a loss of tolerance. The activated CD8αα αβ IELs did not show antigen-specific cytotoxicity nor did they promote an inflammatory response. Instead, the responding CD8αα αβ IELs secreted enhanced levels of TGF-β. The constitutive expression of measurable levels of mRNA for immunoregulatory cytokines, including IFN-γ, TGF-β, and IL-10, even without specific antigenic stimulation, further underscores that these type b IELs might exert regulatory functions rather than affording immune protection against specific pathogens.

The regulatory ability of these self-reactive T cells was first showed by analysis of gene-targeted animals, including IL-2/IL-2R-deficient mice, which have specific defects in this mucosal T-cell subset. These mice, in which the CD8αα αβ IEL population is reduced, are highly susceptible to gut inflammatory diseases (157, 158).

Direct evidence that CD8αα αβ IELs could regulate immune responses by conventional mucosal T cells was provided by sophisticated adoptive transfer experiments of CD8αα αβ IELs into immune-deficient $Rag^{-/-}$ recipient mice that also received pathogenic $CD4^{+}CD45RB^{high}$ splenocytes (156). In the absence of CD8αα αβ IELs, transferred pathogenic $CD4^{+}$ T cells migrate to the intestine and induce an uncontrolled inflammatory immune response, whereas mice that had previously received CD8αα αβ IELs were protected against this $CD4^{+}$ T-cell-mediated pathology. Surprisingly, $CD4^{+}CD45RB^{high}$ T-cell-induced colitis was prevented by transfer of CD8αα αβ IELs isolated from H-Y TCR transgenic male donor mice (156). This protection, however, was observed only in $H\text{-}2D^{b}$ male recipient mice and not in female mice, indicating that TCR must recognize self-antigen if it is to mediate regulatory function.

CD8αα γδ IELs and immune protection

The antigen specificity of CD8αα γδ IELs is poorly defined, and cell-transfer studies have indicated that the CD8αα γδ IELs have only minimal pathogen-specific activity (159, 160). Nevertheless, in the case of recipient mice infected with *Toxoplasma gondii*, it was shown that successful protection by CD8αβ αβ IELs was, in part, dependent on the presence of CD8αα γδ IELs (160). Although these CD8αα γδ IELs show only minimal pathogen-specific activity, their ability to respond rapidly to unprocessed self-antigens expressed by stressed or transformed ECs and to control the activity of the non-self-reactive CD8αβ αβ IELs has led to the idea that these cells might provide the first line of defense against invading pathogens (54).

Alternatively, because activated γδ IELs can produce keratinocyte growth factor (KGF), which is important for epithelial growth and the repair of damaged tissue, some γδ IELs could be involved in the repair of tissue damage elicited during inflammatory immune responses (161). The synthesis of KGF by γδ IELs has led to the hypothesis that these cells are important for the integrity and healing of the epithelium. Consistent with this hypothesis, the epithelium of the intestine in TCRδ-chain-deficient mice had reduced numbers of crypts (162). By contrast, the epithelium had a higher mitotic index in the presence of γδTCR transgene (163). In addition, TCRδ-chain-deficient mice show increased susceptibility to epithelial damage caused by 2,4,6-trinitrobenzene sulfonic-acid-induced colitis; indeed, the transfer of γδ IELs to TCRδ-chain-deficient mice ameliorated the hapten-induced colitis, which correlated with decreased IFN-γ and TNF-α production and increased TGF-β production by IELs (164).

It remains to be determined if the effect of γδ IELs on ECs is mediated solely by KGF or if other factors are involved. γδ IELs synthesize mRNA for TGF-β1 (18) and TGF-β3 (165), and TGF-β has been reported to aid in the healing of epithelial damage (166). Furthermore, a lack of γδ T cells often results in severe pathological conditions mediated by autoantibodies and/or in destructive cell-mediated immune responses by αβ T cells (167). TCRδ-deficient mice that have been orally infected with *Eimeria vermiformis*, an enterocyte-specific parasite, can efficiently control the infection, but they also show severe bleeding in the small intestine because of uncontrolled immune responses by infiltrating $CD4^{+}$ αβ T cells (168). Transfer of normal IELs to these TCRδ-deficient mice could prevent mucosal injury. These results suggest that γδ IELs exert significant control over the immune responses mediated by mucosal αβ T cells.

Not all immune responses mediated by γδ IELs are beneficial. For example, in the case of celiac disease, it was shown that dysregulated IL-15 secretion by wheat gliadin elicited overexpression of MICA/MICB on ECs and IELs, possibly including γδ T cells. When isolated from patients with active celiac disease, γδ T cells exhibited NKG2D-mediated cytotoxicity of the EC line HT29 possessing MICA (30).

γδ IELs for mucosal IgA responses

γδ IELs appear to be necessary for the induction and maintenance of humoral immune responses in the intestine. Interestingly, in TCRα-chain-deficient mice, an increase in the numbers and overall proportion of γδ IELs is seen after environmental microbial challenge (169), which is accompanied by B-cell maturation and production of high levels of Igs (170–172). Collaboration between B and non-αβ T cells, including γδ T cells as well as ββ TCR^{+} T cells (ββ T cells), sustains the production of germinal centers, lymphoid follicles that ordinarily are the anatomical signature of αβ T-cell and B-cell interactions.

Murine γδ IELs, if administered in the periphery, can enhance antigen-specific IgA responses to orally ingested antigens and thus might be capable of abrogating oral tolerance (173, 174). However, because it is unlikely that γδ IELs recirculate from the intestinal mucosa, their potential role in inducing or suppressing systemic oral tolerance is questionable. Although antibody responses against some T-cell-dependent antigens are impaired in mice lacking αβ T cells, mucosal γδ T cells may enhance antigen-specific as well as polyclonal S-IgA responses in the *lamina propria* of the small intestine (175).

Conclusions and future perspectives

In the harsh environment of the gut, the mucosal immune system must prevent the invasion of pathogenic microorganisms from the lumen while remaining quiescent against commensal microorganisms and food antigens, if disordered immune responses are to be prevented. Remarkably, IELs play a pivotal role in both immune surveillance and immune regulation, helping to maintain intestinal homeostasis by adjusting their immunological function to the different circumstances that prevail in the small and large intestines. However, the origin and development of these key players remain a subject of debate, and so it is crucial that we work to define their precise function and delineate their developmental pathway. Further investigation of IELs in both the small and large intestine will undoubtedly be key to the development of novel mucosal vaccines against infectious diseases and to immune therapy for inflammatory bowel diseases, food allergies, and celiac diseases.

