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# Coordinated Involvement of Mast Cells and T Cells in Allergic Mucosal Inflammation: Critical Role of the CC Chemokine Ligand 1:CCR8 Axis

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CCL1 is the predominant chemokine secreted from IgE-activated human and mouse mast cells in vitro, colocalizes to mast cells in lung biopsies, and is elevated in asthmatic airways. CCR8, the receptor for CCL1, is expressed by ~70% of CD4<sup>+</sup> T lymphocytes recruited to the asthmatic airways, and the number of CCR8-expressing cells is increased 3-fold in the airways of asthmatic subjects compared with normal volunteers. In vivo, CCL1 expression in the lung is reduced in mast cell-deficient mice after aeroallergen provocation. Neutralization of CCL1 or CCR8 deficiency results in reduced mucosal lung inflammation, airway hyperresponsiveness, and mucus hypersecretion to a similar degree as detected in mast cell-deficient mice. Adenoviral delivery of CCL1 to the lungs of mast cell-deficient mice restores airway hyperresponsiveness, lung inflammation, and mucus hypersecretion to the degree observed in wild-type mice. The consequences of CCR8 deficiency, including a marked reduction in Th2 cytokine levels, are comparable with those observed by depletion of CD4<sup>+</sup> T lymphocytes. Thus, mast cell-derived CCL1- and CCR8-expressing CD4<sup>+</sup> effector T lymphocytes play an essential role in orchestrating lung mucosal inflammatory responses. *The Journal of Immunology*, 2007, 179: 1740–1750.

Allergen-induced mast cell activation mediated through cross-linking of FcεRI contributes to the asthmatic response by providing a source of bioactive amines and lipid mediators that induce smooth muscle contraction (1, 2). Ag-

IgE triggering of mast cells induces the secretion of a number of preformed enzymes such as tryptase (3), as well as de novo-generated cytokines, which include TNF-α (4). Although increased mast cell numbers and elevated levels of mast cell-specific products have been reported in the lungs of asthmatic individuals (3, 5), the importance of this cell type in orchestrating lung mucosal inflammation remains highly controversial. However, the recent appreciation that mast cells produce a much broader array of proinflammatory cytokines and chemokines than previously believed has prompted a re-evaluation of the role of the mast cell in allergic diseases as well as a number of autoimmune disorders (6–8). Interestingly, in the context of bronchial asthma, histopathological analysis of the airway mucosa in asthmatic subjects has revealed that mast cell numbers are increased in the bronchial smooth muscle layer, the number of which correlate with the degree of airway hyperresponsiveness (AHR)<sup>13</sup> (9), suggesting that mast cells may play a broader role than merely mediating smooth muscle bronchospasm. However, although mast cell-mediated responses have been central in our understanding of asthma pathology for the last 30 years, more recently attention has been focused on understanding the role and function of subsets of CD4<sup>+</sup> T lymphocytes. Indeed, an accumulated body of evidence has suggested that CD4<sup>+</sup> T lymphocytes in the lung mucosa of asthmatic individuals produce Th2 cytokines that include IL-4, IL-5, IL-9, and IL-13 that orchestrate the lung inflammatory response (10). Thus, the relative importance of mast cells and/or CD4<sup>+</sup> T lymphocytes in

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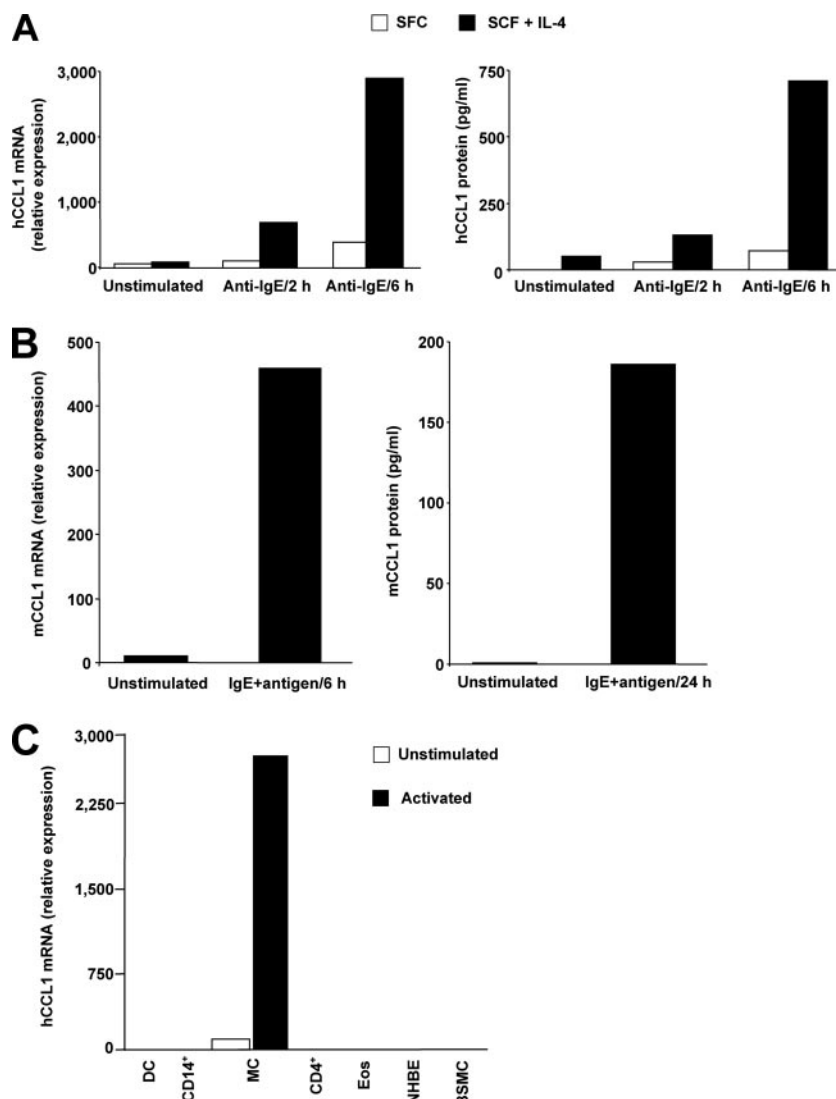
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<sup>13</sup> Abbreviations used in this paper: AHR, airway hyperresponsiveness; Penh, enhanced pause; BAL, bronchoalveolar lavage; wt, wild type; AB/PAS, Alcian blue/periodic acid Schiff; DIG, digoxigenin; SCF, stem cell factor; i.n., intranasal(ly).

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**FIGURE 1.** CCL1 expression by cultured mast cells. *A*, Human mast cells were cultured in the presence of SCF alone (□) or SCF plus IL-4 (■). CCL1 mRNA expression (*left panel*) and protein levels (*right panel*) in cell culture supernatants were determined before and after FcεR1 cross-linkage at the indicated time points. *B*, Murine mast cells were cultured in the presence of SCF. CCL1 mRNA expression (*left panel*) and protein levels (*right panel*) in cell culture supernatants were determined before and after Ag-induced FcεR1 activation at the indicated time points. *C*, CCL1 mRNA expression by unstimulated human mast cells (□) and FcεR1 activated human mast cells (SCF plus IL-4) (■) in comparison with dendritic cells (DC), monocytes (CD14<sup>+</sup>), CD4<sup>+</sup> T lymphocytes (CD4<sup>+</sup>), eosinophils (Eos), normal human bronchial epithelial cells (NHBE), and bronchial smooth muscle cells (BSMC) that have been activated with anti-CD40, LPS, anti-CD3/anti-CD28, IL-5, IL-4/IL-13, and IFN-γ, respectively.

regulating inflammation and AHR remains contradictory. Indeed, studies performed in experimental animals using either mice genetically deficient in mast cells or CD4<sup>+</sup> T lymphocytes suggested that T cell-driven and not IgE-dependent mast cell activation plays a critical and unique role in lung inflammatory responses (11, 12). However, more recent studies performed in mast cell-deficient animals have suggested an important role of these cells in not only lung mucosal inflammation (8), but also a number of autoimmune organ-specific responses including the CNS and joint (6, 7).

