuclear cells from individual tissues were isolated by gentle teasing using needles through 40- μ m nylon mesh.

Analysis of cytokine production by CD4⁺ T cells

For the purification of CD4⁺ T cells, isolated mononuclear cells were incubated with CD4 (L3T4) MicroBeads (Miltenyi Biotec) at 4°C for 30 min. CD4⁺ cells were sorted by autoMACS (Miltenyi Biotec) and suspended in complete RPMI 1640 medium containing 10% FBS, 5 μ M 2-ME, 10 U/ml of penicillin, and 100 μ g/ml streptomycin. Cells were then cultured at a density of 1×10^5 cells/well in the presence of 1 mg/ml OVA with T cell-depleted and irradiated splenic feeder cells (5×10^5 cells/well) in round-bottom 96-well microculture plates for 48 to 96 h (11).

To determine whether each DC subset preferentially directed naive CD4⁺ T cells to develop a Th1 or Th2 cytokine profile, mononuclear cells harvested from mice with AR were first incubated with CD11c MicroBeads (Miltenyi Biotec) and sorted using autoMACS to enrich the CD11c⁺ population. Cells were then stained with allophycocyanin-conjugated anti-CD11c (HL3; BD Pharmingen), PE-conjugated anti-CD8 α (53-6.7; BD Pharmingen), and FITC-conjugated anti-CD11b (M1/70; BD Pharmingen) to collect CD11c⁺CD8 α ⁺CD11b⁺ cells (m-DCs) and CD11c⁺CD8 α ⁺CD11b⁺ cells (lymphoid DCs: l-DCs) by FACSria (BD Biosciences). Naive T cells were isolated from the spleen of DO11.10 TCR transgenic mice and stained with FITC-conjugated anti-CD62L (MEL-14; BD Pharmingen), PE-conjugated anti-CD44 (IM7; BD Pharmingen), and allophycocyanin-conjugated anti-CD4 (L3T4) (RM4-5; BD Pharmingen) using FACSria. Tregs were sorted from spleen cells as a CD3⁺CD4⁺CD25⁺ population using FACSria by staining with FITC-conjugated anti-CD3 ϵ (145-2C11; BD Pharmingen), PE-conjugated anti-CD4 (BD Pharmingen) and allophycocyanin-conjugated anti-CD25 (PC61; BD Pharmingen). DCs of each subset were initially cultured at a density of 1×10^4 cells/well, with naive CD4⁺CD44^{int}CD62L^{high} cells (1×10^5 cells/well) isolated from the spleen of DO11.10 TCR transgenic mice in the presence of human IL-2 and OVA (1 mg/ml) with or without Tregs (2×10^4 cells/well) for 7 days. Cells were then washed and re-stimulated with OVA in the presence of irradiated splenic feeder cells (9×10^5 cells/well) for 48 h (29, 30). For the cytokine neutralization assay, anti-IL-10 mAb (JES5-2A5) (10 μ g/ml), anti-TGF- β mAb (1D11) (10 μ g/ml) or rat IgG (Sigma-Aldrich) (10 μ g/ml) was added to the culture. Culture supernatants were collected and

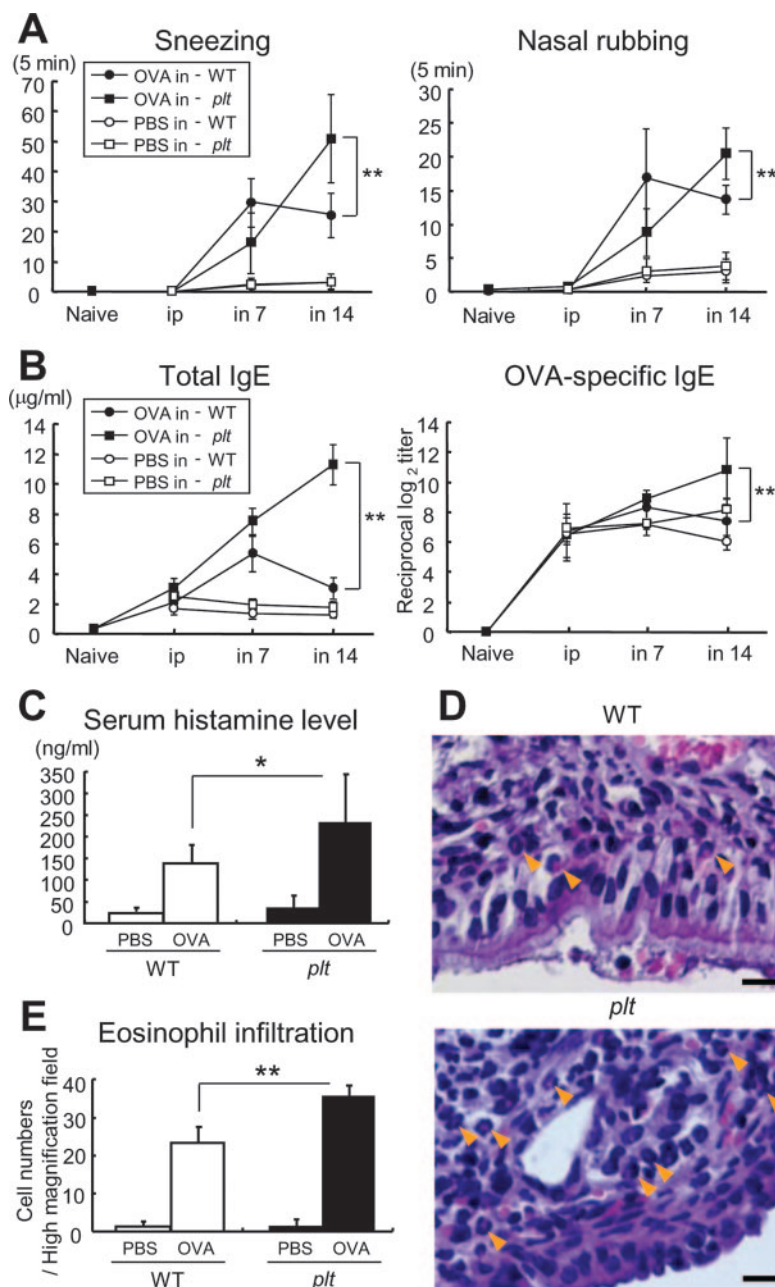


FIGURE 1. Nasal symptoms and Ag-specific allergic responses in mice with allergic rhinitis (AR). WT mice and *plt* mice were nasally challenged with OVA for 14 consecutive days following systemic sensitization. **A**, Sneezing and nasal rubbing were observed and counted for 5 min at 4 different time points: 1) before receiving any sensitization (naive, on day -1); 2) after three rounds of i.p. sensitization (on day 20); 3) after seven nasal challenges ("in 7", on day 27); and 4) after the last nasal challenge ("in 14", on day 34). **B**, Total and OVA-specific IgE levels in serum were assayed by sandwich ELISA. OVA-specific IgE Abs are expressed as reciprocal log₂ titers. **C**, Serum histamine levels were determined by sandwich ELISA. **D**, Nasal tissue was subjected to H&E staining. The arrowheads point to eosinophils. The scale of the bar is 20 μm. **E**, Total numbers of eosinophils infiltrated into the bottom of the nasal septum were counted. These data are representative of three independent experiments containing three to five mice in each group. Significance was evaluated by an unpaired *t* test. *, *p* < 0.05; **, *p* < 0.01 vs WT mice.

examined for the production of cytokines (IL-4, IL-5, IL-13, and IFN-γ) by cytokine ELISA kits (R&D Systems).