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Regulatory Role of Lymphoid Chemokine CCL19 and CCL21 in the Control of Allergic Rhinitis[1]

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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 $CD8\alpha^-CD11b^+$ 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 FcεR, 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,

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[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 $CD8\alpha^-CD11b^+$ 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 $I\text{-}A^d$ (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 μg of OVA (Grade V; Sigma-Aldrich) with 1 mg of aluminum hydroxide hydrate gel (Alum) (LSL Co.) in 200 μl of PBS on days 0, 7, and 14. Thereafter, mice were challenged by nasal administration of either 500 μg of OVA in 20 μl of PBS (for AR group) or 20 μl 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 H_2SO_4. 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 $\log_2$ 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 μm 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 (×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-μm 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 μM 2-ME, 10 U/ml of penicillin, and 100 μg/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-CD8α (53-6.7; BD Pharmingen), and FITC-conjugated anti-CD11b (M1/70; BD Pharmingen) to collect $CD11c^+CD8\alpha^-CD11b^+$ cells (m-DCs) and $CD11c^+CD8\alpha^+CD11b^-$ cells (lymphoid DCs: l-DCs) by FACSAria (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 FACSAria. Tregs were sorted from spleen cells as a $CD3^+CD4^+CD25^+$ population using FACSAria by staining with FITC-conjugated anti-CD3ε (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 μg/ml), anti-TGF-β mAb (1D11) (10 μg/ml) or rat IgG (Sigma-Aldrich) (10 μg/ml) was added to the culture. Culture supernatants were collected and

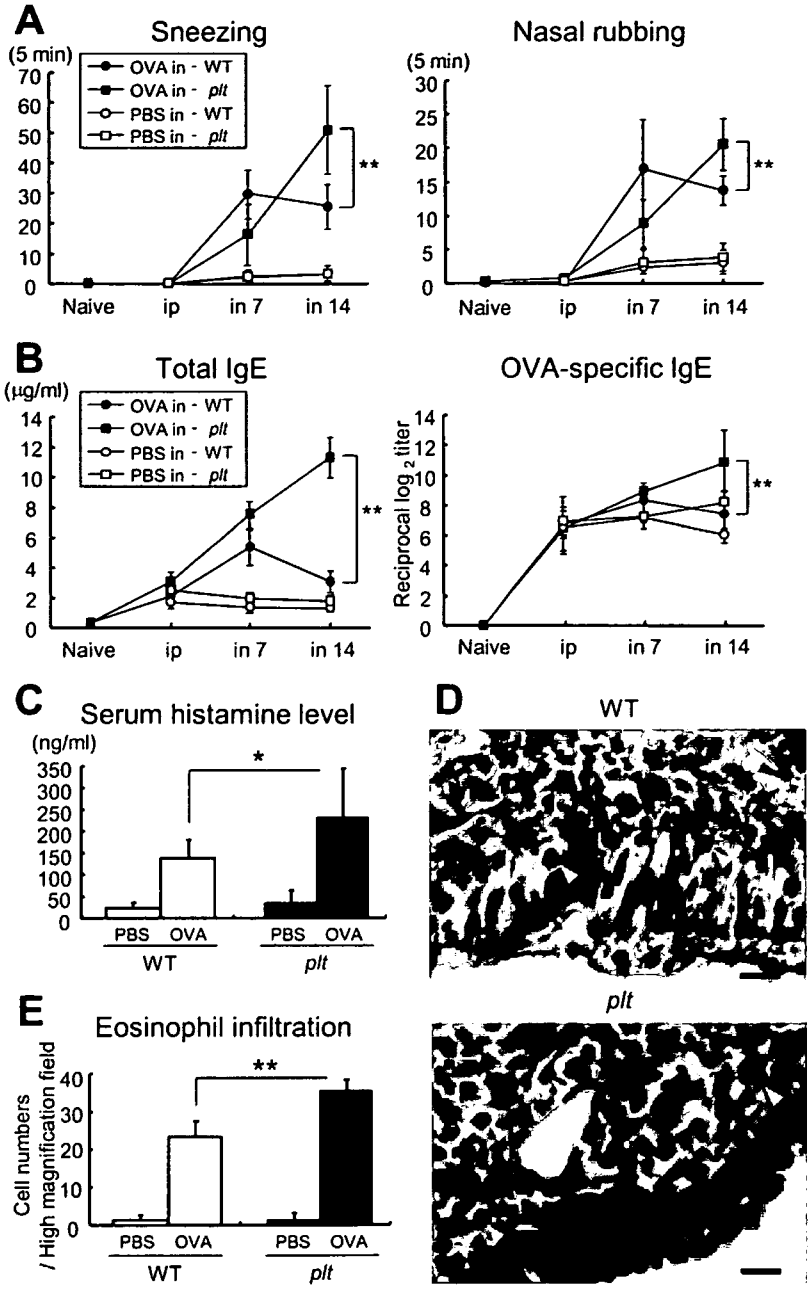

FIGURE 1. Nasal symptoms and Ag-specific allergic responses in mice with allergic rhinitis (AR). WT mice and *plt* mice were nasally challenged with OVA for 14 consecutive days following systemic sensitization. *A*, Sneezing and nasal rubbing were observed and counted for 5 min at 4 different time points: 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 $\log_2$ 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′-TATTGCATGGCCTCTGTAACCGCAAGG-3′); the oligonucleotide primers specific for GATA-3 (sense, 5′-CATGCGTGAGGAGTCTCCAA-3′; antisense, 5′-GGAATGCAGACACCACCTCG-3′): the GATA-3 detection FITC-labeled probe (5′-GGGCTTCATGATACTGCTCCTGCG AAA-3′) and the Lightcycler Red 640-labeled hybrid probe (5′-ACGCA AGTAGAAGGGGTCGGAGGAACTC-3′); and the oligonucleotide primers specific for GAPDH (sense, 5′-TGAACGGGAAGCTCACTGG-3′; antisense, 5′-TCCACCACCCTGTTGCTGTA-3′): the GAPDH detection FITC-labeled probe (5′-CTGAGGACCAGGTTGTCTCCTGCGA-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-CD3ε, 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