Chemokines are chemoattractant cytokines instrumental in mediating the migration of leukocytes across the vascular endothelium by activating seven transmembrane G-protein-coupled receptors (13). Chemokine receptors are expressed on distinct populations of cells and control not only basal cell trafficking required for immune homeostasis, but also regulate cell recruitment during inflammatory responses (13). In recent years, subsets of T lymphocytes, including Th1 and Th2 effector cells have been described to use different chemokine receptors to govern cell migration and to regulate distinct adaptive immune responses (14). In this regard, Th1 effector cells express the chemokine receptors CCR5, CXCR3, and CXCR6 (15, 16) and preferentially migrate to CCL3 (MIP-1α), CXCL10 (IFN-γ-inducible protein 10), and CXCL16, respectively, and have

been implicated in delayed-type hypersensitivity. In contrast, Th2 effector cells express CCR3 (17), CCR4 (18), CCR8 (18–20), as well as chemoattractant receptor-homologous molecule expressed on Th2 cells (21) and migrate in response to CCL3 (eotaxin), CCL22/CCL17 (macrophage-derived chemokine/thymus- and activation-regulated chemokine), CCL1, as well as the arachidonate metabolite PGD<sub>2</sub>, respectively, and have been suggested to control cell migration during allergic responses. However, the relative importance of these receptors in regulating the migration of specific subsets of Th2 effector cells into the lungs during an allergic response *in vivo* remains unknown.

In the present study, we provide evidence to suggest that both mast cells and CD4<sup>+</sup> T lymphocytes are required for lung mucosal inflammation. We propose that the mechanisms underlying this mast cell-CD4<sup>+</sup> T lymphocyte axis is determined by mast cell-derived CCL1 and a subset of CD4<sup>+</sup> T lymphocytes expressing CCR8. CCR8-positive lymphocytes are preferentially recruited from the periphery into the lungs of asthmatic individuals, driven by elevated CCL1 levels produced almost exclusively from mast cells and basophils. Deletion of CCR8 or inhibition of CCL1 reduces mast cell-dependent eosinophilic inflammation, Th2 cytokine production, and mucus gene dysregulation, and suggests a novel mechanism in allergic inflammation.

## Materials and Methods

### RNA extraction and expression analysis

Total RNA from cells in culture was extracted by single-step method using the Qiagen RNA extraction kit RNeasy system (Qiagen). Total RNA from mouse lungs was extracted by single-step method using RNA STAT-60 (Tel-Test). Expression profiles were determined by real-time PCR analysis (TaqMan) as described (22). Probe/primer sequences were as follows: hCCL1 (forward, CCATTGTGGGCTCTGGAAA; reverse, GGAATGGTGTAGGGCTGGTAGT; probe, CACATGGCTTCACCTGTCCCCGA), mCCL1 (forward, TTCCCCTGAAGTTTATCCA GTGTT; reverse, TGAACCCACGTTTTGTAGTTGAG; probe, TCC TGTCTCTTGGATGCGTGCCTTCT), Gob-5 (forward, CACTAAGGT GGCCTACCTCCAA; reverse, GTGAGCTCGCTTGAATGCTGTAT; probe, AAAGCCAACCTTAGCCGTGCCTGG), mL-4 (forward, GGC ATTTTGAACGAGTCA; reverse, AGGACGTTTGGCACATCC A; probe, CTCCTGTCATGGCGTCCCTTCT), mL-13 (forward, GG AGCTGAGCAACATCACACAA; reverse, GCGGCCAGGTCCACAC T; probe, ACCAGACTCCCCTGTGCAACGGC), and mL-5 (PE probe mix: sequence unavailable).

### Cell purification and activation

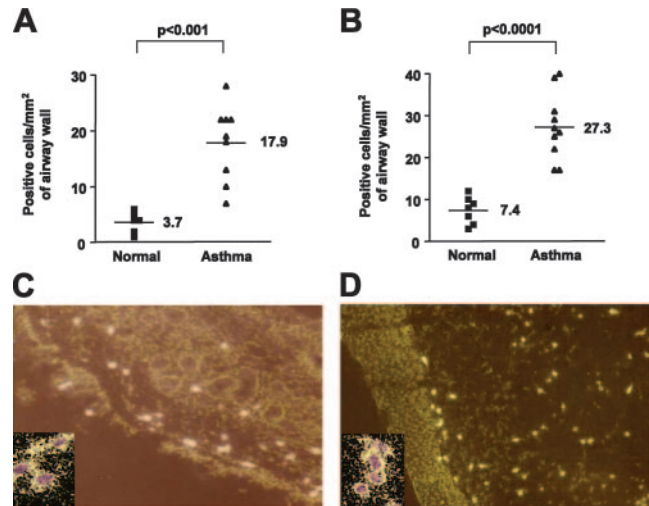
PBMCs were isolated from buffy coats or whole blood by density centrifugation with Ficoll. Leukocyte subsets were further purified by positive selection using the Miltenyi Biotec's MACs system (microbeads) following the manufacturer's instructions. Normal human bronchial epithelial cells and bronchial smooth muscle cells were purchased from Clonetics. Human mast cells were prepared from cord blood, sensitized, and activated as described (23). Mouse mast cells were prepared from bone marrow and activated as described (24).

### Mice and in vivo procedures

Eight-week-old C57BL/6J mice and genetically mast cell-deficient WBB6F<sub>1</sub>-Kit<sup>W/Kit<sup>W/v</sup></sup> (W/W<sup>v</sup>) (25, 26) mice and congenic normal WBB6F<sub>1</sub>-/-/+ (wild-type (wt)) littermates were purchased from The Jackson Laboratory and kept in a pathogen-free mouse facility. CCR8-deficient mice were generated as described (27). The mouse model of lung inflammation used here consists of a sensitization phase (OVA, 10 µg/200 µl PBS/mouse i.p. on each of seven alternate days starting on day 1) (Sigma-Aldrich) and an induction of the response phase (OVA, 20 µg/20 µl/mouse intranasally (i.n.) on days 40, 43, and 46). PBS (i.p. and/or i.n.) was administered to mice as a negative control. For the blocking experiments, mice also received 75 µg/mouse of neutralizing mAb 4B6 against CCL1 (anti-CCL1 Ab) (i.p. 30 min before OVA provocation on days 40, 43, and 46). For the CD4<sup>+</sup> T cell depletion experiments, mice also received 150 µg/mouse of GK1.5 Abs (anti-CD4) (28) (i.p. on days 36 and 38). OVA-treated control mice were injected with the same amount of control Ab (rat IgG) at the same time points indicated during treatment. Eight mice were included per group and per experiment. Mice were assessed for AHR 24 h following the last OVA challenge and for lung inflammation and mucus production 48 h following the last OVA challenge. The degree of AHR expressed as enhanced pause (Penh) was measured 24 h after the last OVA provocation by recording respiratory pressure curves by whole-body plethysmography (Buxco Technologies) in response to inhaled methacholine (Sigma-Aldrich) as described (22). Bronchoalveolar lavage (BAL) was performed as described (12). BAL supernatant was used for ELISA. For the administration of CCL1 in the lung, adenovirus carrying the CCL1 gene or control virus were i.n. delivered to isoflurane-anesthetized W/W<sup>v</sup> mice and wt mice 24 h before the first OVA challenge (6 × 10<sup>8</sup> pfu in 25 µl).

### Lung cell isolation and flow cytometry

Forty-eight hours after the last OVA challenge, lungs were perfused with 10 ml of PBS, removed, minced, and incubated in 4 ml of complete RPMI 1640 containing 600 U of type 1A collagenase (Sigma-Aldrich; 37°C, 1 h). The recovered lung tissue was dissociated and filtered through a cell strainer (70-µm pore size) to prepare a single-cell suspension. Cells were washed twice, resuspended, and used for staining. A total of 5 × 10<sup>6</sup> cells in 100 µl of staining buffer (PBS containing 0.1% FCS) was stained with 4 µl of Alexa 647-CCL1 (4 nM) as described in detail elsewhere (20) and incubated at 37°C for 1 h in a humidified incubator. Cells were then placed on ice and stained with FITC anti-mouse NK-1.1 (clone PK136), PE anti-mouse CD44 (clone IM7), PerCP-Cy5.5 anti-mouse CD4 (clone RM4-5), and anti-mouse CD16/CD32 mAb (clone 2.4G2) for 30 min, washed twice, and fixed with BD Biosciences FACS lysing solution. Flow cytometry was performed on a FACSCalibur (BD Biosciences), and data were analyzed using the FlowJo software (Tree Star). All Abs were purchased from BD

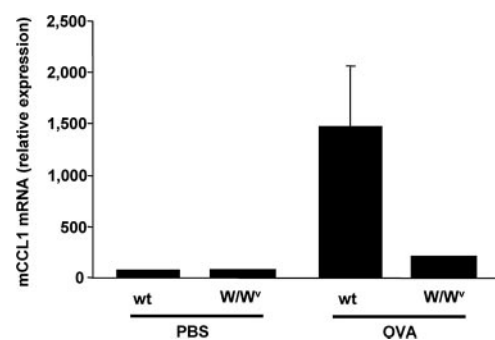


**FIGURE 2.** CCR8 and CCL1 mRNA in human asthma. In situ hybridization was performed on lung biopsies of mild-to-moderate asthmatics and healthy age-matched volunteers using CCR8- and CCL1-specific probes. The number of positive cells per square millimeter of airway wall was determined. *A*, Numbers of CCL1<sup>+</sup> cells in asthmatic (Asthma, *n* = 9) and healthy volunteers (Normal, *n* = 6). *B*, Numbers of CCR8<sup>+</sup> cells in asthmatic (Asthma, *n* = 10) and healthy volunteers (Normal, *n* = 7). *C*, CCL1-positive cells appear bright in a representative section of an asthmatic individual. *Inset*, Double-positive cells for CCL1 (in situ hybridization) and tryptase (immunocytochemistry) indicate that the majority of double-positive cells are mast cells. *D*, CCR8<sup>+</sup> cells appear bright in a representative section from an asthmatic individual. *Inset*, CCR8 (in situ hybridization)/CD4 (immunocytochemistry) double-positive cells.