RT-PCR

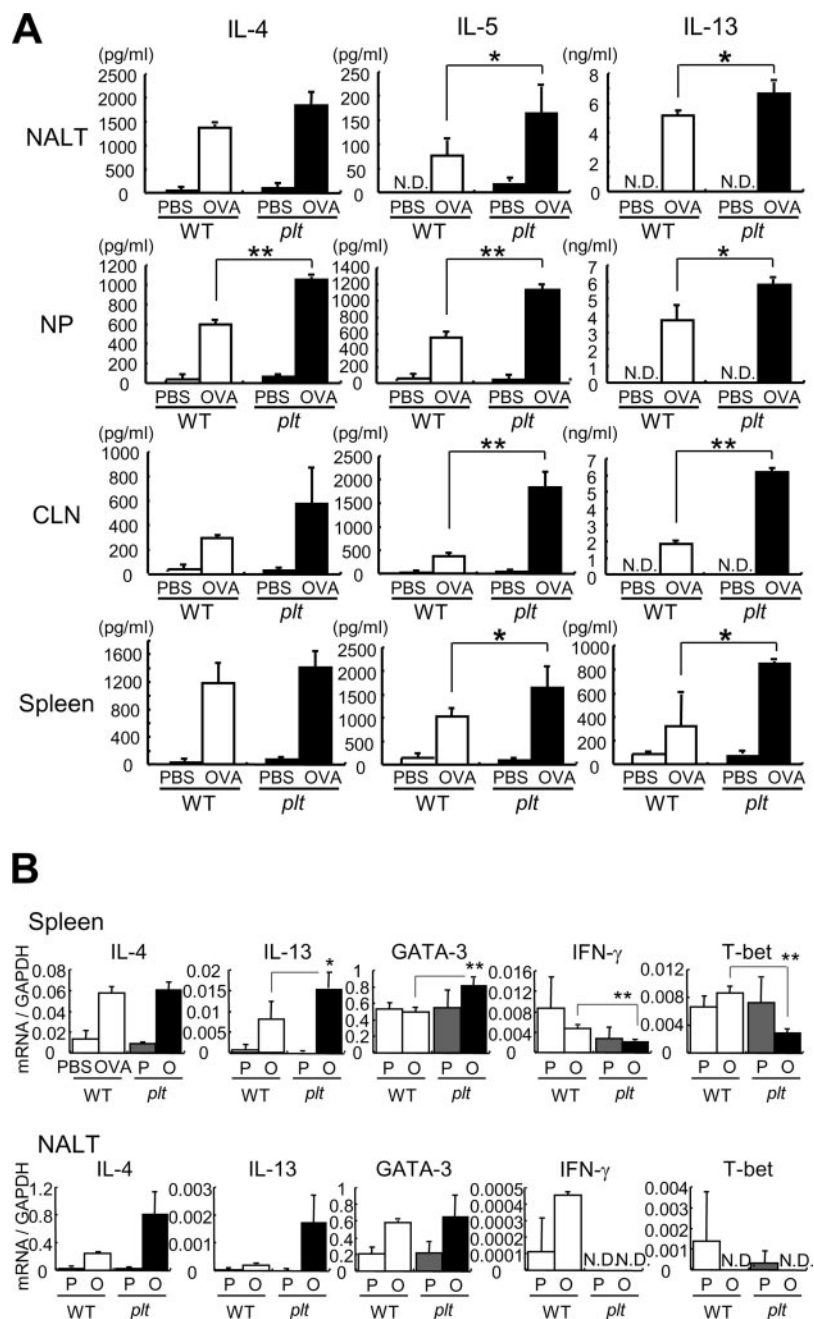
Total RNA was extracted using TRIzol reagent (Invitrogen). DNase digestion of the extracted RNA was performed before cDNA synthesis. We conducted reverse transcription using Omniscript Reverse Transcriptase (Qiagen) and Oligo d(T)₁₆ (Applied Biosystems), as well as quantitative real-time PCR using LightCycler (Roche Diagnostics) with LightCycler-FastStart DNA Master Hybridization probes (Roche Diagnostics). The primers and hybrid probes for real-time PCR were as follows: the oligonucleotide primers specific for IL-13 (sense, 5'-AGCATGGTATGGAG TGTGGA-3'; antisense, 5'-GTGGGCTACTTCGATTTTGG-3'); the IL-13 detection FITC-labeled probe (5'-TGCAATGCCATCTACAGGAC CCAGAGG-3') and the Lightcycler Red 640-labeled hybrid probe (5'-TATTGCATGGCCTCTGTAAACCGCAAGG-3'); the oligonucleotide primers specific for GATA-3 (sense, 5'-CATGCGTGAGAGTCTCCAA-3'; antisense, 5'-GGAATGCAGACACCACCTCG-3'); the GATA-3 detection FITC-labeled probe (5'-GGGCTTCATGATAGTCTCTGCG AAA-3') and the Lightcycler Red 640-labeled hybrid probe (5'-ACGCA AGTAGAAGGGGTCCGAGGAAGTCTC-3'); and the oligonucleotide

primers specific for GAPDH (sense, 5'-TGAACGGGAAGCTCACTGG-3'; antisense, 5'-TCCACCACCCTGTTGCTGTA-3'); the GAPDH detection FITC-labeled probe (5'-CTGAGGACCAGGTTCTCTCTGCGA-3') and the Lightcycler Red 640-labeled hybrid probe (5'-TTCAACAGCAA CTCCCACTCTTCCACC-3'). They were designed and produced by Nihon Gene Research Laboratories. A Lightcycler-primer/probes set (Nihon Gene Research Laboratories) was used for the amplification of the cDNA of IL-4, IFN-γ, and T-bet. Messenger RNA expression levels for specific genes were normalized as a ratio relative to GAPDH.

Flow cytometric analysis

For the flow cytometric analysis, mononuclear cells isolated from several tissues were first incubated with anti-CD16/CD32 (2.4G2; BD Pharmingen) to block nonspecific binding of Abs to the Fcγ III and Fcγ II receptors, and then stained with each Ab. Allophycocyanin- (or PE)-conjugated anti-CD11c, FITC-conjugated anti-CD11b, and PE-conjugated anti-CD8α were used to analyze DCs. For the analysis of Tregs, cells were stained with FITC-conjugated anti-CD3e, FITC- or PE-conjugated anti-CD4, and allophycocyanin-conjugated anti-CD25. In some experiments, a PE anti-mouse/rat Foxp3 staining set (FJK-16s; eBioscience) or biotin-conjugated anti-CCR7 (4B12; eBioscience) with a streptavidin-PE conjugate (BD

FIGURE 2. Th2 cytokine production, and Th1/Th2 cytokine and associated transcriptional factor mRNA expression from CD4⁺ T cells isolated from mice with AR. **A**, Culture supernatants of CD4⁺ T cells of NALT, NP, CLN, and spleen from mice with AR were assessed for Th2 cytokine production levels by ELISA. **B**, Th1 and Th2 cytokine and associated transcriptional factor-specific mRNA expression in spleen, and NALT was determined by quantitative real-time PCR analysis. The expression of each cytokine was normalized to the expression of GAPDH. Representative results from three independent experiments containing three mice in each group are shown. Significance was evaluated by an unpaired *t* test. *, *p* < 0.05; **, *p* < 0.01 vs WT mice. N.D., not detected. P, PBS-treated; O, OVA-treated.



Pharmingen) was used. Compensation was carefully performed in each tissue in accordance with the published instructions (31, 32). Nonviable cells were excluded using a Via-Probe (7-amino-actinomycin D; BD Pharmingen). Stained cells were then analyzed using a FACSCalibur flow cytometer (BD Biosciences) with CellQuest software (BD Biosciences).