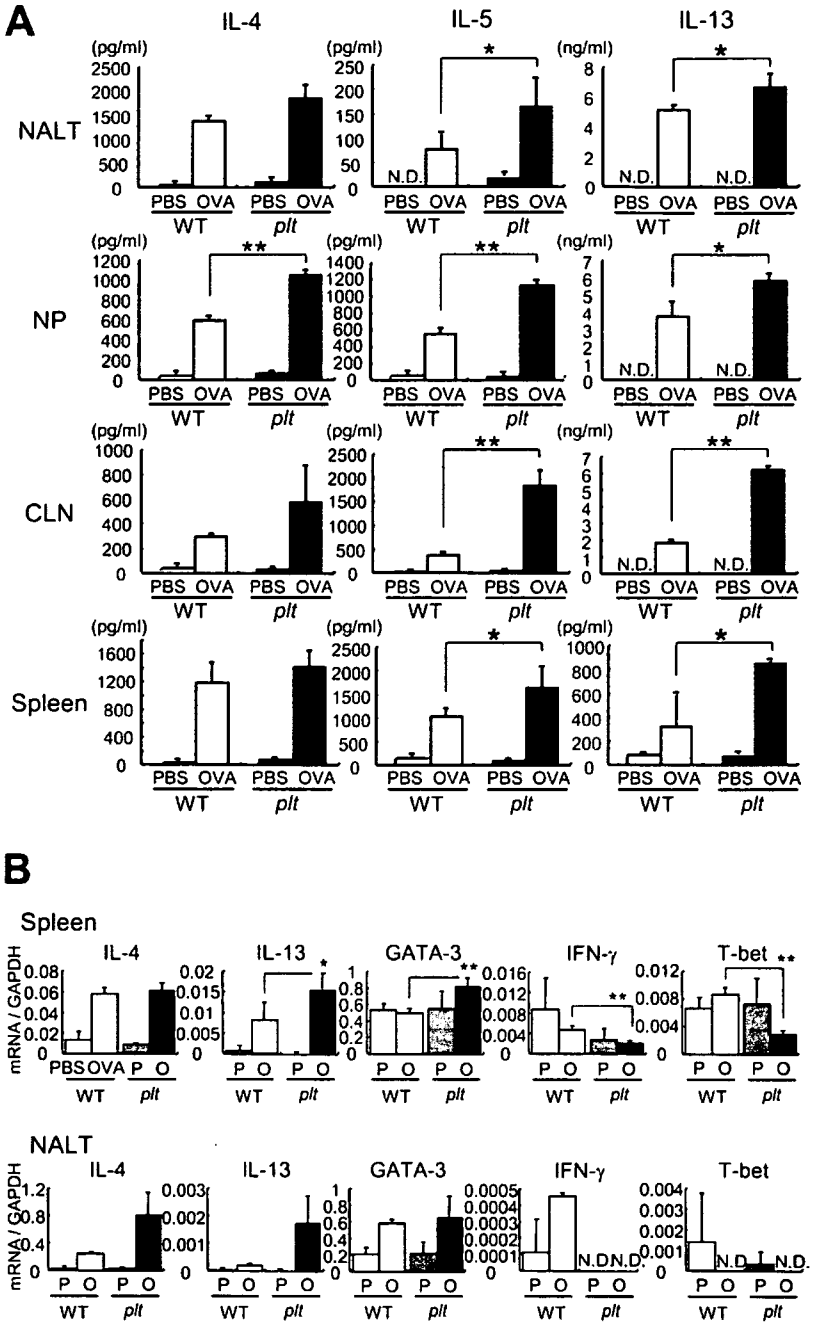

FIGURE 2. Th2 cytokine production, and Th1/Th2 cytokine and associated transcriptional factor mRNA expression from $CD4^+$ T cells isolated from mice with AR. *A*, Culture supernatants of $CD4^+$ T cells of NALT, NP, CLN, and spleen from mice with AR were assessed for Th2 cytokine production levels by 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′-CCTTGTCTCGAGCCACCATGGCCCCCCGTGTGACCCCAC-3′; antisense, 5′-AGCCTCGAATTCTCAAGACACAGGGCTCCTTCTGG-3′) and CCL21-Ser (sense, 5′-CCTTGTCTCGAGCCTCAACTCAACCACA ATCATGGC-3′; antisense, 5′-AGCCTCGAATTCCTATCCTCTTGAG GGCTGTGTC-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 electrophoresis 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 μg of OVA with 1 mg of Alum on days 0, 7, and 14, followed by nasal challenge with 500 μg 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 μg 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 ± SE and evaluated by an unpaired Student's *t* test. Values of $p < 0.05$ were assumed to be statistically significant.

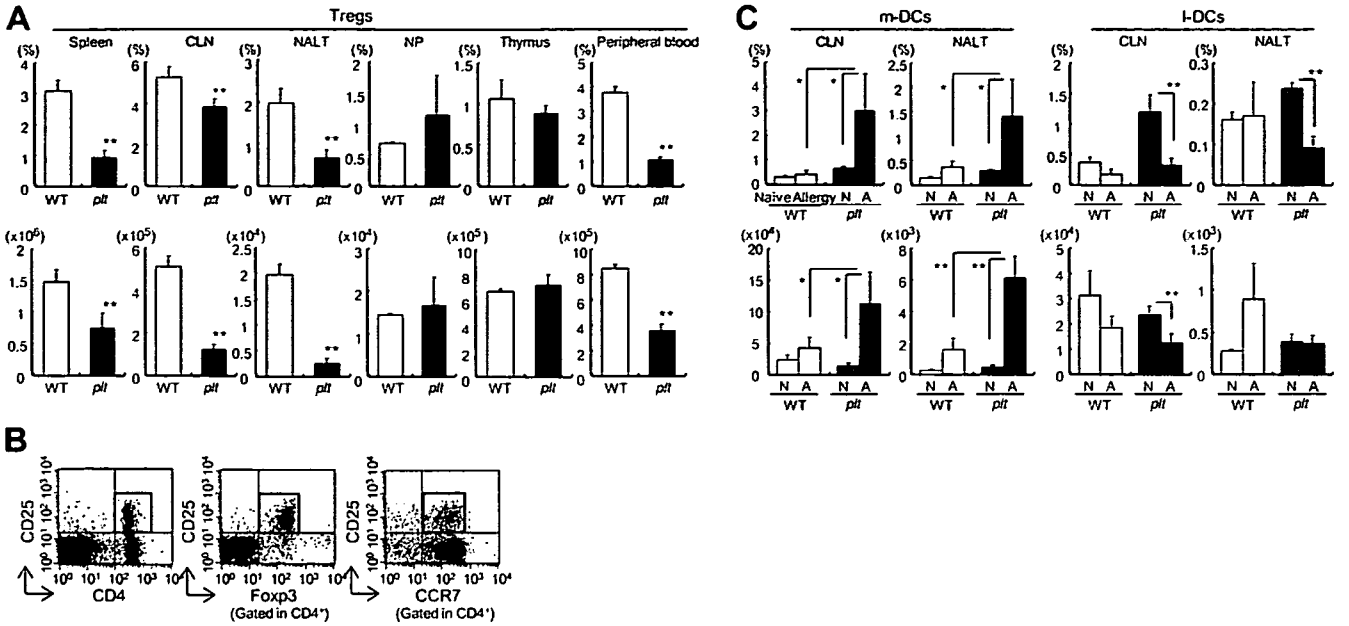

FIGURE 3. Flow cytometric analysis of Tregs and DCs in mice with AR. *A*, The frequency (upper) and absolute number (lower) of Tregs in naive mice were determined and calculated using a flow cytometer. *B*, Representative flow cytometric analysis data of Foxp3 and CCR7 expression in Tregs using mononuclear 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