Pharmingen. The number of CD4<sup>+</sup>CD44<sup>+</sup>CCR8<sup>+</sup>NK-1.1<sup>−</sup> memory T lymphocytes was determined.

### Immunohistochemical phenotyping and quantitation of leukocytes

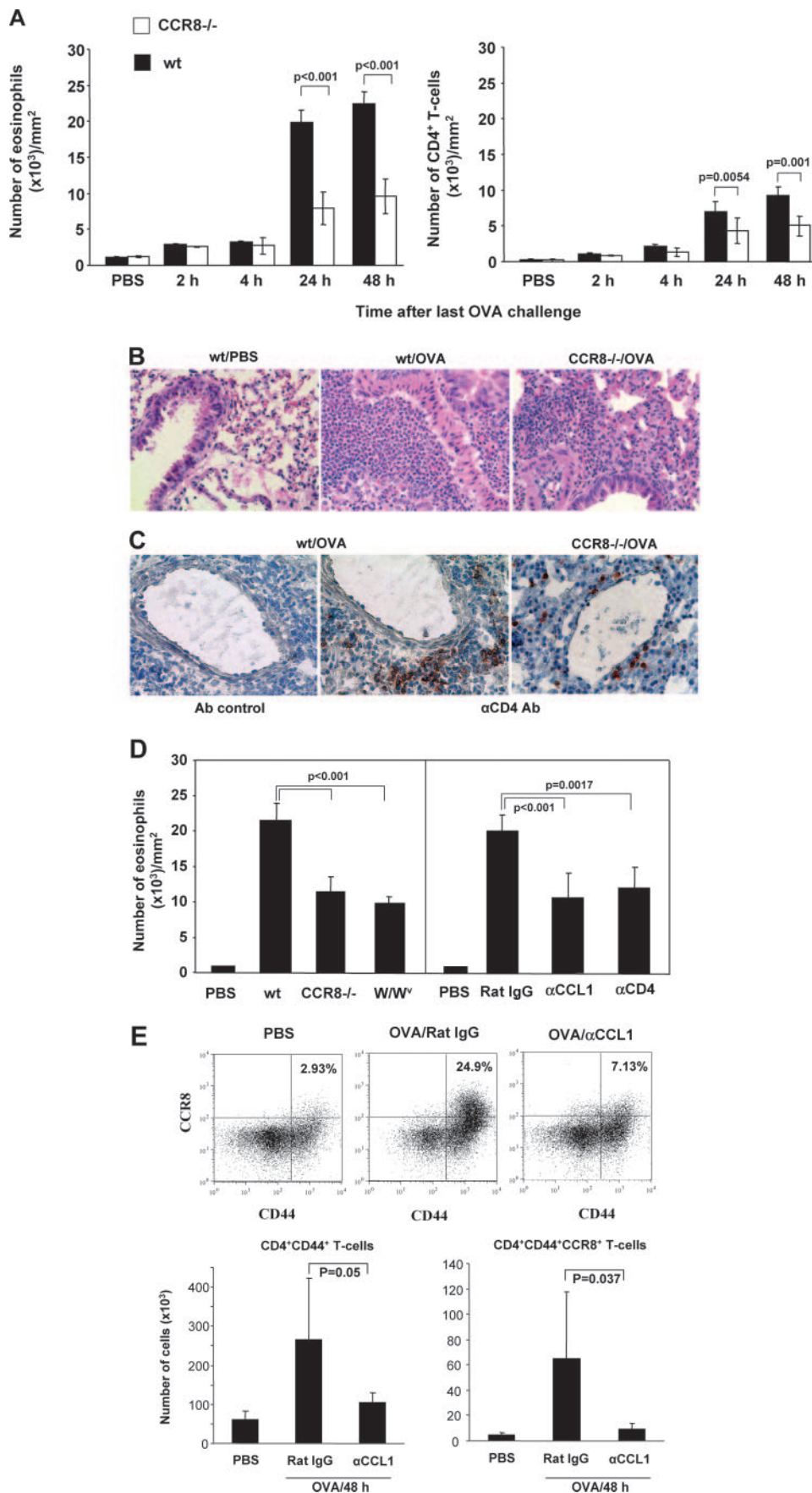
Lung sections from the different experimental groups of mice were prepared as described (12). Briefly, lungs were fixed in 10% neutral buffered formalin (Mallinckrodt Baker) and paraffin embedded. Sections (4 µm) were cut onto microscope slides and stained with H&E according to standard protocols. To determine the sizes of pulmonary infiltrates in the experimental groups of mice, the area of lung tissue covered by infiltrate was calculated in sections at low power (×100) using NIH Image 1.56. At least six fields of at least 0.01 mm<sup>2</sup> were scanned and the mean percentage area was determined for each mouse of each experimental group, relative to the control mice for each experiment. To determine the number of eosinophils,



**FIGURE 3.** CCL1 expression in mast cell-deficient mice. Forty-eight hours after the last OVA challenge, CCL1 mRNA expression was determined by quantitative PCR in whole lung of mast cell-deficient mice (W/W<sup>v</sup>) and wt littermates. PBS-treated mice were included as controls. The data shown are representative of three independent experiments.



**FIGURE 4.** Lung inflammation in the absence of CCR8/CCL1-mediated signals, in mast cell-deficient mice and in CD4 T lymphocyte-depleted mice. **A**, The total number of peribronchial and perivascular eosinophils and CD4<sup>+</sup> T lymphocytes was determined in lung sections from *CCR8*<sup>-/-</sup> mice (□) and wt littermates (■) at the time points indicated after the last OVA challenge. PBS-treated mice were included as negative controls. For eosinophil counts, sections were stained with H&E, and eosinophils were identified based on their morphological appearance. CD4<sup>+</sup> T lymphocyte numbers were determined by counting positive cells after anti-CD4 staining. Cell numbers were determined as described in *Materials and Methods*. Bars represent the mean values of eight mice per group  $\pm$  SD ( $n = 2$ ). **B**, Representative images of lung sections stained with H&E of PBS-treated wt mice (wt/PBS) and wt or *CCR8*<sup>-/-</sup> mice 48 h after the last OVA challenge (wt/OVA and *CCR8*<sup>-/-</sup>/OVA, respectively). **C**, Lung sections of wt mice or *CCR8*<sup>-/-</sup> mice 48 h after the last OVA challenge stained with an isotype Ab control (Ab control) or anti-CD4 Ab. The brown staining indicates CD4<sup>+</sup> T lymphocytes. **D**, Eosinophil numbers were compared 48 h after the last OVA challenge in *CCR8*<sup>-/-</sup> mice (*CCR8*<sup>-/-</sup>), mast cell-deficient mice (*W/W<sup>u</sup>*), anti-CCL1-treated mice (anti-CCL1), CD4<sup>+</sup> T lymphocyte-depleted mice (anti-CD4), and the respective wt littermates (wt) or control Ab-treated mice (Rat IgG). PBS-treated wt mice (PBS) were used as negative controls. The number of eosinophils was determined as described in *Materials and Methods*. **E**, Effects of anti-CCL1 treatment on the number of infiltrating CCR8<sup>+</sup>CD4<sup>+</sup>CD44<sup>+</sup> memory T lymphocytes in the lung 48 h after the last OVA challenge. CD4<sup>+</sup> T lymphocytes derived from PBS (PBS), control Ab (Rat IgG), and anti-CCL1 (anti-CCL1)-treated mice were stained for CD4, CD44, and CCR8 as indicated in *Materials and Methods*. Bars represent the mean values of six to eight mice per group  $\pm$  SD.



slides were stained with H&E (Fisher Diagnostics). CD4<sup>+</sup> T lymphocytes were identified by anti-CD4 Ab (clone RM4-5; BD Pharmingen) staining. The number of eosinophils per square millimeter or CD4<sup>+</sup> T lymphocytes

per square millimeter was determined by counting stained cells, respectively, within peribronchiolar infiltrates in five randomly selected high power ( $\times 400$ ) fields per section (total area of 1 mm<sup>2</sup>).