Nasal CCL19/CCL21 DNA treatment

CCL19 and CCL21-Ser cDNAs were amplified by PCR using cDNA from whole spleen cells of naive BALB/c mice as a template. The oligonucleotide primers were as follows: the primers specific for CCL19 (sense, 5'-CCTTGTCTCGAGCCACCATGGCCCCCGTGTGACCCAC-3'; antisense, 5'-AGCCTCGAATTCTCAAGACACAGGGCTCCTTCTGG-3') and CCL21-Ser (sense, 5'-CCTTGTCTCGAGCCTCAACTCAACCACAATCATGGC-3'; antisense, 5'-AGCCTCGAATTCTATCTCTTGGGGCTGTGTC-3', with underlining indicating the *Xho*I and *Eco*RI restriction enzyme site). Plasmid DNA encoding either CCL19 or CCL21-Ser was constructed by the ligation of CCL19 or CCL21-Ser cDNA, respectively, into a pIRES2-EGFP vector (BD Biosciences Clontech). The empty vector pIRES2-EGFP (mock DNA) was used as a control. The plasmid DNAs and the mock DNA control were amplified in *E. coli* and purified

using an EndoFree Plasmid Maxi kit (Qiagen). For the detection of CCL19/CCL21 expression, each plasmid was transfected into COS-7 (CRL-1651; ATCC) in Opti-MEM (Invitrogen) by electroporation using Gene Pulser Xcell (Bio-Rad). After 48 h, culture supernatants were collected and chemokine levels were determined using a commercial ELISA kit (R&D Systems). Mice were sensitized by i.p. injection of 25 μ g of OVA with 1 mg of Alum on days 0, 7, and 14, followed by nasal challenge with 500 μ g of OVA for 14 consecutive days from day 21 to 34 for the induction of AR. As a nasal CCL19/CCL21 DNA treatment, mice were nasally administered with an additional 100 μ g of plasmid, mock DNA, or PBS on days -1, 6, and 13 (24 h before systemic sensitization) and from day 20 to 33 (before nasal challenge). Nasal symptoms were observed, and sera and mononuclear cells in several tissues were harvested for further examination.

Statistical analysis

Data were expressed as mean $\pm$ SE and evaluated by an unpaired Student's *t* test. Values of *p* < 0.05 were assumed to be statistically significant.

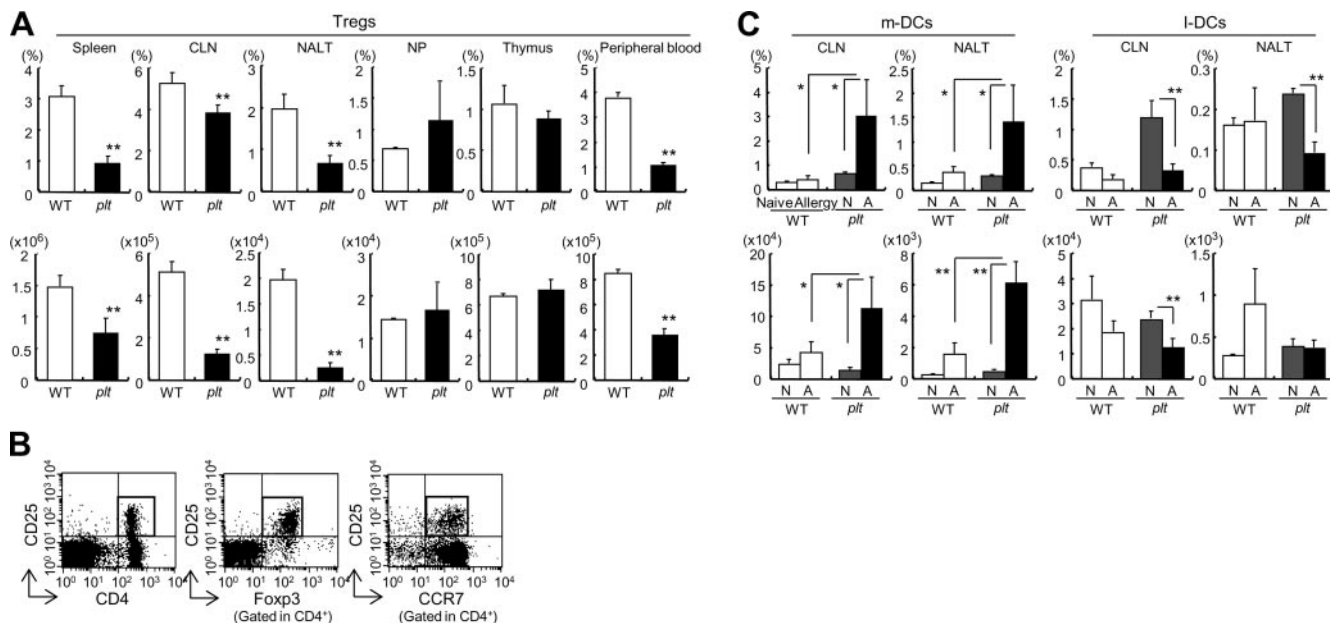


FIGURE 3. Flow cytometric analysis of Tregs and DCs in mice with AR. *A*, The frequency (upper) and absolute number (lower) of Tregs in naive mice were determined and calculated using a flow cytometer. *B*, Representative flow cytometric analysis data of Foxp3 and CCR7 expression in Tregs using mononuclear cells obtained from spleen of WT mice. *C*, The frequency (top) and absolute number (bottom) of DCs in both naive mice and mice with AR were determined. Results were obtained from three independent experiments containing 3 to 5 mice in each group (*A* and *C*). Significance was evaluated by an unpaired *t* test. *, *p* < 0.05; **, *p* < 0.01 vs WT mice. N, naive; A, allergy.

Results

Induction of severe allergic symptoms and Ag-specific IgE production in *plt* mice

To clarify the role played by the lymphoid chemokines CCL19 and CCL21 in the control of allergic diseases in the upper respiratory tract, the murine AR model was employed (25). Systemically primed wild-type (WT) BALB/c mice and *plt* mice were nasally challenged with OVA for 14 consecutive days with no significant symptomatic difference between WT and *plt* mice seen through the seventh nasal challenge (Fig. 1*A*). After 14 days of continuous exposure, however, markedly more severe nasal symptoms were observed in *plt* than in WT mice (Fig. 1*A*). As would be expected given the worsened nasal symptoms observed in *plt* mice, the serum of these mice showed significantly higher levels of OVA-specific IgE and of total IgE Abs than did that of identically treated WT mice (Fig. 1*B*). To assess the extent of immediate AR-associated reactions, serum histamine levels were measured by ELISA and *plt* mice were found to produce significantly higher levels of histamine than WT mice (Fig. 1*C*). When the nasal tissues of nasally challenged *plt* and WT mice were histologically compared, *plt* nasal tissue showed higher numbers of infiltrated eosinophils, a signature trait of the delayed phase of the allergic reaction (Fig. 1, *D* and *E*). These findings suggest that the clinical symptoms of inhaled Ag-induced AR escalate in the absence of the lymphoid chemokines CCL19 and CCL21.

Th2 responses were enhanced in *plt* mice

Given the exacerbated Ag-specific allergic responses observed in *plt* mice to nasally administered Ag, we hypothesized that Th2 responses would also be enhanced in these mice. To test this hypothesis, we compared the Th1/Th2 cytokine synthesis profiles of *plt* and WT mice after chronic exposure to nasal allergens. Thus, Th1 and Th2 cytokine production was measured in vitro by OVA stimulation of CD4⁺ T cells isolated from the CLN, the regional LN of the upper airway, in nasally challenged *plt* and WT mice. As

one might expect, no evidence of Th1 cytokine IFN- γ synthesis was found in *plt* mice with AR (data not shown). In contrast, significantly higher levels of the Th2 cytokines IL-5 and IL-13 were observed in CLN isolated from nasally challenged *plt* than from WT mice (Fig. 2*A*). Higher levels of IL-5 and IL-13 were also noted in CD4⁺ T cells isolated from the site of allergic reactions, i.e., NALT and NP, of *plt* than of WT mice. An identical pattern of elevated IL-5 and IL-13 production was also noted in systemically (spleen-) derived CD4⁺ T cells of *plt* mice with AR. As a rule, levels of IL-4, the other known Th2 cytokine, tended to be higher in *plt* mice nasally exposed to allergens, but that increase did not reach statistical significance when compared with WT mice (Fig. 2*A*). However, the NP of *plt* mice, where major local allergic responses were occurring, showed a more vigorous synthesis of IL-4 and an increase over WT IL-4 levels that reached statistical significance. The hypothesis that Th2 responses were dominant in nasally challenged *plt* mice received further support from the analysis of the levels of Th1-/Th2-associated transcription factor and of cytokine-specific mRNA. Higher levels of GATA-3- and IL-13-specific mRNA expression were noted in spleen of *plt* mice than in that of WT mice, although no significant difference was observed in IL-4-specific mRNA expression levels (Fig. 2*B*). In contrast, the mRNA expression of Th1 transcription factor T-bet and IFN- γ was low or undetectable in *plt* mice with AR. These results suggest that inhaled allergens trigger an aberrant Th2 immunological environment at both inductive (e.g., NALT) and effector (e.g., NP) sites in *plt* mice.

