

RESEARCH ARTICLE

Murine lung eosinophil activation and chemokine production in allergic airway inflammation

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Eosinophils play important roles in asthma and lung infections. Murine models are widely used for assessing the functional significance and mechanistic basis for eosinophil involvements in these diseases. However, little is known about tissue eosinophils in homeostasis. In addition, little data on eosinophil chemokine production during allergic airway inflammation are available. In this study, the properties and functions of homeostatic and activated eosinophils were compared. Eosinophils from normal tissues expressed costimulation and adhesion molecules B7-1, B7-2 and ICAM-1 for Ag presentation but little major histocompatibility complex (MHC) class II, and were found to be poor stimulators of T-cell proliferation. However, these eosinophils expressed high levels of chemokine mRNA including C10, macrophage inflammatory protein (MIP)-1 α , MIP-1 γ , MIP-2, eotaxin and monocyte chemoattractant protein-5 (MCP-5), and produced chemokine proteins. Eosinophil intracellular chemokines decreased rapidly with concomitant surface marker downregulation upon *in vitro* culturing consistent with piecemeal degranulation. Lung eosinophils from mice with induced allergic airway inflammation exhibited increased chemokines mRNA expression and chemokines protein production and upregulated MHC class II and CD11c expression. They were also found to be the predominant producers of the CCR1 ligands CCL6/C10 and CCL9/MIP-1 γ in inflamed lungs. Eosinophil production of C10 and MIP-1 γ correlated with the marked influx of CD11b^{high} lung dendritic cells during allergic airway inflammation and the high expression of CCR1 on these dendritic cells (DCs). The study provided baseline information on tissue eosinophils, documented the upregulation of activation markers and chemokine production in activated eosinophils, and indicated that eosinophils were a key chemokine-producing cell type in allergic lung inflammation. *Cellular & Molecular Immunology* advance online publication, 12 July 2010; doi:10.1038/cmi.2010.31

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INTRODUCTION

Eosinophils play significant roles in allergic diseases, in parasitic, viral and bacterial infections, and in a variety of hypereosinophilic diseases (reviewed in Refs. 1 and 2). They secrete cytotoxic granule cationic proteins such as major basic protein (MBP), eosinophil cationic protein, eosinophil peroxidase and eosinophil-derived neurotoxin which mediate tissue damage and dysfunction. In addition, eosinophils produce cytokines, chemokines, reactive oxygen species, arachidonic acid metabolites and other mediators that are involved in tissue inflammation. By expressing major histocompatibility complex (MHC) II molecules and costimulation molecules, eosinophils have also been shown to present Ag to T cells.³ Eosinophilia has long been considered a significant pathological component of bronchial asthma (reviewed in Refs. 4 and 5). Eosinophils were found in lungs, sputum and bronchoalveolar lavages of asthma patients. Lung eosinophil infiltration in autopsy samples of asthma patients has been reported as early as 1908⁶ and eosinophil numbers in lung correlate well with asthma severity.⁷ However, the involvement of eosinophils in the pathological conditions of the general asthma population has been vigorously

debated.^{8–10} Aside from asthma involvement, eosinophilia has been shown to be clearly involved in other disease manifestations. Anti-IL-5 monoclonal antibody (mAb) therapy has been successful in improving disease severity in a number of hypereosinophilic diseases.^{11–13}

Studies of peripheral blood T-cell lines derived from asthma patients have shown that the disease is a consequence of T helper (Th) 2-biased responses to allergens.¹⁴ Further studies in human and mouse have supported the Th2-biased paradigm in asthma.⁴ Studies on the role of eosinophilia in allergic airway hyper-responsiveness (AHR) showed that IL-5 produced by Th2 cells is the single most important pathogenic factor for mediating eosinophil development, and that anti-IL-5 mAb blockade or IL-5 deficiency abrogates AHR in a mouse strain-dependent manner.^{4,15–18} Because reducing eosinophilia represents a promising strategy for asthma therapy in many quarters, clinical trials using humanized anti-IL-5 mAbs (Mepolizumab and SCH55700) have been performed. With patients selected on the basis of only their asthma status, little efficacy was observed except for a trend towards improved baseline forced expiratory volume at 1 s.^{19–21} The trial results were inconclusive because anti-IL-5 mAbs depleted airway eosinophil

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numbers by only 55% in patient lungs.²² The positive outcomes of two recent clinical trials involving the use of anti-IL-5 mAbs on selected populations with refractory eosinophilic asthma have generated considerable enthusiasm and interest.^{10,23,24} mAb treatment significantly reduced the frequency of asthma exacerbations and Asthma Quality of Life Questionnaire scores and allowed prednisone sparing despite reduction of eosinophil numbers in bronchial biopsies by only 68%. The results support an important role of eosinophils in a subset of severe and difficult-to-treat asthma patients. Though other important asthma indicators such as symptoms, forced expiratory volume at 1 s after bronchodilator use and AHR were not significantly changed in these patients, improved anti-IL-5 mAb therapy can be achieved by altering the mAb dose, frequency or duration, or by combination therapy with other modalities to further reduce eosinophil numbers in lungs.