### Mucus production

Forty-eight hours after the last OVA challenge, fixed (10% neutral buffered formalin; Mallinckrodt Baker) and paraffin-embedded sections from experimental groups of mice and controls were stained with Alcian blue/periodic acid Schiff (AB/PAS) according to standard methods to analyze Goblet cell-induced mucus secretion.

### Measurement of protein cytokine production

Serial dilutions of BAL fluid samples were assayed using commercial ELISA kits for IL-4, IL-13 (Endogen), and CCL1 (R&D Systems). Absorbance values were converted to concentrations of each factor in the BAL fluid (picograms per milliliter or nanograms per milliliter) by interpolation in the respective standard curve.

### Gob-5 *in situ* hybridization

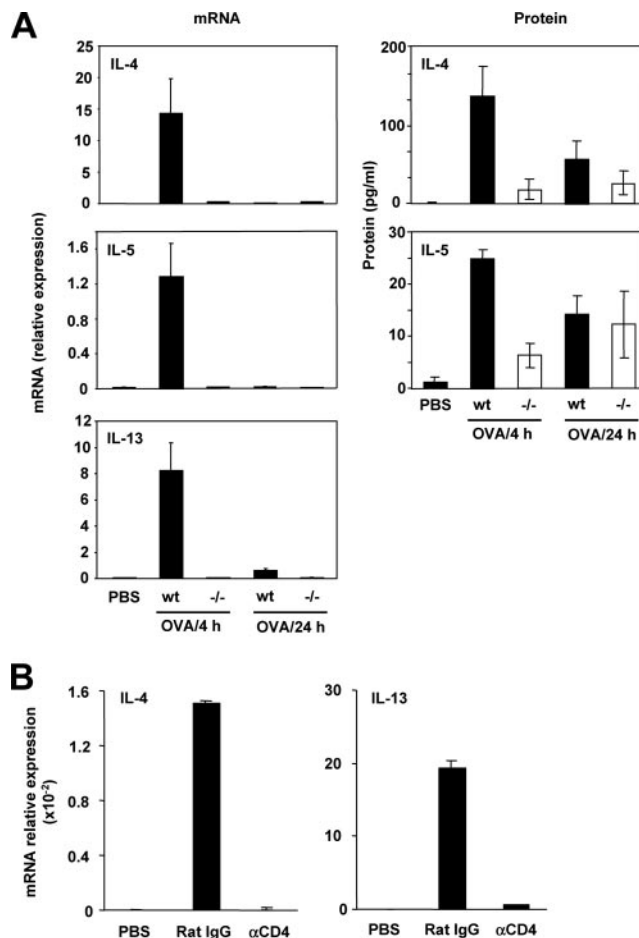
Cryostat sections (6–15  $\mu$ m thick) were thawed at room temperature ( $\sim$ 1 h), fixed in 4% formalin/PBS for 10 min, and treated with 100 mM tetraethylammonium/0.25% acetic anhydride at room temperature for 10 min. Then, sections were allowed to prehybridize in hybridization buffer (50% formamide, 5 $\times$  SSC, 0.3 mg/ml yeast t-RNA, 0.1 mg/ml heparin, 1 $\times$  Denhardt's solution, 0.1% Tween 20, 0.1% CHAPS, 5 mM EDTA (pH 8.0)) for 2–3 h at 60°C. T7 sense and antisense probes were synthesized following the LigoScribe kit (Ambion), and digoxigenin (DIG) labeled using the Roche DIG RNA labeling kit (Roche Applied Science) as per manufacturer's instructions. After washing in PBS, probes were denatured at 80°C for 3 min and chilled on ice before the addition of hybridization buffer at a final concentration of 1 ng/ $\mu$ l. Each section was overlaid with 100  $\mu$ l of hybridization/RNA mix and hybridized overnight at 50°C in a humidity chamber. After hybridization, slides were washed in warm 0.2 $\times$  SSC for 20 min at 50°C, and blocked in PBT (1 $\times$  PBS, 2 mg/ml BSA, 0.1% Triton X-100)/20% heat-inactivated sheep serum for 30–60 min. Slides were incubated with preabsorbed anti-DIG (AP) Ab (Fab) (Roche Applied Science) for 2 h and detected with NBT/5-bromo-4-chloro-3-indolyl phosphate substrate according to the manufacturer's instructions. The detection reaction was stopped after overnight incubation, and slides were washed in TE buffer and mounted with aqueous mounting medium. DNA primers used to generate the Gob-5 DNA/RNA probe (426 bp) were as follows: Gob-5, forward, 5'-CGC TGA TGT CCT TGT ATC AAC A-3'; Gob-5, reverse, 5'-CAG AAT TCA ACC ACA GAA TTG A-3'.

### Generation of anti-CCL1 mAb

Female Wistar-Kyoto rats, 6–8 wk old, were immunized i.p. with 100  $\mu$ g each of synthetic murine CCL1 protein (produced at Millennium Pharmaceuticals) prepared in CFA at day 1 and in IFA at days 14 and 28. The rats were then boosted at day 49 with 100  $\mu$ g of murine CCL1 protein in PBS, and 3 days after the spleens were harvested and fused to SP2/0 myeloma cells. The fusion was screened by ELISA using plates coated with murine CCL1 protein. Hybridomas producing anti-CCL1 mAbs were then subcloned by limiting dilution. Ab 4B6 was selective against plate-bound human CCL1, murine CCL27, murine CCL28, and murine CXCL9 and selectively inhibited murine CCL1-dependent (6 nM) chemotaxis of murine CCR8-transfected L1.2 cells with an IC<sub>50</sub> of 2.3 nM. Clone 4B6 is of the IgG1 isotype as determined with a standard isotyping kit.

### CCR8 and CCL1 *in situ* hybridization

Bronchial biopsies were obtained from 10 mild-to-moderate asthmatics (forced expiratory volume in 1 s, 80–103%) and 7 control age-matched subjects, following ethical approval from the Ethics Committee of the Montreal Chest Hospital (Montreal, Quebec, Canada). All of the asthmatic subjects were atopic; none of them was receiving steroids or any other anti-inflammatory drugs at the time of biopsy. The average age of the patients ranges from 25 to 41 years old and none has any significant respiratory problem. Tissue was fixed in 4% freshly prepared paraformaldehyde for 2 h and washed with PBS/glucose before sectioning. Sections were hybridized with cRNA probes that were constructed from cDNA coding for CCR8 and CCL1 using standard method (29). Briefly, the probes were labeled with S<sup>35</sup> or digoxigenin-UTP in the presence of SP6 and T7 polymerase. Following hybridization, the mRNA-cRNA signal was developed. To identify the phenotype of positive cells expressing CCR8 or CCL1, simultaneous *in situ* hybridization and immunocytochemistry were performed as described previously (30). The sections were first hybridized with a modified protocol (31) and were stained with mAbs before the development of mRNA-cRNA signal. The following Abs were used: CD4 (Th cells), tryptase (mast cells), and BB1 (basophils).