Low numbers of naturally occurring Tregs but elevated numbers of m-DCs under allergic conditions are characteristic of *plt* mice

To further elucidate the immunopathological mechanisms underlying the exacerbated allergic responses observed in *plt* mice, we next set out to determine whether the regulatory network formed by Tregs and DCs was altered in nasally challenged *plt* mice. Flow cytometric analysis revealed a lower frequency of and decreased

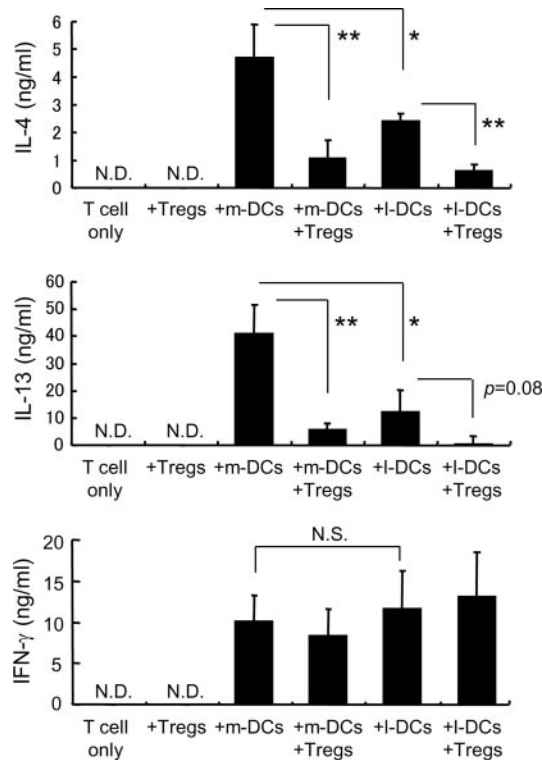


FIGURE 4. Role played by each DC subset in the induction of Ag-specific T cell responses. The production levels of IL-4, IL-13, and IFN- γ were measured by ELISA in culture supernatants of naive T cells from DO11.10 OVA-TCR transgenic mice cocultured with or without Tregs and/or DCs. Data are representative of three separate experiments. Significance was evaluated by an unpaired *t* test. *, $p < 0.05$; **, $p < 0.01$. N.S., not significant.

numbers of CD4⁺CD25⁺ T cells in the secondary lymphoid tissues such as NALT and in the peripheral blood of naive *plt* than of naive WT mice (Fig. 3A). Because these CD4⁺CD25⁺ T cells expressed Foxp3, they were considered to be Tregs (Fig. 3B). Interestingly, most of these Tregs observed in both *plt* and WT mice expressed CCR7 (Fig. 3B). As Treg levels did not change after the induction of AR (data not shown), these Tregs were considered to be naturally occurring. As CD8 α ⁺CD11b⁺ m-DCs and CD8 α ⁺CD11b⁺ l-DCs are reported to have immunomodulatory roles in Th1/Th2 cytokine production (21–23), our next flow cytometric analysis was aimed at DC subsets located in the various tissues of *plt* mice with severe AR. The most significant changes observed were an increased frequency of m-DCs residing in secondary lymphoid tissues such as CLN and NALT of nasally challenged *plt* mice with AR ($0.66 \pm 0.08\%$ to $3.02 \pm 1.51\%$ and $0.30 \pm 0.02\%$ to $1.41 \pm 0.74\%$, respectively; Fig. 3C) and an elevated total number of m-DCs (Fig. 3C). In contrast, the frequency and number of m-DCs in nonsensitized *plt* mice were comparable to those seen in WT mice (Fig. 3C). Taken together, these findings suggest that, under CCL19- and CCL21-deficient conditions, severe AR is associated with a reduction in naturally occurring Tregs and an increase in the frequency and the number of m-DCs in the nasal mucosa-associated lymphoid tissues.

m-DCs induce a predominantly Th2 environment

Inasmuch as the secondary lymphoid tissues of *plt* mice with severe nasal allergic responses showed increased numbers of m-DCs, we focused our next experiment on the role of this DC subset in the development of AR. When cultured with naive CD4⁺ T cells isolated from OVA-TCR transgenic mice in the presence of OVA, m-DCs isolated from the CLN of WT mice with AR produced significantly higher levels of IL-4 and IL-13 than did l-DCs isolated from the same mice (Fig. 4). When Tregs isolated from WT mice were added to Th2-leaning cultures of m-DC and CD4⁺ T

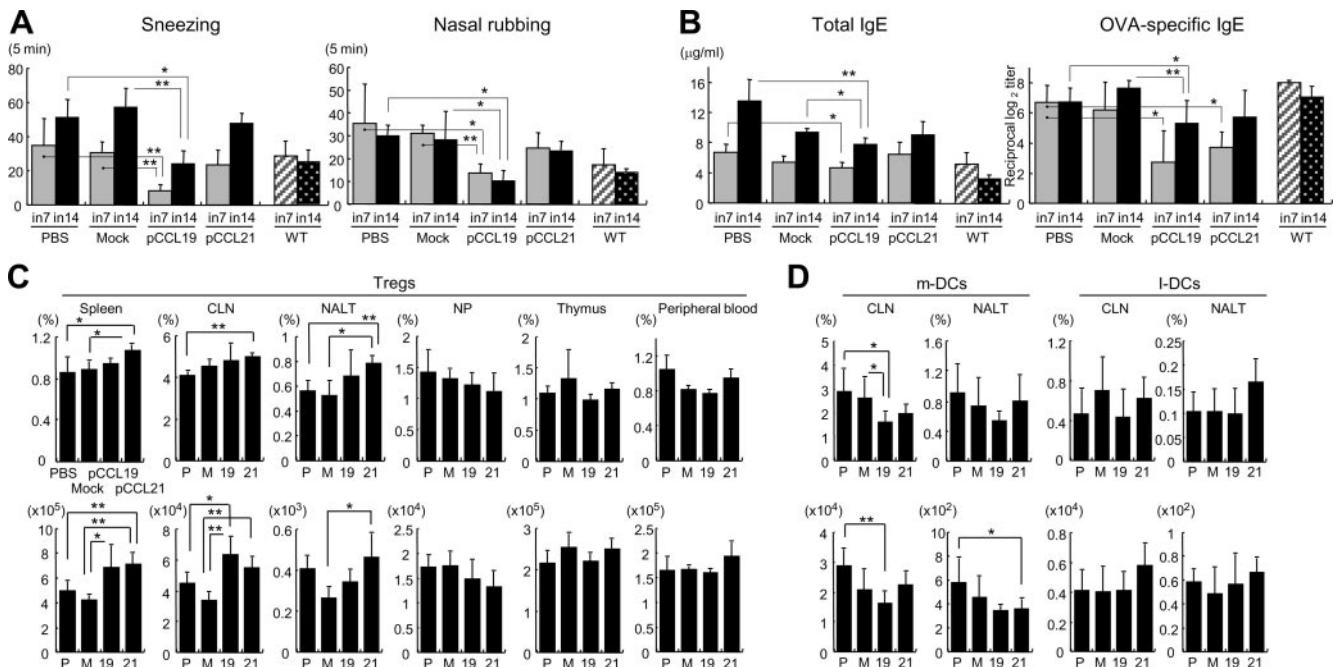


FIGURE 5. Allergic symptoms and the number of Tregs and DCs in *plt* mice treated by nasal administration of plasmids encoding CCL19/CCL21-Ser DNA. Nasal symptoms (A) and total and OVA-specific IgE levels in serum (B) were assessed as previously described at two different time points: 1) after 7 nasal challenges (“in 7”, on day 27) and 2) after the last nasal challenge (“in 14”, on day 34). C, The final frequency (top) and absolute number (bottom) of Tregs were determined and calculated by flow cytometric analysis. D, The final frequency (top) and absolute number (bottom) of DCs in CLN and NALT was determined. These data were obtained from two to three independent experiments containing 3 to 5 mice in each group. Significance was evaluated by an unpaired *t* test. *, $p < 0.05$; **, $p < 0.01$. P, PBS-treated; M, mock DNA-treated; 19, pCCL19-treated; 21, pCCL21-treated.