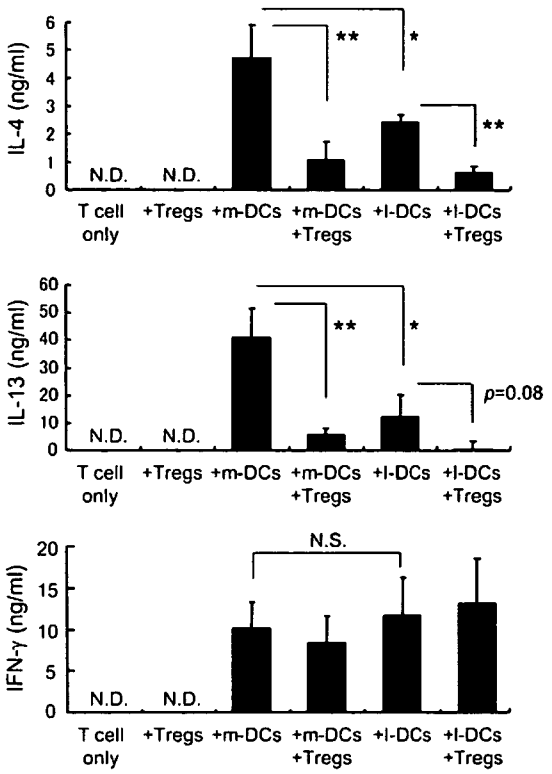

FIGURE 4. Role played by each DC subset in the induction of Ag-specific T cell responses. The production levels of IL-4, IL-13, and IFN-γ were measured by ELISA in culture supernatants of naive T cells from DO11.10 OVA-TCR transgenic mice cocultured with or without Tregs and/or DCs. Data are representative 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. 3*A*). Because these $CD4^+CD25^+$ T cells expressed Foxp3, they were considered to be Tregs (Fig. 3*B*). Interestingly, most of these Tregs observed in both *plt* and WT mice expressed CCR7 (Fig. 3*B*). As Treg levels did not change after the induction of AR (data not shown), these Tregs were considered to be naturally occurring. As $CD8\alpha^-CD11b^+$ m-DCs and $CD8\alpha^+CD11b^-$ 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 ± 0.08% to 3.02 ± 1.51% and 0.30 ± 0.02% to 1.41 ± 0.74%, respectively; Fig. 3*C*) and an elevated total number of m-DCs (Fig. 3*C*). In contrast, the frequency and number of m-DCs in nonsensitized *plt* mice were comparable to those seen in WT mice (Fig. 3*C*). 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

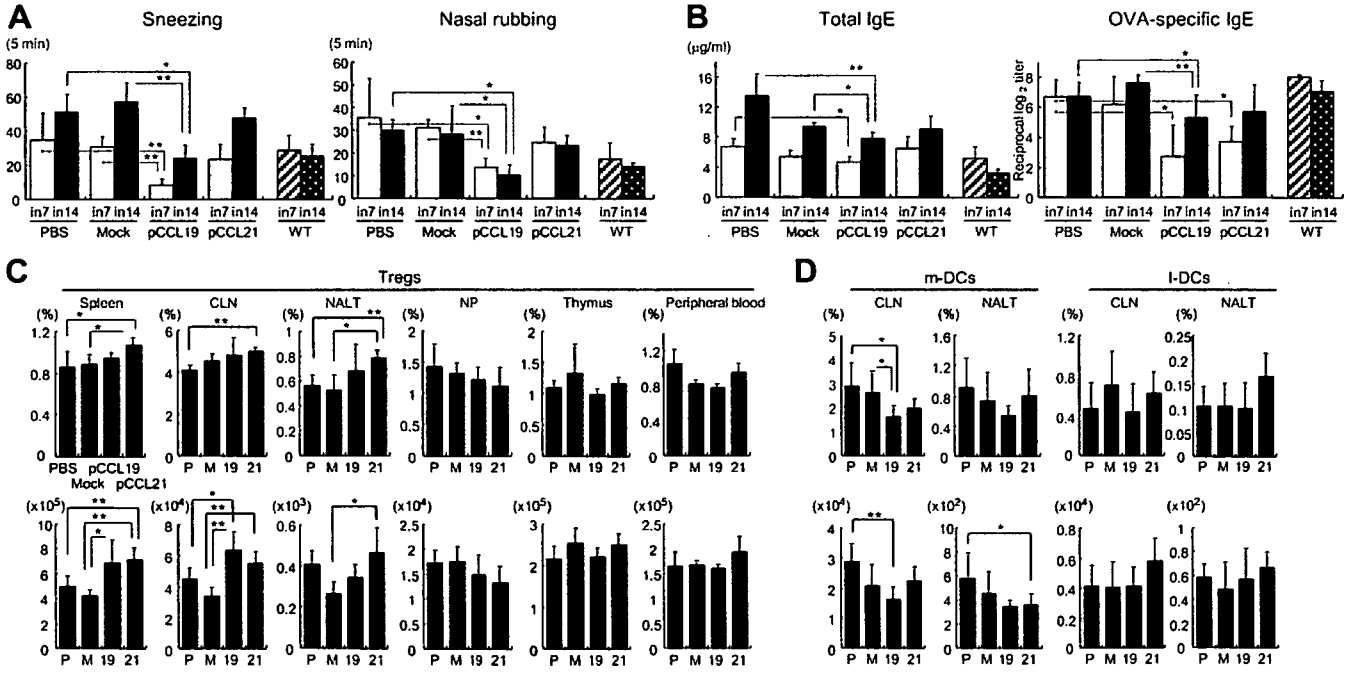

FIGURE 5. Allergic symptoms and the number of Tregs and DCs in *plt* mice treated by nasal administration of plasmids encoding CCL19/CCL21-Ser DNA. Nasal symptoms (*A*) and total and OVA-specific IgE levels in serum (*B*) were assessed as previously described at two different time points: 1) after 7 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.