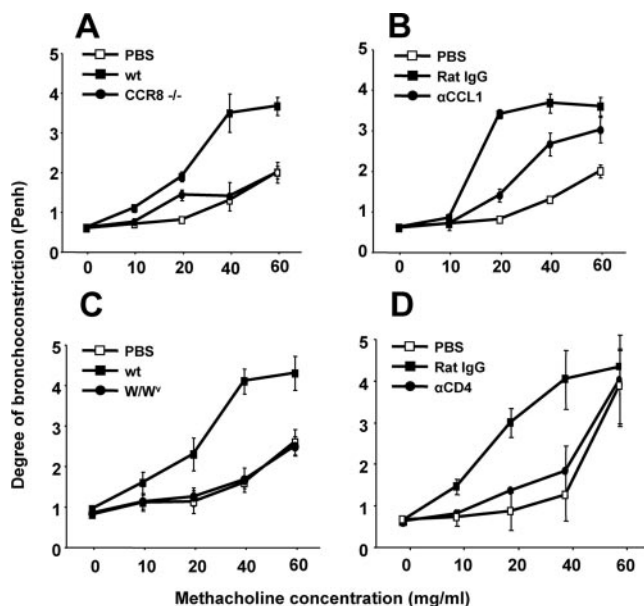


**FIGURE 5.** Th2 cytokine levels in the inflamed lung. *A*, Whole-lung mRNA levels and BAL fluid protein levels of the cytokines IL-4, IL-5, and IL-13 were determined in *CCR8*<sup>-/-</sup> mice (□) and wt littermates (■) at the indicated time points after the last OVA challenge by quantitative PCR and ELISA, respectively. PBS-treated wt mice were used as negative controls (PBS). *B*, IL-4 and IL-13 mRNA expression in the lungs of OVA-challenged mice (4 h after last OVA challenge) after CD4<sup>+</sup> T lymphocyte depletion (anti-CD4) or treatment with an isotype Ab control (Rat IgG). Lungs from PBS-treated controls (PBS) were also included. One representative experiment of three ( $n = 8$  mice/group) is shown.

## Results

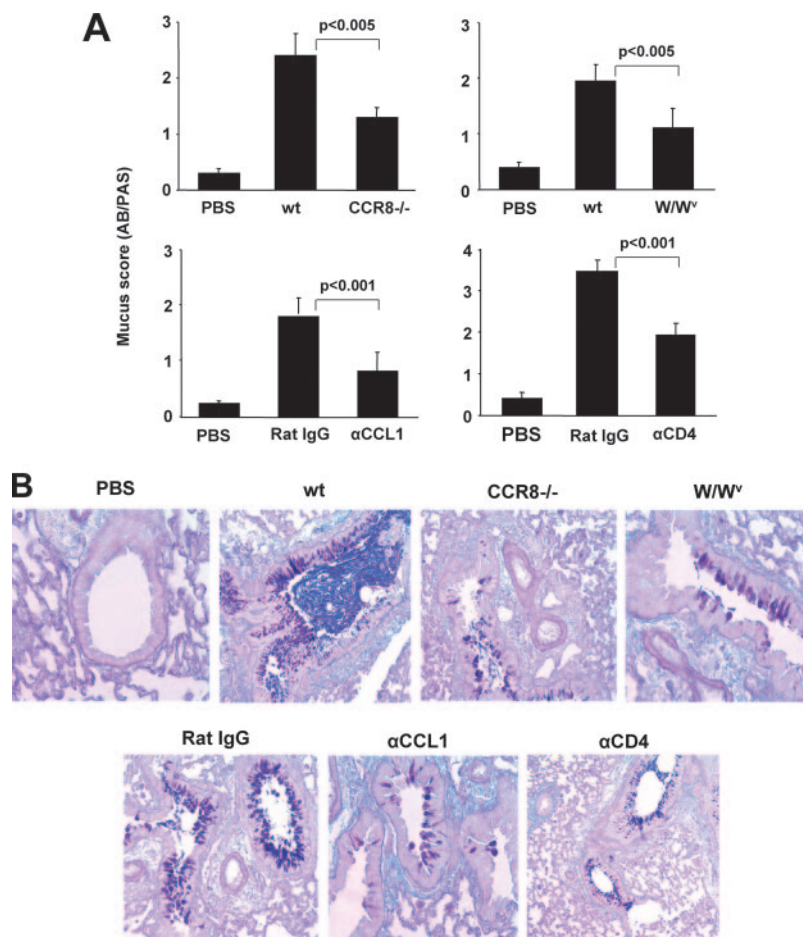
### IgE-activated mast cells are the predominant source for CCL1

In an attempt to identify genes regulated by FcεRI-activated human mast cells, we conducted microarray experiments and identified CCL1 as the chemokine with the highest up-regulation (264-fold) 2 h after FcεRI activation, followed by CXCL8 (22-fold) and CCL20 (12-fold) (our unpublished observations). We next assessed CCL1 mRNA and protein expression by cultured mast cells at different times after IgE activation using quantitative PCR and ELISA, respectively. CCL1 mRNA levels were significantly increased at 2 and 6 h after anti-IgE cross-linking compared with controls (Fig. 1A, left panel). Highest CCL1 mRNA levels were detected in human mast cells cultured in the presence of stem cell factor (SCF) and IL-4, 6 h after activation (Fig. 1A, left panel). In a similar fashion, CCL1 mRNA expression by cultured murine mast cells was strongly up-regulated 6 h after Ag-induced IgE cross-linking (Fig. 1B, left panel). There was a clear correlation between mRNA expression and CCL1 protein levels in supernatants from cultured mast cells. CCL1 protein levels reached  $\sim$ 700 pg/ml at 6 h after FcεRI activation, a  $\sim$ 25-fold increase over the



**FIGURE 6.** Induction of AHR in the absence of CCR8-mediated signals. A–D, AHR was evaluated 24 h after the last OVA challenge in CCR8<sup>-/-</sup> mice (CCR8<sup>-/-</sup>) (A), anti-CCL1-treated wt mice (anti-CCL1) (B), mast cell-deficient mice (W/W<sup>v</sup>) (C), and anti-CD4-treated wt mice (anti-CD4) (D). Results are shown as Penh before (0 mg/ml) and after methacholine provocation (10, 20, 40, and 60 mg/ml). Each symbol represents the mean of the indicated experimental group. Rat IgG was used as an Ab control (Rat IgG). OVA-sensitized mice challenged with PBS or OVA were used as a negative control (PBS) or positive control (wt), respectively. One representative experiment of six performed, with eight animals per experimental group, is shown.

**FIGURE 7.** Mucus hypersecretion in the absence of CCR8-mediated signals. A, A semiquantitative scoring system was used to determine the degree of mucus hypersecretion (0, no or few cells positive; 1, some cells in large airways only; 2, most cells in large airways; 3, most cells in large airways and few cells in small airways; 4, most cells in large and small airways; 5, mucus plug). Mucus production was evaluated 48 h after the last OVA challenge in CCR8<sup>-/-</sup> mice (CCR8<sup>-/-</sup>), mast cell-deficient mice (W/W<sup>v</sup>), wt littermate controls (wt), mice treated with anti-CCL1 Ab (anti-CCL1), mice treated with the CD4<sup>+</sup> T lymphocyte-depleting Ab GK1.5 (anti-CD4), and mice treated with Ab isotype control (Rat IgG). PBS-treated wt mice were used as the negative control (PBS). Bars represent mean values of eight mice per group  $\pm$  SD. One representative experiment of six performed is shown. B, Representative lung sections from the different experimental and control groups of mice stained for mucus (AB/PAS).



levels detected in unstimulated human mast cells (Fig. 1A, right panel). More than 200-fold higher CCL1 protein levels (187 pg/ml) were also detected in the supernatants of murine mast cells 24 h after activation compared with unstimulated cells (Fig. 1B, right panel). We next examined the capacity of mast cells to produce CCL1 in comparison with other cell types. As shown in Fig. 1C, activated mast cells are the predominant human cell type expressing CCL1 mRNA in vitro.

#### Increased expression of CCL1 and CCR8 in the lungs of asthmatic individuals

To evaluate the potential of mast cells to express CCL1 mRNA in lung biopsies of allergic asthmatics, we performed in situ hybridization. The number of CCL1-positive cells per square millimeter of airway wall in asthmatics (17.9 cells/mm<sup>2</sup>) was significantly higher compared with control subjects (3.7 cells/mm<sup>2</sup>) (Fig. 2A). CCL1 mRNA-positive cells were mainly present in the subepithelial layer and within the smooth muscle area (Fig. 2C). Double staining showed that the majority of CCL1-positive cells were tryptase-producing mast cells (Fig. 2C, inset). Fifty to 65% of the mast cells present within the mucosa were CCL1 positive and >45% of the basophils, as determined by costaining for BB-1, were also positive for CCL1. Increased expression of CCL1 mRNA in asthmatic lung biopsies was also accompanied by elevated numbers of CCR8 mRNA-expressing cells as shown by in situ hybridization. The number of cells positive for CCR8 per square millimeter of the airway wall was significantly higher in asthmatics (27.3 cells/mm<sup>2</sup>) compared with control subjects (7.4 cells/mm<sup>2</sup>) (Fig. 2B). The cells were mostly located within the subepithelial layer, although some of them appear to be present