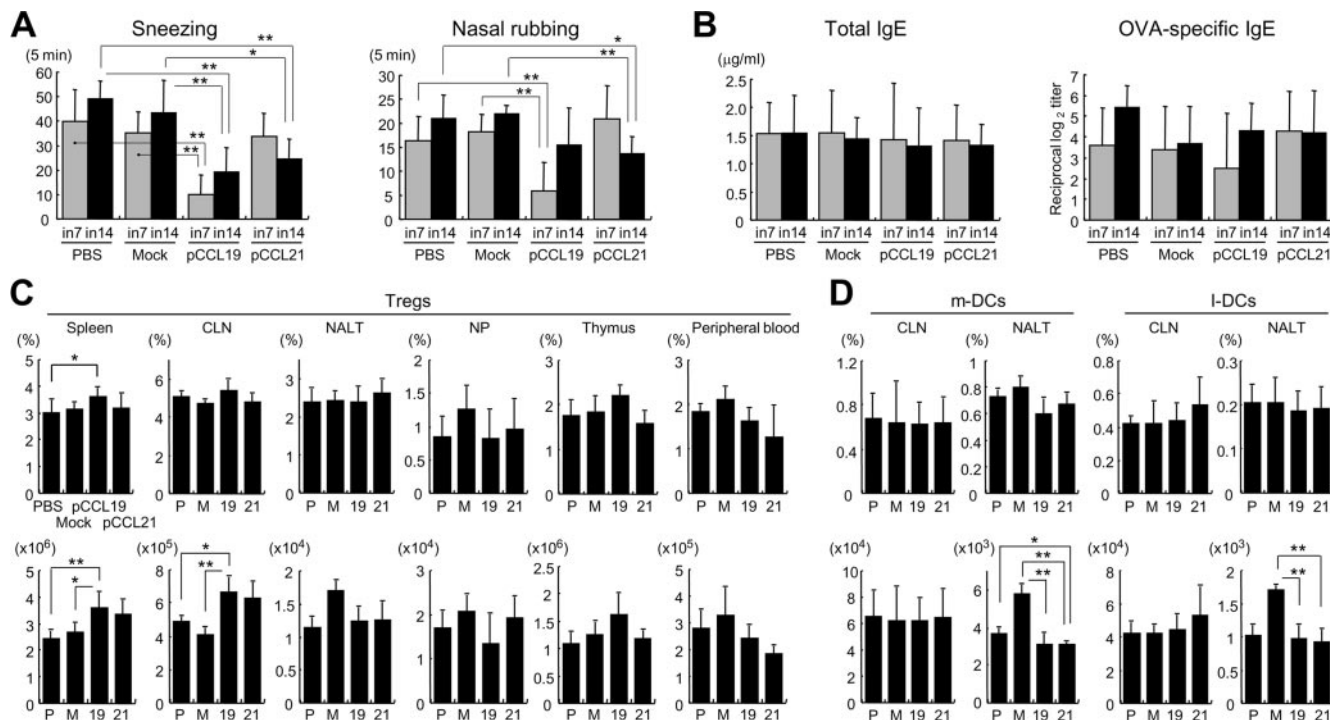


FIGURE 6. Allergic symptoms and the number of Tregs and DCs in WT mice treated by nasal administration of plasmids encoding CCL19/CCL21-Ser DNA. Nasal symptoms (A) and total and OVA-specific IgE levels in serum (B) were assessed at two different time points: 1) after 7 nasal challenges ("in 7", on day 27) and 2) after the last nasal challenge ("in 14", on day 34). C, The final frequency (top) and absolute number (bottom) of Tregs were determined by flow cytometric analysis. D, The final frequency (top) and absolute number (bottom) of DCs in CLN and NALT were determined. These data were obtained from three independent experiments containing 3 to 6 mice in each group. Significance was evaluated by an unpaired *t* test. *, *p* < 0.05; **, *p* < 0.01.

cells, Th2 cytokine production was suppressed. Tregs from *plt* mice possessed a similar capacity to suppress m-DC-induced IL-4 and IL-13 production (data not shown). The addition of two neutralizing Abs, anti-IL-10 Ab and anti-TGF- β Ab, did not inhibit Treg function, suggesting that Tregs suppress Th2 production independently of suppressive cytokines such as IL-10 and TGF- β (data not shown). No significant difference was observed in IFN- γ production between CD4⁺ T cells cocultured with m-DCs and those cocultured with I-DCs (Fig. 4). These data demonstrate that m-DCs are key players in Th2 cytokine production and Tregs are key players in its suppression.

Nasal administration of plasmids encoding CCL19 DNA altered DC population and suppressed allergic symptoms in *plt* mice

Because *plt* mice show enhanced allergic responses, we next sought to determine whether artificial reconstitution of lymphoid chemokines using plasmids encoding CCL19 DNA (pCCL19) and CCL21-Ser DNA (pCCL21) would lead to the inhibition of nasal allergic responses. For the assessment of protein production, pCCL19 and pCCL21 were transfected into COS-7 cells and the production of CCL19 and CCL21 was confirmed in culture supernatants (data not shown). When *plt* mice were treated with controls (PBS or mock DNA) or these chemokine plasmids together with AR induction, significantly milder nasal clinical symptoms were observed in *plt* mice treated with pCCL19 than in mice treated with PBS or mock DNA (Fig. 5A). These milder clinical symptoms were similar to those observed in AR-induced WT mice without any treatment. Both total IgE- and OVA-specific IgE Abs were also significantly lower in pCCL19-treated *plt* mice than in control mice (Fig. 5B). Following pCCL21 treatment, some lessening of exaggerated allergic symptoms and IgE production were noted, but, with the exception of the level of Ag-specific IgE, observed

differences did not reach statistical significance when compared with control-treated mice (Fig. 5, A and B).

Flow cytometric analysis revealed that the frequency of Tregs was higher in spleen, CLN and NALT of *plt* mice treated with pCCL21 than in control *plt* mice (Fig. 5C). In mice treated with pCCL19, the frequency of Tregs also increased in CLN and NALT compared with control-treated mice, but no statistical difference was observed (Fig. 5C). The frequency of m-DCs in CLN of *plt* mice treated with pCCL19 was considerably reduced when compared with control-treated mice (Fig. 5D). The frequency of m-DCs was also lower in NALT of mice treated with pCCL19 and in CLN of mice treated with pCCL21 than in control mice, but the reduction did not reach statistical differences (Fig. 5D).

When the nasal chemokine plasmid treatment was also tested in WT mice, the reduction of nasal symptoms could be observed; however, serum IgE levels did not change when WT mice were treated with pCCL19/pCCL21 (Fig. 6, A and B). Nasal pCCL19 treatment induced a higher frequency and increased number of Tregs in spleen as well as CLN (Fig. 6C). The total number of m-DCs in NALT was reduced when WT mice were treated with pCCL19/pCCL21 (Fig. 6D). Taken together, these data suggest that CCL19 and CCL21 increase the number of Tregs and simultaneously inhibit the pathological function of m-DCs in allergic diseases.