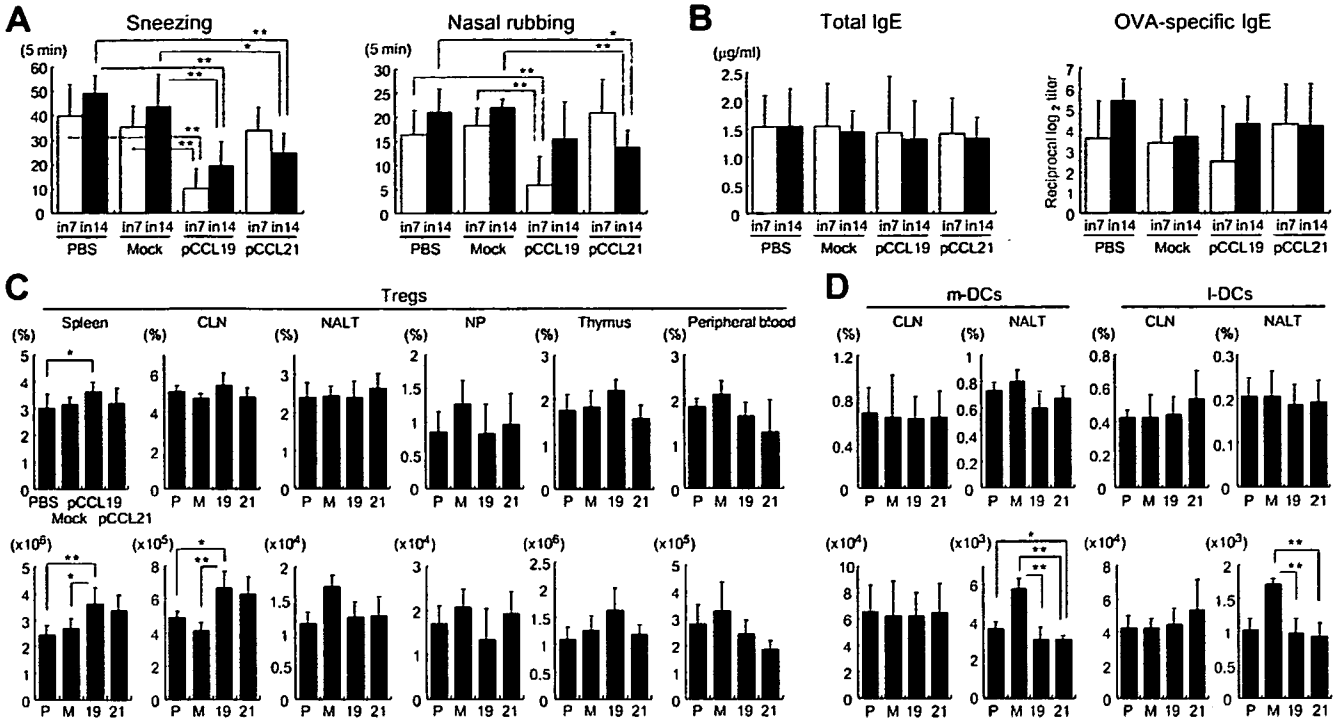

FIGURE 6. Allergic symptoms and the number of Tregs and DCs in WT mice treated by nasal administration of plasmids encoding CCL19/CCL21-Ser DNA. Nasal symptoms (*A*) and total and OVA-specific IgE levels in serum (*B*) were assessed at two different time points: 1) after 7 nasal challenges ("in 7", on 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 l-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. 5*A*). 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. 5*B*). 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. 5*C*). 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. 5*C*). The frequency of m-DCs in CLN of *plt* mice treated with pCCL19 was considerably reduced when compared with control-treated mice (Fig. 5*D*). 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. 5*D*).

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. 6*C*). The total number of m-DCs in NALT was reduced when WT mice were treated with pCCL19/pCCL21 (Fig. 6*D*). 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-1β, 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-1β 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. 3*A*), 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.

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Disclosures

The authors have no financial conflict of interest.

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CUTTING EDGE

Cutting Edge: *Tlr5*$^{-/-}$ Mice Are More Susceptible to *Escherichia coli* Urinary Tract Infection[1]

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Although TLR5 regulates the innate immune response to bacterial flagellin, it is unclear whether its function is essential during in vivo murine infections. To examine this question, we challenged Tlr5$^{-/-}$ ***mice transurethrally with*** Escherichia coli. ***At 2 days postinfection, wild-type mice exhibited increased inflammation of the bladder in comparison to*** Tlr5$^{-/-}$ ***mice. By day 5 postinfection,*** Tlr5$^{-/-}$ ***mice had significantly more bacteria in the bladders and kidneys in comparison to wild-type mice and showed increased inflammation in both organs. In addition, flagellin induced high levels of cytokine and chemokine expression in the bladder that was dependent on TLR5. Together, these data represent the first evidence that TLR5 regulates the innate immune response in the urinary tract and is essential for an effective murine in vivo immune response to an extracellular pathogen. The Journal of Immunology,*** 2007, 178: 4717–4720.

Toll-like receptors are a family of germline-encoded innate immune receptors that recognize pathogen-associated molecular patterns, such as bacterial flagellin (TLR5), LPS (TLR4), and lipopeptides (TLR1/2/6) (1). Expression of TLRs varies among cells and tissues, suggesting that individual TLRs may regulate distinct pathogen and organ-specific roles in host defense to different pathogens (1). We previously discovered that the ligand for TLR5 is bacterial flagellin, the most abundant protein in the whip-like tails of flagellated bacteria (2). We defined the TLR5 recognition site on flagellin and found that it is conserved among a wide variety of flagellated bacteria (3), although select bacterial species possess unique flagellin molecules that evade TLR5 recognition (4). TLR5 is expressed in epithelial cells of the airways, intestine, and urogenital tract, as well as on hemopoietic cells of the innate and adaptive immune system and has recently been shown to be involved in the transport of flagellated *Salmonella typhimurium* from the intestinal tract to the mesenteric lymph nodes (5).

Many important pathogenic bacteria, both Gram-positive and Gram-negative, are flagellated. Flagellated uropathogenic *E. coli* (UPEC)[4] cause 70–90% of all urinary tract infections (UTI), and their pathogenesis involves contact between bacteria and the epithelial cell surface of the urogenital tract, a site of TLR5 expression in humans (6). UPEC colonize the urethra and ascend to the bladder, where they can persist at high levels (7). In addition to cystitis in the bladder, UPEC may ascend to the kidney and cause serious complications, including pyelonephritis and bacteremia (8). *E. coli* is recognized by several TLRs, including TLRs 2, 4, 5, and 11, and likely also TLR9. Previous studies indicate that TLR4 and TLR11 regulate susceptibility to UTIs (9–11). However, it is not currently known whether TLR5 is critical for host defense to UTIs or whether there is sufficient TLR redundancy to obviate its requirement.