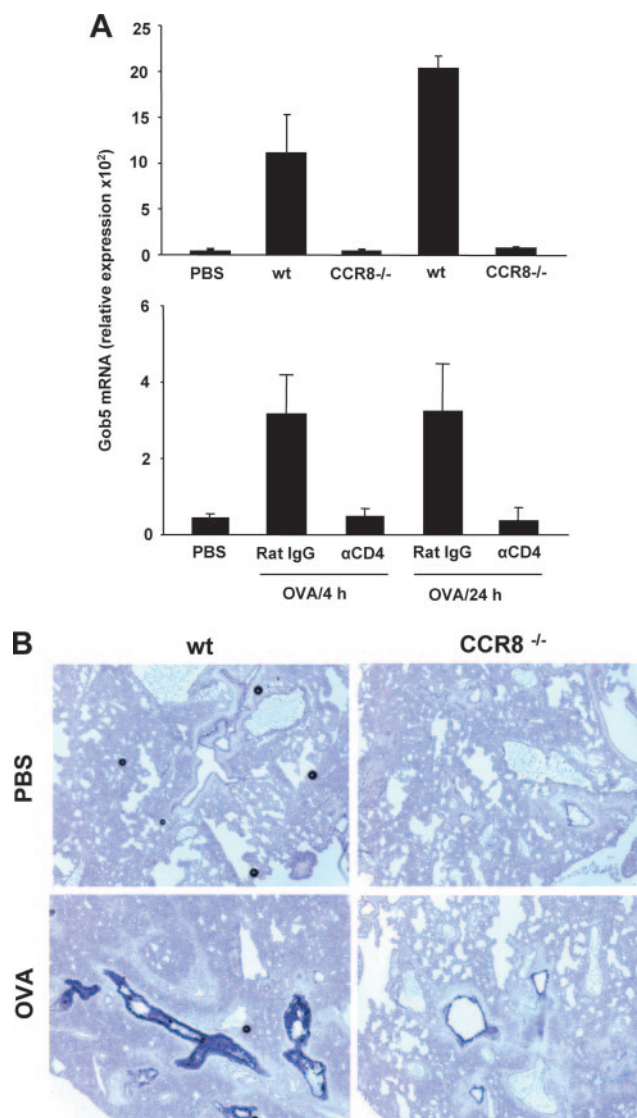
within the epithelium (Fig. 2D). Using simultaneous in situ hybridization and immunocytochemistry, we showed that most of the CCR8-positive cells were CD4<sup>+</sup> T lymphocytes (Fig. 2D, inset). We were able to demonstrate that ~70% of the CD4<sup>+</sup> T lymphocytes that infiltrate the mucosa were CCR8 positive.

#### Lung inflammation in the absence of CCR8-mediated signals

The increased numbers of CCL1-positive mast cells and CCR8-expressing CD4<sup>+</sup> T lymphocytes in asthmatic lung biopsies prompted us to further investigate the functional consequences of CCR8 deficiency in a mast cell-dependent mouse model of allergic airway inflammation (8). In this model, mast cells are essential contributors in the development of lung inflammation and AHR (Ref. 8 and data provided herein) and represent a main source of CCL1 as demonstrated by a significant reduction in CCL1 mRNA expression in the lungs of mast cell-deficient mice (*W/W<sup>v</sup>*) 48 h after the last allergen (OVA) challenge (Fig. 3). Using the same model, we evaluated the degree of lung inflammation at different time points following the final OVA challenge by counting the numbers of eosinophils and CD4<sup>+</sup> T lymphocytes in the lung interstitium of wt and *CCR8*<sup>-/-</sup> mice. Fig. 4A shows a progressive increase in eosinophil and CD4<sup>+</sup> T lymphocyte recruitment in wt mice that peaks at 48 h. At that time, OVA-treated *CCR8*<sup>-/-</sup> mice showed a ~50% reduction in lung eosinophilia and a 20–30% reduction in CD4<sup>+</sup> T lymphocyte accumulation when compared with wt littermates (Fig. 4A). Inflammatory cells in wt mice were located to perivascular and peribronchial areas as shown by H&E and CD4 staining (Fig. 4, B and C). Eosinophil and T lymphocyte recruitment into the airway lumen, as assessed by enumerating the number of cells in the BAL fluid, was not elevated in wt and *CCR8*<sup>-/-</sup> mice at any time point analyzed during OVA treatment as described previously (8).

To better understand the relative contribution of mast cells and CD4<sup>+</sup> T lymphocytes in the inflammatory response, we compared *CCR8*<sup>-/-</sup> mice with *W/W<sup>v</sup>* or CD4 T lymphocyte-depleted wt mice. Fig. 4D shows that the reduction in lung eosinophilia was indistinguishable between *CCR8*<sup>-/-</sup> mice and mast cell-deficient mice (*W/W<sup>v</sup>*, ~50%) or mice that have been depleted of CD4 T lymphocytes before OVA challenge (anti-CD4, ~38%). In addition, treatment of wt mice with the neutralizing anti-CCL1 mAb 4B6 (anti-CCL1) decreased the number of lung eosinophilia (~42%), suggesting that functional inhibition of CCR8/CCL1 interaction during challenge is sufficient to inhibit lung inflammation to a similar degree as observed in *CCR8*<sup>-/-</sup> mice (Fig. 4D). H&E-stained lung sections from the different groups of mice showed that, at 48 h after the last OVA administration, interstitial infiltrates in perivascular and peribronchiolar areas were reduced in size by ~50% in OVA-treated *CCR8*<sup>-/-</sup>, *W/W<sup>v</sup>* mice, anti-CCL1 Ab-treated mice, and anti-CD4 Ab-treated mice when compared with their respective OVA-treated wt controls (data not shown). Thus, mast cell deficiency or CCL1 neutralization resulted in a reduction of lung inflammation similar to CCR8 deficiency or CD4<sup>+</sup> T lymphocyte depletion.

To further explore the effects of CCL1 neutralization on the recruitment of CD4 T lymphocytes into the lung, we administered the neutralizing anti-CCL1 mAb during airway challenge and investigated the phenotype of the infiltrating CD4 T cells by flow cytometry. Our data clearly demonstrate that anti-CCL1 mAb treatment dramatically reduced both the number of CD4<sup>+</sup>CD44<sup>+</sup> memory T lymphocytes (~70%) and more specifically the number of CCR8<sup>+</sup>CD4<sup>+</sup>CD44<sup>+</sup> memory T lymphocytes in the lung (Fig. 4E).



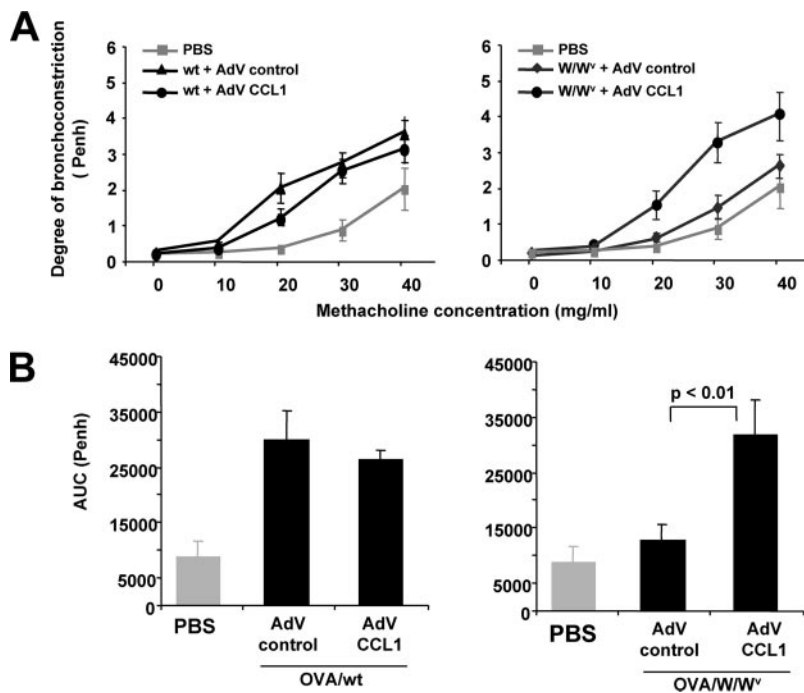
**FIGURE 8.** Expression of the mucus-associated chloride channel Gob-5. **A**, Four hours (OVA/4 h) and 24 h (OVA/24 h) after the last OVA challenge, Gob-5 mRNA expression was examined in the lungs of *CCR8*<sup>-/-</sup> mice (*CCR8*<sup>-/-</sup>) and mice treated with the CD4<sup>+</sup> T lymphocyte-depleting Ab GK1.5 (anti-CD4). Rat IgG-treated mice (Rat IgG) and PBS-treated mice (PBS) were used as negative controls. One representative experiment (*n* = 8 mice/group) of two performed is shown. **B**, In situ hybridization using a Gob-5-specific probe shows strong Gob-5 gene expression (dark blue) in the bronchial epithelium of wt, but not *CCR8*<sup>-/-</sup> mice, 24 h after the last OVA challenge. PBS-treated wt and *CCR8*<sup>-/-</sup> mice were used as negative controls.