Discussion

Because our recent study demonstrated that lymphoid chemokines play a crucial role in the maturation of NALT, a major commander tissue for the upper respiratory mucosal immune system (5), we set out in this study to elucidate the roles of the lymphoid chemokines CCL19 and CCL21 in the development of allergic diseases associated with the nasal cavity and other upper airway tract tissues

using a murine AR model. Constitutively produced by stromal cells in the T cell area of lymphoid tissues, in the endothelial cells of high endothelial venules, and in lymphatic vessels, these two chemokines are involved in homeostatic lymphocyte trafficking and DC migration as well as in the distribution of cells in and the development of organized lymphoid tissues (1). After up-regulating CCR7 expression, DCs migrate via lymphatic vessels from peripheral tissues into T cell areas of the secondary lymphoid tissues, which constitutively express CCL19 and CCL21 for the priming of naive T cells expressing CCR7 (1–3). Primed CD4⁺ T cells down-regulate CCR7 expression and differentiate into Th1, Th2 or nonpolarized memory T cells before circulating or migrating to the periphery for the initiation of Ag-specific immune response (33). Despite such major immunological contributions by these chemokines to secondary lymphoid tissue development and immune cell trafficking, limited information exists as to their involvement in allergic states of the upper respiratory tract such as AR. By using *plt* mice defective in CCL19 and CCL21-Ser but still capable of producing CCL21-Leu from the endothelium of lymphatic vessels (8), we have directly shown that CCL19 and CCL21 regulate the inhibition of nasal allergic responses. It has been further demonstrated that *plt* mice show delayed but enhanced T cell responses in a contact sensitivity model (8). It has also been shown that enhanced Th2-mediated allergen-induced lung inflammation is observed in *plt* mice using an asthma model (34, 35). Our current study is in agreement with these previous findings and demonstrates in a murine model of allergy in the upper respiratory tract that Th2 allergic responses are aggravated when CCL19 and CCL21-Ser are deficient. It also shows that AR development is prevented by the reintroduction of the chemokines using the corresponding plasmids via the nasal route. Together with the original studies (8, 34, 35), our study suggests that the lymphoid chemokine family of CCL19 and CCL21 regulates the inhibition of hypersensitivity responses including allergy.

Under CCL19- and CCL21-deficient conditions, m-DCs were significantly increased in the upper airway-associated lymphoid tissues of the CLN and NALT of mice with AR. Indeed, m-DCs isolated from the CLN of WT mice with AR induced considerably higher levels of IL-4 and IL-13 but not of IFN- γ production from cocultured naive T cells when compared with l-DCs. Interestingly, the nature of the immune response (Th1 and/or Th2 responses) depends in part on the specific subsets of DCs involved and their point of origin (17). For example, m-DCs isolated from the spleen and/or Peyer's patches are capable of inducing Ag-specific T cells to produce Th2 cytokines, while l-DCs induce Th1 responses (29, 36). Furthermore, Th2 responses are generated when bone marrow-derived m-DCs are transferred into the airway, leading to eosinophil infiltration in the asthma model (37). Lymphoid chemokines can also enhance the functioning of DCs; bone marrow-derived DCs stimulated with CCL19/CCL21 produce inflammatory cytokines such as IL-1 β , TNF- α and IL-12 at almost comparable levels to those stimulated with LPS and anti-CD40 Ab (38). Indeed, CCL19-activated DCs selectively mediate the induction of Th1 responses (38). Taken together, these findings strongly suggest that the absence of CCL19/CCL21 during DC Ag presentation strongly favors the creation of a Th2-dominant environment that is conducive to the establishment of airway allergy.

Chemokines other than CCL19/CCL21 may help account for the increase in m-DCs noted in *plt* mice. For instance, CCR6 is required for the recruitment of m-DCs toward mucosal surfaces expressing its ligand CCL20 (39), and the CCL20-CCR6 signal is crucial for airway immune responses. The proinflammatory cytokines TNF- α and IL-1 β and the Th2 cytokines IL-4 and IL-13 can

stimulate human bronchial epithelial cells to produce CCL20 (40). CCR6 knockout mice demonstrate a diminished allergic response and reduced peribronchial eosinophil accumulation and IgE production (41). These findings suggest that other chemokines in addition to CCL19/CCL21 may be involved in the recruitment of m-DCs in *plt* mice with AR.

In general, undesired T cell-mediated responses are down-regulated by Ag-specific inducible Tregs and/or naturally occurring CD4⁺CD25⁺Foxp3⁺ Tregs. Down-regulation is mediated by the anti-inflammatory cytokines IL-10 and TGF- β and/or by cell-to-cell contact with coinhibitory molecules such as cytotoxic T lymphocyte-associated Ag-4 (CTLA-4), B and T lymphocyte attenuator, and PD-1 (22–24, 42). The frequency of Ag-specific Tregs expressing the surface molecules CTLA-4 and PD-1 and secreting IL-10 and TGF- β is higher in healthy individuals than in allergic individuals (43). Indeed, healthy immune responses to allergens depend upon a proper balance between allergen-specific Tregs and allergen-specific Th2 cells, with a disruption of that balance characterizing disease states like allergies (43). The murine colitis model can be used to demonstrate that the mediation of the inflammatory immune response by naturally occurring Tregs depends upon CTLA-4 (44). B and T lymphocyte attenuator and PD-1 are crucial in limiting the duration of acute allergic airway inflammation and act as terminators of established immune responses (42). In addition, naturally occurring Tregs are capable of inhibiting DC function directly. Depletion of these Tregs resulted in worsening airway hyperresponsiveness as the result of the exaggerated Th2 cytokine production caused by altered pulmonary DC function (45). In the murine asthma model, *in vivo* transfer of Ag-specific Tregs reduced airway hyperresponsiveness, eosinophil recruitment and Th2 responses in an IL-10-dependent manner (46). Th2 responses were elevated when m-DCs and naive T cells were cocultured in the presence of Ag, but suppressed upon the addition of Tregs (Fig. 4). These data suggest that naturally occurring Tregs play a critical role in inhibiting the Th2 response, probably by directly suppressing T cell responses and/or by indirectly suppressing the m-DC function that favors Th2 responses. Moreover, neither anti-IL-10 Ab nor anti-TGF- β Ab treatment impaired Treg-mediated Th2 suppression, suggesting that this suppression was independent of the inhibitory cytokines IL-10 and TGF- β (our unpublished observation). However, cell-to-cell interaction is required for the suppression of aberrant Th2 responses by Tregs.

CCL19/CCL21-Ser is not produced by *plt* mice, but CCL21-Leu is produced by their lymphatic vessels (1). As a result, activated m-DCs and naive T cells expressing CCR7 can migrate to and accumulate in secondary lymphoid tissues in *plt* mice. To this end, CCL21-Leu has been shown to act as a chemoattractant for CCR7-expressing DCs (2, 7). Because Tregs are significantly reduced in *plt* mice with AR (Fig. 3A), the capacity of Tregs to inhibit m-DC function and thereby suppress Th2 responses may also be impaired. However, a reduction in Tregs was observed in both naive and diseased *plt* mice, suggesting that the remnant Treg populations might be Ag-nonspecific, naturally occurring Tregs. The origin and development of naturally occurring Tregs in the thymus are still poorly understood. Generally, upon TCR-mediated positive selection, developing thymocytes relocate within the thymus from the cortex to the medulla for further differentiation and selection before export to the periphery (47). The CCR7 signal is essential for the migration of single-positive thymocytes from the cortex to the medulla and for the optimal emigration of T cells from the thymus to the periphery in newborn but not in adult mice (47). In *plt* mice, mature single-positive thymocytes rarely migrate from the cortex to the medulla, but, paradoxically T cell export

from the thymus into peripheral blood is not impaired (47). Therefore, one can speculate that *plt* mice have an impaired ability to maintain naturally occurring Tregs in the periphery (blood circulation) once they exit the thymus, but further studies are needed to shed light on this issue.

Nasal administration of pCCL19 results in the inhibition of AR development in *plt* mice. To the best of our knowledge, the current study provides the first evidence that intranasal pCCL19 treatment suppresses AR-associated allergic responses. In recent studies, plasmid DNA can be used in vivo as an adjuvant to enhance Ag-specific immune response (48) or as a therapeutic tool to alter undesired immunopathological conditions (49, 50). Intranasal codelivery of plasmids encoding the DNA of CCR7 ligands and plasmid DNA or recombinant vaccinia virus encoding HSV-gB increases HSV-gB-specific serum IgG and vaginal IgA levels, thereby enhancing protective immunity against HSV-1 infection (48). In contrast, nasally administered plasmids encoding IL-12 DNA can be used to treat not only airway hyperresponsiveness in asthma but even large intestinal inflammation in allergic diarrhea (49, 50). Hino et al. (50) have also demonstrated that GFP⁺ signals are preferentially colocalized within DCs in the NALT, spleen, and intestine after nasal administration of GFP-DNA. This finding suggests that mucosal DCs take up plasmids deposited in the nasal cavity and then migrate to distant lymphoid tissues. Our model has not yet enabled us to elucidate the mechanism underlying the decrease in m-DCs in the CLN and NALT following pCCL19/pCCL21 treatment. However, it is possible to speculate that replacement of these chemokines in *plt* mice enhanced Treg function, thereby inhibiting the accumulation of m-DCs in the CLN and NALT. Alternatively, it is also possible that the administration of the lymphoid chemokine plasmid either triggers a shift from Th2 to benign responses in m-DCs or simply restores their capacity for migration.