Materials and Methods

Mice, bacteria, and TLR agonists

Tlr5$^{-/-}$ mice (strain designation B6.129P2-*Tlr5*^{tm1Aki}) were derived and backcrossed to a C57BL/6 background for eight generations as previously described (5). Wild-type (WT) control mice were from a C57BL/6 background (The Jackson Laboratory). *E. coli* strain CFT073, from a patient with acute pyelonephritis (American Type Culture Collection), was grown in Luria-Bertani (LB) medium in static culture at 37°C for 48 h. Expression of type 1 pili was confirmed for each experiment by testing for yeast agglutination (12). Flagellin was purified from *S. typhimurium* as described in Ref. 3 and was heated to 70°C for 15 min to monomerize it. Contaminating endotoxin was removed by passage through a 100-kDa molecular mass cutoff filter (Millipore) followed by endotoxin removal on a polymixin B column (Pierce). The resulting flagellin did not show detectable endotoxin by *Limulus* assay (Cambrex). Ultrapure LPS was purchased from List Biologicals.

Real-time PCR

RNA was extracted from organs with TRIzol (Invitrogen Life Technologies), DNase treated with TURBO DNA-free (Ambion), and cDNA produced with Superscript II (Invitrogen Life Technologies). Real-time PCR was performed with TaqMan Fast (Applied Biosystems) on an Applied Biosystems Prism 7900 HT. Primer/probe sets for elongation factor 1α (EF1α) were designed with

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[4] Abbreviations used in this paper: UPEC, uropathogenic *E. coli;* UTI, urinary tract infection; EF1α, elongation factor 1α; CHO, Chinese hamster ovary; LB, Luria-Bertani; IQR, interquartile range; WT, wild type.

Primer Express 1.0 (PerkinElmer) with a 5′-FAM and 3′-TAMRA modification (Biosearch Technologies). Primer/probe sets for mouse TLR5 and cytokines/chemokines were purchased from Applied Biosystems. Threshold cycle (Ct) values were transformed by $1/2^{Ct}$, and then normalized to EF1α for each organ.

NF-κB luciferase reporter assay

Chinese hamster ovary (CHO) K1 cells stably expressing mouse TLR5 and NF-κB luciferase reporter constructs (3) were stimulated with heat-killed bacteria for 4 h and assayed for luciferase activity. Assays were done in duplicate, and the experiment was repeated three times. Percent fold induction was calculated by dividing the luciferase values for each bacterial dose by the maximal luciferase value for the bacteria in each experiment.

UTI model of infection

The Institute for Systems Biology and Osaka University Institutional Animal Care and Use Committees approved all animal protocols. Forty-eight-hour static cultures of *E. coli* CFT073 were resuspended in cold PBS at 1×10^9 CFU/ml. Anesthetized mice were inoculated transurethrally with 5×10^7 *E. coli* in 50 μl, and urethras were coated with collodion (Sigma-Aldrich) (13). Six hours postinfection, collodion was removed by blotting with acetone. At each time point, organs were homogenized in 1 ml of 0.025% Triton X-100/PBS and plated on LB-agar to enumerate CFUs.

Statistical analysis

Comparisons were made with a two-tailed Mann-Whitney *U* test or a Student's *t* test. A $p \leq 0.05$ was considered to be significant. Statistics were calculated with PRISM4 (GraphPad).

Histology

Bladders and kidneys were fixed in 10% formalin-buffered saline and embedded in paraffin. Four-micrometer sections were cut, stained with H&E, and examined by a pathologist blinded to mouse genotype.

Results and Discussion

$Tlr5^{-/-}$ *mice are more susceptible to* E. coli *urinary tract infection*

To test our hypothesis that TLR5 is critical for host defense against *E. coli* UTIs, we first examined bladder and kidney tissue for TLR5 expression. We extracted RNA from tissues of C57BL/6 mice and evaluated expression levels by real-time PCR (Fig. 1*A*). TLR5 was expressed in both bladder and kidney, which suggested that it may regulate critical aspects of the immune response during UTI. We next examined whether $Tlr5^{-/-}$ mice were more susceptible to urinary tract infections. When grown in static culture, uropathogenic *E. coli* forms type 1 pili (12) that enhance adherence to bladder epithelia and increase bladder colonization. We first determined whether growth in static culture resulted in flagellin expression. CHO cells stably expressing mouse TLR5 and a NF-κB-dependent luciferase reporter construct responded to statically grown heat-killed bacteria in a dose-dependent manner, detecting fewer than 8000 bacteria, a multiplicity of infection of ~0.1 (Fig. 1*B*). Control CHO cells expressing the pEF6 vector alone did not respond to bacteria (data not shown).

To test the role of TLR5 during infection in vivo, we inoculated WT and $Tlr5^{-/-}$ mice transurethrally with 5×10^7 CFU of statically cultured *E. coli*. Bladder and kidneys were harvested 4 h, 24 h, 2 days, and 5 days after infection and the number of CFU in each organ was determined. At early time points, no difference was seen in bladder CFU between WT and $Tlr5^{-/-}$ mice (Fig. 1*C*). In contrast, although all mice remained infected at day 5, the number of CFU per bladder was reduced in WT mice but rose dramatically in $Tlr5^{-/-}$ mice. WT mice had a median of 475 CFU/bladder (interquartile range (IQR): 212.5–685) in comparison to $Tlr5^{-/-}$ mice with a median of 7.7×10^5 CFU/bladder (IQR: 9.0×10^4–4.1×10^6, $p < 0.0001$ by Mann-Whitney *U* test) (Fig. 1*C*).

We next examined whether the *E. coli* disseminated to the kidney. Bacteria were present in the kidneys of both WT and TLR5-deficient animals, and no significant CFU differences were observed between WT and $Tlr5^{-/-}$ mice in the kidney at early time points (Fig. 1*D*). By day 5 after infection, however, no bacteria were detected in WT kidneys, but $Tlr5^{-/-}$ mice had a median of 6.5×10^2 CFU/kidney (IQR: 0- 6.5×10^4, $p = 0.0274$ by Mann-Whitney *U* test) with six of nine infected mice showing kidney counts (Fig. 1*D*). Together, these data suggest that $Tlr5^{-/-}$ mice are unable to control bacterial replication and cannot clear the infection from the kidneys by day 5.