#### Th2 cytokine expression in the absence of CCR8-mediated signals

Because CCR8 deficiency only provoked a moderate reduction (20–30%) in the number of CD4<sup>+</sup> T lymphocytes in the lung, we were curious about changes in the production of Th2 cytokines involved in allergic lung inflammation. Fig. 5A shows that, at 4 h after the last OVA administration, IL-4, IL-5, and IL-13 mRNA levels were increased in the lungs of wt but not in those of *CCR8*<sup>-/-</sup> mice. Increases in IL-4 and IL-5 mRNA expression correlated with increased levels of protein as determined by ELISA in the BAL fluid, being 145 ± 30 and 26 ± 3 pg/ml in wt and 12 ± 10 and 7 ± 3 pg/ml in *CCR8*<sup>-/-</sup> mice, respectively (Fig. 5A). At 24 h after the last OVA administration, cytokine mRNA and protein levels returned to steady-state levels and were similar in both



**FIGURE 9.** Adenoviral expression of CCL1 in the lungs of mast cell-deficient mice restores AHR. *A*, AHR was evaluated 24 h after the last OVA challenge in wt and mast cell-deficient mice ( $W/W^v$ ) treated with control adenovirus (AdV control) or CCL1 coding adenovirus (AdV CCL1). Results are shown as Penh before (0 mg/ml) and after methacholine provocation (10, 20, 30, and 40 mg/ml). Each symbol represents the mean of the indicated experimental group. OVA-sensitized mice challenged with PBS were used as a negative control (PBS). One representative experiment with eight animals per experimental group is shown. *B*, Quantitative comparison of the data shown in *A* by calculating the area under the curves (AUC).



experimental groups (Fig. 5A). These data indicate that, in the absence of *CCR8*, IL-4, IL-5, and IL-13 expression in the inflamed lung are strongly reduced.

To determine the contribution of recruited  $CD4^+$  T lymphocytes to cytokine production, we determined the degree of IL-4 and IL-13 mRNA expression in the lungs of  $CD4^+$  T lymphocyte-depleted mice. Both IL-4 and IL-13 mRNA levels were significantly increased in the lungs of Ab control (Rat IgG)-treated mice at 4 h after the last OVA challenge, but not in anti- $CD4$  Ab-treated mice at the same time point of analysis (Fig. 5B). In summary, these results suggest that  $CD4^+$  T lymphocytes expressing *CCR8* are the main producers of IL-4 and IL-13 in the inflamed lung.

#### AHR in the absence of *CCR8*-mediated signals

To evaluate whether the reduction in lung inflammation in the absence of *CCR8*-mediated signals is accompanied by a decrease in AHR, we treated mice with increasing concentrations of methacholine and evaluated the degree of AHR in all treatment groups 24 h following the final OVA challenge. In *CCR8* $^{-/-}$  mice (Fig. 6A), AHR was reduced to the level measured in PBS-treated control mice, whereas CCL1 neutralization significantly, but not completely reduced AHR (Fig. 6B). This could be due to an incomplete inhibition of CCL1, the treatment period, or the existence of a yet-to-be-identified ligand for *CCR8* other than CCL1. Interestingly, AHR in OVA-treated  $W/W^v$  mice (Fig. 6C) and in wt mice after  $CD4^+$  T lymphocyte depletion (Fig. 6D) was reduced to a similar degree as measured in the absence of *CCR8*. The data presented here underline the role of *CCR8*-mediated signals for the induction of AHR triggered by mast cell-dependent mechanisms.

#### Mucus production in the absence of *CCR8*-mediated signals

To analyze whether differences in lung inflammation and Th2 cytokine levels in response to OVA were accompanied by changes in the degree of mucus overproduction, we quantified mucus by AB/PAS staining in lung sections from OVA-treated wt, *CCR8* $^{-/-}$ ,  $W/W^v$ , anti-CCL1 Ab, and anti- $CD4$  Ab-treated mice. OVA-induced mucus production was gradually increased postchallenge until reaching a maximum at 48 h after the last OVA administra-

tion (our unpublished data). As shown in Fig. 7, all OVA-treated groups showed increased levels of mucus when compared with PBS-treated controls. However, when AB/PAS-stained lung sections from OVA-treated *CCR8* $^{-/-}$ ,  $W/W^v$ , anti-CCL1 Ab, and anti- $CD4$  Ab-treated mice were analyzed, a clear decrease in the frequency of epithelial cells producing mucus as well as in the number of airways showing mucus plugs was observed in all groups to a similar degree: *CCR8* $^{-/-}$ , ~50%;  $W/W^v$ , ~44%; anti-CCL1 Ab, ~52%; anti- $CD4$  Ab, ~43% (Fig. 7). These differences were even more pronounced when we compared mRNA expression levels for the mucus associated chloride channel Gob-5 in *CCR8* $^{-/-}$  and anti- $CD4$  Ab-treated mice with control mice. In both experimental groups, Gob-5 mRNA levels remained unchanged and similar to PBS controls at 4 and 24 h after the last OVA challenge. However, Gob-5 mRNA levels were increased ~500-fold in the respective wt controls at all time points investigated (Fig. 8A). Similarly, strong signals for Gob-5 were detected in bronchial epithelium of OVA-challenged wt mice compared with *CCR8* $^{-/-}$  mice or PBS controls by in situ hybridization using a Gob-5-specific probe (Fig. 8B). The data presented here underline the role of *CCR8* in mucus production triggered by mast cell-dependent mechanisms.

#### Administration of CCL1 adenovirus restores the phenotype in mast cell-deficient mice

Our data presented herein strongly support a role of mast cells, CCL1, and *CCR8* $^+CD4^+$  T lymphocytes in the induction of mucosal inflammation and AHR. To demonstrate that these events are sequential and associated, we hypothesized that the defects in inflammation and AHR observed in the absence of mast cells should be reversed by administration of exogenous CCL1. To address this issue, we administered CCL1 by adenoviral delivery to the airways both in wt as well as in mast cell-deficient mice. Transient expression of CCL1 by adenovirus increased BAL levels of CCL1 to 329 pg/ml in wt mice and 352.5 pg/ml in mast cell-deficient mice 24 h after the last OVA challenge, whereas levels remained under the detection limit when a control virus was used. Similar to data shown in previous experiments, allergen provocation augmented

AHR to a similar degree in wt mice treated with CCL1 adenovirus (AdV CCL1) or control adenovirus (AdV control) (Fig. 9). In contrast, i.n. administration of AdV CCL1, but not AdV control, in mast cell-deficient mice fully reversed impaired AHR (Fig. 9). Indeed, the level of AHR in mast cell-deficient mice treated with AdV CCL1 was identical with that seen in wt animals (Fig. 9B). Likewise, AdV CCL1 in mast cell-deficient mice also reversed the impaired eosinophilic inflammation and mucus secretion (data not shown). Taken together, these data demonstrate that mast cell-derived CCL1 plays an essential role in orchestrating the inflammatory response in the airways after allergen exposure.

## Discussion

To further understand the potential role that mast cells may play in orchestrating the inflammatory response, we initially performed microarray analysis of IgE-activated human mast cells and determined the profile of chemokines produced. From the 42 chemokines that were investigated, CCL1 was regulated at the mRNA level 10-fold greater than any other chemokine (our unpublished observation). Interestingly, mast cells primed in the presence of IL-4 produced even higher levels of CCL1, raising the interesting hypothesis that a "local Th2 environment" further primes mast cells for CCL1 production. These data support previous observations in both murine and human mast cells that CCL1 is the most abundant chemokine produced after IgE cross-linking (32–34).

CCL1 has previously been reported to be produced from activated T lymphocytes, in particular Th1 effector cells, as well as IgG- and LPS-activated monocytes (35, 36). However, our data would suggest that mast cells are the predominant cellular source of CCL1, expressing CCL1 mRNA at levels 100-fold higher than in T lymphocytes and monocytes *in vitro*.