In summary, we demonstrated enhanced allergic responses in *plt* mice lacking the lymphoid chemokines CCL19 and CCL21-Ser. We also showed that these lymphoid chemokines are involved in the recruitment of CCR7 expressing naturally occurring Tregs in the secondary lymphoid tissues and help suppress the pathological Th2 environment induced by m-DCs during the development of AR. Taken together, these findings underline the importance of the lymphoid chemokines CCL19/CCL21 as regulatory molecules for the control of allergic disease.

Acknowledgments

We thank Dr. H. Nakano in Department of Medicine, Division of Cardiology, Duke University Medical Center for technical advice on mating *plt* mice. We thank the members of our laboratory for their technical advice and discussions. We also thank Dr. K. McGhee, Medical College of Georgia for editorial help.

Disclosures

The authors have no financial conflict of interest.

References

- Gunn, M. D., K. Tangemann, C. Tam, J. G. Cyster, S. D. Rosen, and L. T. Williams. 1998. A chemokine expressed in lymphoid high endothelial venules promotes the adhesion and chemotaxis of naive T lymphocytes. *Proc. Natl. Acad. Sci. USA* 95: 258–263.
- Gunn, M. D., S. Kyuwa, C. Tam, T. Kakiuchi, A. Matsuzawa, L. T. Williams, and H. Nakano. 1999. Mice lacking expression of secondary lymphoid organ chemokine have defects in lymphocyte homing and dendritic cell localization. *J. Exp. Med.* 189: 451–460.
- Förster, R., A. Schubel, D. Breitfeld, E. Kremmer, I. Renner-Müller, E. Wolf, and M. Lipp. 1999. CCR7 coordinates the primary immune response by establishing functional microenvironments in secondary lymphoid organs. *Cell* 99: 23–33.
- Ohl, L., G. Henning, S. Krautwald, M. Lipp, S. Hardtke, G. Bernhardt, O. Pabst, and R. Förster. 2003. Cooperating mechanisms of CXCR5 and CCR7 in development and organization of secondary lymphoid organs. *J. Exp. Med.* 197: 1199–1204.
- Fukuyama, S., T. Nagatake, D. Y. Kim, K. Takamura, E. J. Park, T. Kaisho, N. Tanaka, Y. Kuroki, and H. Kiyono. 2006. Cutting edge: uniqueness of lymphoid chemokine requirement for the initiation and maturation of nasopharynx-associated lymphoid tissue organogenesis. *J. Immunol.* 177: 4276–4280.
- Kim, C. H., L. M. Pelus, J. R. White, and H. E. Broxmeyer. 1998. Differential chemotactic behavior of developing T cells in response to thymic chemokines. *Blood* 91: 4434–4443.
- Nakano, H., and M. D. Gunn. 2001. Gene duplications at the chemokine locus on mouse chromosome 4: multiple strain-specific haplotypes and the deletion of secondary lymphoid-organ chemokine and EBI-1 ligand chemokine genes in the *plt* mutation. *J. Immunol.* 166: 361–369.
- Mori, S., H. Nakano, K. Aritomi, C. R. Wang, M. D. Gunn, and T. Kakiuchi. 2001. Mice lacking expression of the chemokines CCL21-ser and CCL19 (*plt* mice) demonstrate delayed but enhanced T cell immune responses. *J. Exp. Med.* 193: 207–218.
- Kiyono, H., and S. Fukuyama. 2004. NALT- versus Peyer's-patch-mediated mucosal immunity. *Nat. Rev. Immunol.* 4: 699–710.
- 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: 3346–3353.
- Yanagita, M., T. Hiroi, N. Kitagaki, S. Hamada, H. O. Ito, H. Shimauchi, S. Murakami, H. Okada, and H. Kiyono. 1999. Nasopharyngeal-associated lymphoreticular tissue (NALT) immunity: fimbriae-specific Th1 and Th2 cell-regulated IgA responses for the inhibition of bacterial attachment to epithelial cells and subsequent inflammatory cytokine production. *J. Immunol.* 162: 3559–3565.
- Tamura, S., E. Hatori, T. Tsuruhara, C. Aizawa, and T. Kurata. 1997. Suppression of delayed-type hypersensitivity and IgE antibody responses to ovalbumin by intranasal administration of *Escherichia coli* heat-labile enterotoxin B subunit-conjugated ovalbumin. *Vaccine* 15: 225–229.
- Yura, M., I. Takahashi, S. Terawaki, T. Hiroi, M. N. Kweon, Y. Yuki, and H. Kiyono. 2001. Nasal administration of cholera toxin (CT) suppresses clinical signs of experimental autoimmune encephalomyelitis (EAE). *Vaccine* 20: 134–139.
- Unger, W. W., W. Jansen, D. A. Wolvers, A. G. van Halteren, G. Kraal, and J. N. Samsom. 2003. Nasal tolerance induces antigen-specific CD4⁺CD25⁺ regulatory T cells that can transfer their regulatory capacity to naive CD4⁺ T cells. *Int. Immunol.* 15: 731–739.
- Gelfand, E. W. 2004. Inflammatory mediators in allergic rhinitis. *J. Allergy Clin. Immunol.* 114: S135–S138.
- Skoner, D. P. 2001. Allergic rhinitis: definition, epidemiology, pathophysiology, detection, and diagnosis. *J. Allergy Clin. Immunol.* 108: S2–S8.
- Kelsall, B. L., and M. Rescigno. 2004. Mucosal dendritic cells in immunity and inflammation. *Nat. Immunol.* 5: 1091–1095.
- Kalinski, P., and M. Moser. 2005. Consensual immunity: success-driven development of T-helper-1 and T-helper-2 responses. *Nat. Rev. Immunol.* 5: 251–260.
- McGuirk, P., C. McCann, and K. H. Mills. 2002. Pathogen-specific T regulatory 1 cells induced in the respiratory tract by a bacterial molecule that stimulates interleukin 10 production by dendritic cells: a novel strategy for evasion of protective T helper type 1 responses by *Bordetella pertussis*. *J. Exp. Med.* 195: 221–231.
- Kelsall, B. L., and F. Leon. 2005. Involvement of intestinal dendritic cells in oral tolerance, immunity to pathogens, and inflammatory bowel disease. *Immunol. Rev.* 206: 132–148.
- Sakaguchi, S., N. Sakaguchi, M. Asano, M. Itoh, and M. Toda. 1995. Immunologic self-tolerance maintained by activated T cells expressing IL-2 receptor α -chains (CD25): breakdown of a single mechanism of self-tolerance causes various autoimmune diseases. *J. Immunol.* 155: 1151–1164.
- Taylor, A., J. Verhagen, C. A. Akdis, and M. Akdis. 2005. T regulatory cells and allergy. *Microbes Infect.* 7: 1049–1055.
- Hawrylowicz, C. M., and A. O'Garra. 2005. Potential role of interleukin-10-secreting regulatory T cells in allergy and asthma. *Nat. Rev. Immunol.* 5: 271–283.
- Shevach, E. M. 2002. CD4⁺CD25⁺ suppressor T cells: more questions than answers. *Nat. Rev. Immunol.* 2: 389–400.
- Iwasaki, M., K. Saito, M. Takemura, K. Sekikawa, H. Fujii, Y. Yamada, H. Wada, K. Mizuta, M. Seishima, and Y. Ito. 2003. TNF- α contributes to the development of allergic rhinitis in mice. *J. Allergy Clin. Immunol.* 112: 134–140.
- Kweon, M. N., M. Yamamoto, M. Kajiki, I. Takahashi, and H. Kiyono. 2000. Systemically derived large intestinal CD4⁺ Th2 cells play a central role in STAT6-mediated allergic diarrhea. *J. Clin. Invest.* 106: 199–206.
- Morafo, V., K. Srivastava, C. K. Huang, G. Kleiner, S. Y. Lee, H. A. Sampson, and A. M. Li. 2003. Genetic susceptibility to food allergy is linked to differential TH2-TH1 responses in C3H/HeJ and BALB/c mice. *J. Allergy Clin. Immunol.* 111: 1122–1128.
- Fukuyama, S., T. Hiroi, Y. Yokota, P. D. Rennert, M. Yanagita, N. Kinoshita, S. Terawaki, T. Shikina, M. Yamamoto, Y. Kuroki, and H. Kiyono. 2002. Initiation of NALT organogenesis is independent of the IL-7R, LT β R, and NIK signaling pathways but requires the Id2 gene and CD3⁺CD4⁺CD45⁺ cells. *Immunity* 17: 31–40.
- Iwasaki, A., and B. L. Kelsall. 2001. Unique functions of CD11b⁺, CD8 α ⁺, and double-negative Peyer's patch dendritic cells. *J. Immunol.* 166: 4884–4890.
- Sato, A., M. Hashiguchi, E. Toda, A. Iwasaki, S. Hachimura, and S. Kaminogawa. 2003. CD11b⁺ Peyer's patch dendritic cells secrete IL-6 and induce IgA secretion from naive B cells. *J. Immunol.* 171: 3684–3690.