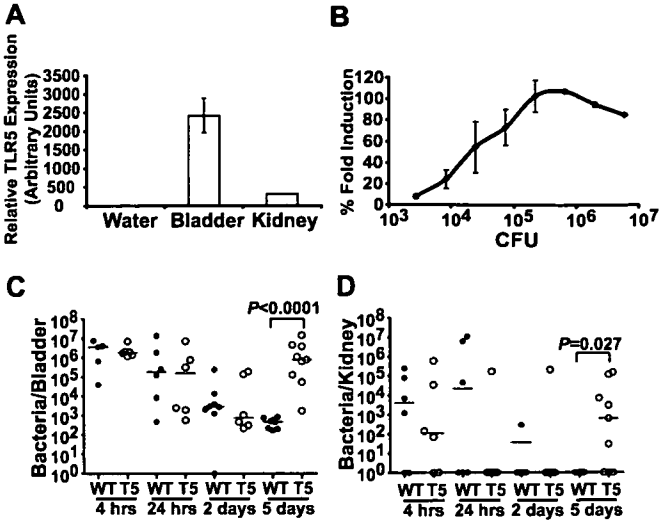

FIGURE 1. $Tlr5^{-/-}$ mice are more susceptible to *E. coli* UTI. *A*, Total RNA was isolated from bladders and kidneys of two WT and two $Tlr5^{-/-}$ mice. cDNA was prepared and real-time PCR analysis was performed. TLR5 mRNA levels are expressed as a ratio to EF1α mRNA expression. Data are representative of two experiments, each performed in triplicate. Error bars, 1 SD. *B*, NF-κB luciferase activity (percent fold induction) for CHO cells stably expressing mouse TLR5 and NF-κB luciferase reporter constructs. Cells were stimulated at a range of bacterial doses and data are from one representative experiment of three independent experiments run in duplicate. Error bars, 1 SD. *C* and *D*, *E. coli* bacterial counts in the bladder (*C*) and kidney (*D*) of WT and $Tlr5^{-/-}$ (T5) mice. Mice were inoculated transurethrally with 5×10^7 *E. coli* CFT073 and 5 days later bladder and kidneys were removed. Organ homogenates were plated on LB agar to enumerate the CFUs per organ. Colony counts were averaged from two plates per mouse. The Mann-Whitney *U* test was used to determine the *p* values for CFU differences. Median values are depicted with a line.

$Tlr5^{-/-}$ *mice exhibit decreased inflammation at 2 days postinfection*

We next examined histologic sections from WT and knockout mice by light microscopy to determine the pathologic consequences of TLR5 deficiency. Bladders and kidneys from WT and $Tlr5^{-/-}$ mice exhibited similar levels of inflammation at 4 and 24 h after infection (data not shown). In contrast, at 2 days postinfection, $Tlr5^{-/-}$ mice showed decreased inflammation in the bladder relative to WT mice (Fig. 2*A*). WT mice exhibited prominent submucosal edema and infiltration of the submucosa and epithelium by leukocytes. There was no significant inflammation in the kidneys from WT or $Tlr5^{-/-}$ mice at the 2-day time point (data not shown).

By day 5 postinfection when TLR5-deficient mice showed increased bacterial counts, the situation was reversed, with prominent inflammation in the TLR5-deficient animals. WT mice showed minimal to no inflammation in the bladder and kidney, and bacteria were not visible in the lumen of these organs (Fig. 2, *B* and *C*). In contrast, bladders from $Tlr5^{-/-}$ mice showed prominent submucosal edema with leukocyte infiltration into the submucosa and invasion of the epithelial layer, as well as focal microabscess formation and accumulation of leukocyte-rich exudates on the bladder surface (Fig. 2*B*). Bacteria were readily visible and present predominantly on the surface of the $Tlr5^{-/-}$ urothelium without prominent evidence of enclosure within the umbrella cells of the bladder, such as the intracellular

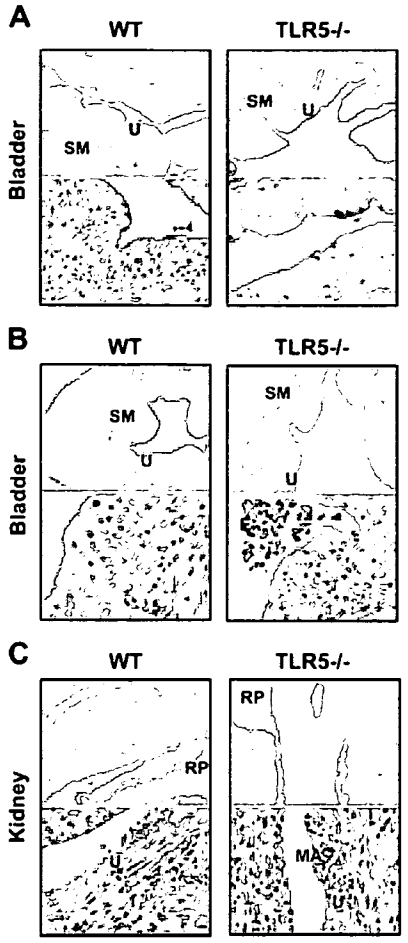

FIGURE 2. *Tlr5*$^{-/-}$ mice exhibit decreased inflammation at 2 days postinfection. *A–C*, H&E-stained histologic sections of mouse bladder (*A* and *B*) and renal pelvis (*C*) at 2 days (*A*) and 5 days (*B* and *C*) after infection. SM, Submucosa; U, urothelium; E, leukocyte-rich exudate; RP, renal pelvis; MA, microabscess. The panels were photographed at ×100 (*upper*) and ×400 (*lower*) magnification.

bacterial communities described by others (14). A similar but less pronounced pattern of inflammation was also present in the *Tlr5*$^{-/-}$ kidneys at day 5, and primarily involved the urothelium in the renal pelvis with focal extension into the interstitium of the renal medulla (Fig. 2*C* and data not shown). Together, these data indicate a turning point at 2 days postinfection: *Tlr5*$^{-/-}$ mice manifest decreased inflammation that leads to overwhelming bacterial growth and more severe inflammation by day 5 after infection.