It next became important to determine whether, in individuals with allergic inflammation, mast cells were the principal source of CCL1. Our data show that the number of CCL1 mRNA-positive cells was 5-fold elevated in asthmatic individuals compared with normal subjects. Forty to 60% of CCL1 mRNA-positive cells colocalized with tryptase<sup>+</sup> mast cells and ~45% with BB1<sup>+</sup> basophils. Taken together, these data provide compelling evidence that mast cells (and basophils) are the principal source of CCL1 and raise the possibility that CCL1 may contribute to pathophysiological processes that are characterized by inappropriate mast cell activation. Interestingly, also the number of CCR8<sup>+</sup> cells was increased 4-fold in the lungs of allergic asthmatics and CCR8 expression was associated with ~70% of CD4<sup>+</sup> cells. In contrast, only 15% of CD4<sup>+</sup> memory T lymphocytes express CCR8 in human blood (20). When activated *in vitro*, these cells preferentially produce the Th2 cytokines IL-4, IL-5, IL-9, and IL-13 (20). Together, these observations raise the potential that CCL1-producing mast cells may play a role in the recruitment of a subset of effector T lymphocytes bearing CCR8 that may contribute to lung mucosal inflammation.

Although mast cells can produce a wide array of proinflammatory mediators and chemokines, the role of IgE activation of mast cells in lung mucosal inflammation remains controversial. This discrepancy is based to some extent on *in vivo* experimental model systems using different Ags and/or adjuvants. Immunization and challenge with low-dose soluble Ag primes for a mast cell-dependent inflammatory response, whereas high-dose Ag in adjuvant immunization results in a T lymphocyte-dependent mucosal inflammatory response independent of mast cells (8, 27, 37). To understand the role of mast cells and CCL1 *in vivo*, we next used a mast cell-dependent system (8) and measured CCL1 mRNA in the lung tissue of wt and mast cell-deficient mice. Our data indicate that, in the absence of mast cells, CCL1 mRNA in the lungs was

dramatically reduced, suggesting that indeed mast cells are the principal source of CCL1. At the same time, *in vivo* inhibition of CCL1 in wt mice during aero-allergen challenge by the administration of the neutralizing mAb 4B6 inhibited eosinophilic inflammation of the airways, allergen-induced AHR, and the recruitment of CCR8<sup>+</sup>CD4<sup>+</sup> memory T lymphocytes into the lungs. Importantly, the degree of suppression of airway inflammation with the CCL1-neutralizing mAb 4B6 was comparable with that observed in mast cell-deficient animals. Taken together, our data suggest that mast cell-derived CCL1 is an important regulator of eosinophilic mucosal inflammation and AHR. These data are in part in agreement with work of Bishop and Lloyd (38) where neutralization of CCL1 inhibited eosinophilic inflammation, but not Th2 cell recruitment. The precise explanation for these discrepancies are unclear; however, unlike in the system we have described, inflammation and AHR are driven by mechanisms other than mast cell activation. Indeed, although we report a 10- to 20-fold increase of CCL1 mRNA levels 48 h after the last OVA challenge in the lungs of mice, there are no significant differences in lung CCL1 mRNA levels at 48 h in the model studied by Bishop and Lloyd (38). Similar to our findings, Gombert et al. (39) recently described a role of mast cell-derived CCL1 in atopic dermatitis and suggest an important role for the recruitment of CCR8<sup>+</sup> T lymphocytes and Langerhans-like cells.

Although CCL1 derived from mast cells plays an important role in mucosal inflammation, it is unclear whether this is due to a direct effect on the airways, or to the CCL1-dependent recruitment and activation of CD4<sup>+</sup> T lymphocytes. To address this issue, we next depleted CD4<sup>+</sup> T lymphocytes before allergen provocation. Similar to data reported by ourselves (40) and others (28), even in this model that requires mast cell-dependent events, CD4<sup>+</sup> T lymphocytes are essential for mucosal inflammation. Thus, we propose a mast cell-T lymphocyte axis in lung inflammation that is linked by the production of CCL1. To further address the role of mast cell-derived CCL1, we hypothesized that mast cell-derived CCL1 plays a crucial role in orchestrating lung mucosal inflammation and AHR. Overexpression of CCL1 in mast cell-deficient mice fully restored the reduced inflammatory and AHR phenotype. These data taken together with the observation that CCL1 mRNA is reduced in mast cell-deficient animals suggest that not only are mast cells the source of CCL1 *in vivo*, but also reconstitution of CCL1 is sufficient to restore AHR in mast cell-deficient mice.

CCL1 is the only mammalian ligand identified to date for CCR8 (41). Similar to CCR3 and CCR4, CCR8 has been reported to be preferentially expressed on Th2 compared with Th1 effector cells (17–20). However, although preferential expression of chemokine receptors on distinct Th populations has been reported by several groups, the chemokine receptor usage and association at the single-cell level remains poorly documented (42). Indeed, in the case of CCR4, which was initially proposed to discriminate between Th1 and Th2 effector populations (18), it is now appreciated that, although all cells that produce IL-4 express CCR4, CCR4<sup>+</sup> cells have the capacity to secrete both IL-4 and IFN- $\gamma$  (43). Indeed, subpopulations of CCR4<sup>+</sup> cells that coexpress CXCR3 uniformly produce IFN- $\gamma$  (44).

A number of studies have also investigated the role and contribution of chemokine receptors to the allergic response. Studies performed using CCR3-deficient mice or CCR3 mAbs have shown that, although this pathway is essential for eosinophil migration, CCR3 appears to be largely redundant for the migration of Th2 cells (45). Similarly, analysis of mice deficient in CCR4 does not support an important role for CCR4 in mucosal inflammation and Th2 cell recruitment (46). The role for CCR8 in mediating Th2 cell recruitment also remains controversial. Although Chensue et al.

(47) initially described defective Th2 cell migration in mice deficient in CCR8, studies by other groups, including ourselves (27, 37), have suggested that CCR8 does not contribute to the allergic response. However, based on our hypothesis that mast cell-derived CCL1 mediates lung inflammation through a CD4<sup>+</sup> T lymphocyte-dependent mechanism, we re-evaluated the role of CCR8 in mast cell-driven mucosal inflammation. Our data using Ag-challenged CCR8<sup>-/-</sup> mice support our mast cell/T lymphocyte axis hypothesis in that mice lacking this receptor have reduced numbers of eosinophils in the lungs as well as attenuated AHR. Quite remarkably, in the lungs of CCR8-deficient mice, mRNA levels of IL-4, IL-5, and IL-13 were reduced by >90%. Together with our data showing that CCL1 inhibition during allergen provocation inhibits nearly exclusively the migration of CCR8<sup>+</sup>CD4<sup>+</sup> memory T lymphocytes into the lung, we propose that CCR8<sup>+</sup>CD4<sup>+</sup> memory T lymphocytes that are recruited to the airways following mast cell activation produce the majority of Th2 cytokines that mediate lung mucosal inflammation.

Increased mucus production in the airways of asthmatic individuals has also been reported and is believed to contribute to airway obstruction. Histological analysis of the lungs of either mast cell- or CCR8-deficient animals, or mice treated with either anti-CD4 Ab or anti-CCL1 Ab, all demonstrated reduced mucus secretion in the lungs following allergen exposure. In addition to cytokines such as IL-13 that regulate mucus secretion (48, 49), the recently identified calcium-activated chloride channel family member mGob-5 (hCLCA-1) has been proposed to play an important role in allergen induced mucus production (50). Indeed, CLCA-1 is expressed in the lung epithelium and up-regulated in the lungs of asthmatic individuals (50). Moreover, it has previously been reported that overexpression of CLCA-1 in vitro increases *MUC5AC* gene induction (50). Our results indicate that mice deficient in CCR8 have a dramatic reduction in Gob-5 mRNA. Moreover, in situ hybridization reveals that Gob-5 mRNA is virtually absent in the lung epithelium of CCR8-deficient mice. The reduced gene expression of Gob-5 together with the reduced levels of IL-13 in the lungs provides a molecular explanation for the reduced mucus production observed in the lungs of CCR8-deficient animals.

In summary, we propose a sequential series of events in the lung mucosa that occurs following allergen provocation. IgE-triggered mast cells produce CCL1, which regulates the recruitment of IL-4-, IL-5-, and IL-13-secreting T lymphocytes into the airways through a CCR8-dependent mechanism. Our data suggest that this mast cell/CCL1-CD4<sup>+</sup> T lymphocytes/CCR8 axis is central to the pathogenesis of lung mucosal inflammation and AHR and offer new strategies for therapeutic intervention in diseases such as bronchial asthma as well as other disorders that are characterized by inappropriate mast cell activation.

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## Disclosures

The authors have no financial conflict of interest.

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