31. Herzenberg Leonore, A., J. Tung, W. A. Moore, A. Leonard Herzenberg, and D. R. Parks. 2006. Interpreting flow cytometry data: a guide for the perplexed. *Nat. Immunol.* 7: 681–685.
32. Henri, S., D. Vremec, A. Kamath, J. Waithman, S. Williams, C. Benoist, K. Burnham, S. Saeland, E. Handman, and K. Shortman. 2001. The dendritic cell populations of mouse lymph nodes. *J. Immunol.* 167: 741–748.
33. Bromley, S. K., S. Y. Thomas, and A. D. Luster. 2005. Chemokine receptor CCR7 guides T cell exit from peripheral tissues and entry into afferent lymphatics. *Nat. Immunol.* 6: 895–901.
34. Yamashita, N., H. Tashimo, Y. Matsuo, H. Ishida, K. Yoshiura, K. Sato, N. Yamashita, T. Kakiuchi, and K. Ohta. 2006. Role of CCL21 and CCL19 in allergic inflammation in the ovalbumin-specific murine asthmatic model. *J. Allergy Clin. Immunol.* 117: 1040–1046.
35. Grinnan, D., S. S. Sung, J. A. Dougherty, A. R. Knowles, M. B. Allen, C. E. Rose, H. Nakano, M. D. Gunn, S. M. Fu, and C. E. Rose. 2006. Enhanced allergen-induced airway inflammation in paucity of lymph node T cell (*plt*) mutant mice. *J. Allergy Clin. Immunol.* 118: 1234–1241.
36. Maldonado-López, R., T. De Smedt, P. Michel, J. Godfroid, B. Pajak, C. Heirman, K. Thielemans, O. Leo, J. Urbain, and M. Moser. 1999. CD8 α^+ and CD8 α^- subclasses of dendritic cells direct the development of distinct T helper cells in vivo. *J. Exp. Med.* 189: 587–592.
37. Lambrecht, B. N., M. De Veerman, A. J. Coyle, J. C. Gutierrez-Ramos, K. Thielemans, and R. A. Pauwels. 2000. Myeloid dendritic cells induce Th2 responses to inhaled antigen, leading to eosinophilic airway inflammation. *J. Clin. Invest.* 106: 551–559.
38. Marsland, B. J., P. Böttig, M. Bauer, C. Ruedl, U. Lässig, R. R. Beerli, K. Dietmeier, L. Ivanova, T. Pfister, L. Vogt, et al. 2005. CCL19 and CCL21 induce a potent proinflammatory differentiation program in licensed dendritic cells. *Immunity* 22: 493–505.
39. Iwasaki, A., and B. L. Kelsall. 2000. Localization of distinct Peyer's patch dendritic cell subsets and their recruitment by chemokines macrophage inflammatory protein (MIP)-3 α , MIP-3 β , and secondary lymphoid organ chemokine. *J. Exp. Med.* 191: 1381–1394.
40. Reibman, J., Y. Hsu, L. C. Chen, B. Bleck, and T. Gordon. 2003. Airway epithelial cells release MIP-3 α /CCL20 in response to cytokines and ambient particulate matter. *Am. J. Respir. Cell Mol. Biol.* 28: 648–654.
41. Lukacs, N. W., D. M. Prosser, M. Wiekowski, S. A. Lira, and D. N. Cook. 2001. Requirement for the chemokine receptor CCR6 in allergic pulmonary inflammation. *J. Exp. Med.* 194: 551–555.
42. Deppong, C., T. I. Juehne, M. Hurchla, L. D. Friend, D. D. Shah, C. M. Rose, T. L. Bricker, L. P. Shornick, E. C. Crouch, T. L. Murphy, et al. 2006. Cutting edge: B and T lymphocyte attenuator and programmed death receptor-1 inhibitory receptors are required for termination of acute allergic airway inflammation. *J. Immunol.* 176: 3909–3913.
43. Akdis, M., J. Verhagen, A. Taylor, F. Karamloo, C. Karagiannidis, R. Cramer, S. Thunberg, G. Deniz, R. Valenta, H. Fiebig, et al. 2004. Immune responses in healthy and allergic individuals are characterized by a fine balance between allergen-specific T regulatory 1 and T helper 2 cells. *J. Exp. Med.* 199: 1567–1575.
44. Read, S., R. Greenwald, A. Izcue, N. Robinson, D. Mandelbrot, L. Francisco, A. H. Sharpe, and F. Powrie. 2006. Blockade of CTLA-4 on CD4 $^+$ CD25 $^+$ regulatory T cells abrogates their function in vivo. *J. Immunol.* 177: 4376–4383.
45. Lewkowich, I. P., N. S. Herman, K. W. Schleifer, M. P. Dance, B. L. Chen, K. M. Dienger, A. A. Sproles, J. S. Shah, J. Köhl, Y. Belkaid, and M. Wills-Karp. 2005. CD4 $^+$ CD25 $^+$ T cells protect against experimentally induced asthma and alter pulmonary dendritic cell phenotype and function. *J. Exp. Med.* 202: 1549–1561.
46. Kearley, J., J. E. Barker, D. S. Robinson, and C. M. Lloyd. 2005. Resolution of airway inflammation and hyperreactivity after in vivo transfer of CD4 $^+$ CD25 $^+$ regulatory T cells is interleukin 10 dependent. *J. Exp. Med.* 202: 1539–1547.
47. Ueno, T., F. Saito, D. H. Gray, S. Kuse, K. Hieshima, H. Nakano, T. Kakiuchi, M. Lipp, R. L. Boyd, and Y. Takahama. 2004. CCR7 signals are essential for cortex-medulla migration of developing thymocytes. *J. Exp. Med.* 200: 493–505.
48. Toka, F. N., M. Gierynska, and B. T. Rouse. 2003. Codelivery of CCR7 ligands as molecular adjuvants enhances the protective immune response against herpes simplex virus type 1. *J. Virol.* 77: 12742–12752.
49. Lee, Y. L., Y. L. Ye, C. I. Yu, Y. L. Wu, Y. L. Lai, P. H. Ku, R. L. Hong, and B. L. Chiang. 2001. Construction of single-chain interleukin-12 DNA plasmid to treat airway hyperresponsiveness in an animal model of asthma. *Hum. Gene Ther.* 12: 2065–2079.
50. Hino, A., S. Fukuyama, K. Kataoka, M. N. Kweon, K. Fujihashi, and H. Kiyono. 2005. Nasal IL-12p70 DNA prevents and treats intestinal allergic diarrhea. *J. Immunol.* 174: 7423–7432.