Flagellin induces early expression of proinflammatory genes in the bladder

To identify proinflammatory molecules up-regulated by TLR5 in the bladder that might lead to increased early inflammation in WT mice, we examined the in vivo response to transurethral administration of flagellin and compared this with LPS, another prominent TLR agonist present in *E. coli*. Mice were inoculated transurethrally with 30 μg of flagellin or 10 μg of ultrapure LPS in PBS, and bladders were harvested at 4 h. Real-time PCR was performed on bladder tissue for several proinflammatory cytokines and chemokines. Transurethral inoculation of flagellin up-regulated expression of KC (CXCL1), MIP2 (CXCL2), MCP-1 (CCL2), IL-6, and TNF-α mRNA, but not β-defensin 1 mRNA in WT mice (Fig. 3). As expected, *Tlr5*$^{-/-}$ mice did not respond to flagellin in the bladder. In contrast to flagellin, LPS delivered into the bladders of WT mice did not induce transcription of these proinflammatory genes. These results demonstrate that flagellin induces a robust TLR5-dependent innate immune response in the murine bladder that may account for its critical role in UTI pathogenesis.

Taken together, these data demonstrate that TLR5 plays a crucial role in host defense to UPEC infection by mediating flagellin-induced inflammatory responses in the bladder that limit bacterial replication in both the bladder and kidney. Two additional TLRs, TLR4 and TLR11, have also been shown to play a role in *E. coli*-induced UTI. TLR4-deficient C3H/HeJ mice exhibit a reduced inflammatory response to UPEC and exhibit significantly higher bacterial counts in the bladder and kidneys (9, 15, 16). In contrast to the inflammatory response to flagellin, we found that the bladder was relatively unresponsive to LPS. There is conflicting evidence about whether the urinary epithelium responds to LPS (12, 17–19). Our experiments comparing in vivo delivery of highly purified flagellin and LPS suggest that TLR5 and TLR4 regulate distinct bladder innate immune responses with TLR5 regulating a relatively dominant role initially. TLR11, which is a pseudogene in humans, is expressed in both kidney and bladder epithelial cells of mice (11). Infection of TLR11-deficient mice with the UPEC strain 8NU resulted in approximately equal colonization of the bladders of TLR11-deficient and WT mice, but significantly more bacteria ascended to the kidneys of TLR11-deficient mice (11). Thus, it is possible that TLR5 and TLR11 play complementary roles in the

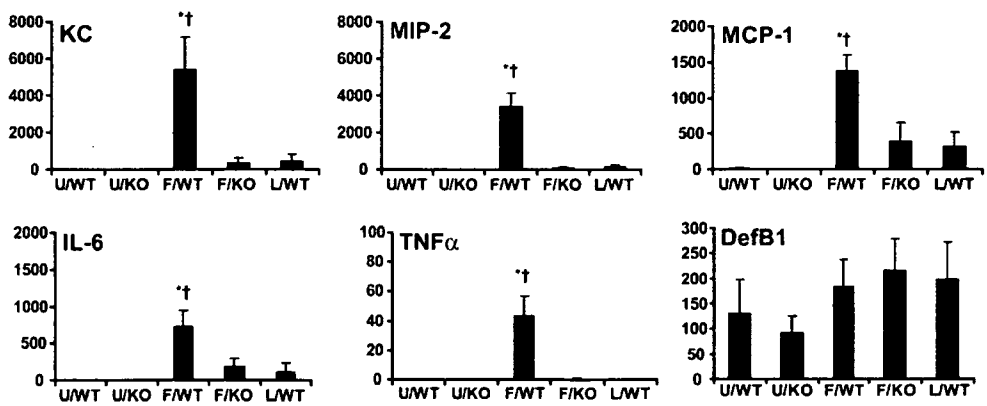

FIGURE 3. Bladder gene expression after transurethral stimulation with TLR agonists. Total RNA was isolated from bladders of unstimulated mice (U) or mice stimulated transurethrally with 30 μg of *S. typhimurium* flagellin (F) or 10 μg of LPS (L). cDNA was prepared and real-time PCR analysis was performed. Data are a combination of two experiments with $n = 2$ mice for the unstimulated WT C57BL/6 and *Tlr5*$^{-/-}$ (KO) mice, $n = 7$ for WT mice stimulated with flagellin, $n = 4$ for *Tlr5*$^{-/-}$ mice stimulated with flagellin, and $n = 7$ for WT mice stimulated with LPS. mRNA levels of cytokines and chemokines are expressed as a ratio to EF1α mRNA expression. *, $p < 0.05$ by Student's *t* test for comparison of flagellin stimulation in WT vs knockout. †, $p < 0.05$ for comparison for flagellin vs LPS stimulation in WT mice.

mouse urinary tract, with TLR5 limiting bladder replication and TLR11 primarily controlling bacterial invasion of the kidney.

Bacterial motility is important for virulence in some models of UTI, which suggests that TLR5-flagellin interactions may be important for bacterial uropathogenesis. Flagellum-negative mutants of *Proteus mirabilis* are significantly less successful at bladder colonization and do not progress to the kidneys as readily (20). Furthermore, two recent studies suggest that flagellar mutants of *E. coli* are less able to colonize the mouse urinary tract (8, 14). Flagellar-based motility may be beneficial in early colonization of the urinary tract, but may not be required for maintenance of infection (8). This is in agreement with a study that demonstrated down-regulation of flagellin genes by *E. coli* CFT073 several days after in vivo infection (21). These studies combined with our results suggest that TLR5 recognition of flagellin is an important component of the innate immune response to *E. coli* during the early stages of UTI when flagellin expression and motility contribute to colonization of the urinary tract.

In addition to TLR5 recognition of extracellular bacterial flagellin, two novel intracellular flagellin receptors have recently been described that are both members of the nucleotide-binding oligomerization domain leucine-rich repeat family (22–24). Naip5 detects flagellin from *Legionella pneumophila* that reaches the macrophage cytosol via the bacteria's type IV secretion system (24, 25). Ipaf detects cytoplasmic flagellin injected into macrophages by the type III secretion system of *S. typhimurium* (22). The roles of these additional flagellin receptors in UTI are not known, but the UPEC strain used in this study, like many other UPEC (26), does not encode a type III secretion system (27). Thus, intracellular flagellin receptors may not be able to compensate for the lack of flagellin recognition by TLR5 in this infection model.

Although TLR5 has been implicated in the innate immune response to mucosal infection (5, 28), there has been limited in vivo data to substantiate this claim. A recent study of $Tlr5^{-/-}$ mice did not find a unique role for TLR5 in defense to *Salmonella* or *Pseudomonas* (29). Our study provides the first evidence that TLR5 regulates a critical and nonredundant role in the innate immune response to a murine infection with extracellular flagellated bacteria. We have previously identified a TLR5 polymorphism present in the general population that results in a stop codon that abrogates TLR5 signaling and is associated with increased susceptibility to Legionnaire's disease (30). These murine studies support a hypothesis that individuals who possess this TLR5 variant will also be more susceptible to UTI.

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Disclosures

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