Animal studies have clarified the mechanistic relationship between AHR and eosinophilia. This relationship has a strong genetic dependence as reflected by strain differences in AHR induction in eosinophil-deficient mice. In C57BL/6 mice, anti-IL-5 mAb treatment or eosinophil deficiency results in the abrogation of allergen-induced AHR.^{16,25,26} Pulmonary eosinophil elicitation of CCR3 ligand CCL11/eotaxin or CCR4 ligands CCL17/thymus- and activation-regulated chemokine (TARC) and CCL22/macrophage-derived chemokine (MDC) production is required for the recruitment of Th2 cells into lungs and AHR induction.^{26,27} In BALB/c mice, AHR induction was unaffected by anti-IL-5 mAb blockade of eosinophil development, IL-5 deficiency, or eosinophil ablation by GATA-1 promoter mutation.^{28–30} However, IL-5/eotaxin dual deficiencies abolished AHR induction and significantly reduced CD4⁺ T-cell production of IL-13 in BALB/c. Both AHR induction and IL-13 production were recovered by airway instillation of eotaxin or eosinophils, indicating that eotaxin is critical for T-cell IL-13 production and AHR induction.³¹ Thus, in mice eosinophils are essential for AHR induction by the eosinophil elicitation of chemokines. Despite the significant role eosinophil chemokine induction plays in airway inflammation and AHR, studies of direct chemokine production by eosinophils are limited in scope and numbers. Human eosinophils have been shown to produce eotaxin 1, CCL-2, -7, -13/monocyte chemoattractant protein (MCP)-1, -3, -4, CCL5/regulated on activation normal T-cell expressed and secreted (RANTES), and CCL3/macrophage inflammatory protein-1 α (MIP-1 α).^{9,32,33} Eosinophil production of chemokines in mouse disease models is poorly studied.

In human eosinophil studies, resting eosinophils are used most frequently because they can be isolated readily from peripheral blood. However, mouse eosinophil studies are limited to the use of activated eosinophils obtained from animals with parasitic or viral infection, with induced airway inflammation, or expressing IL-5 transgenes^{3,15,34–39} and the properties of homeostatic eosinophils in mouse are unclear. The lack of baseline information on gene expression and phenotype in homeostatic mouse eosinophils creates disparities in comparing eosinophil properties between human and mouse, and difficulties in understanding eosinophil activation during inflammation. In this study, lung eosinophils were purified from normal mice and mice with induced airway inflammation for the assessment of the upregulation of surface marker expression and chemokine expression upon eosinophil activation. The results showed that eosinophils upregulate adhesion and costimulation molecule expression and chemokine mRNA and protein expression during lung inflammation. The results support the key role eosinophils play in mediating cellular trafficking during homeostasis and in recruiting CCR1⁺, CCR2⁺, CCR3⁺ or CCR5⁺ cells into lungs during allergic lung inflammation.

MATERIALS AND METHODS

Materials

mAbs conjugated with FITC, phycoerythrin, allophycocyanin, Alexa fluor 647 or allophycocyanin-Alexa fluor 750 were purchased from eBioscience (San Diego, CA, USA) except the following: anti-Siglec-F, CCR3 and integrin β_7 (clone M293) were from BD Biosciences (San Jose, CA, USA); anti-F4/80 (clone CL:A3-1) was from Biolegend; and anti-I-A was from Southern Biotech (Birmingham, AL, USA). Chemokines and affinity-purified polyclonal antichemokine antibodies (Abs) were from Peprotech (Rocky Hill, NJ, USA). Alexa fluor 555- or 647-conjugated secondary Abs used for confocal microscopy, 7-amino-actinomycin D, fluorescein and Caltag calibration beads were from Invitrogen (Carlsbad, CA, USA). Collagenase D and DNase I were from Roche (Nutley, NJ, USA). Ovalbumin (OVA) and methacholine were from Sigma (St Louis, MI, USA).

Mouse immunization

To induce AHR and eosinophilia, BALB/c mice were immunized essentially as described.³⁹ On day 2, 2.5×10^6 DO11.10 anti-OVA-specific T-cell antigen receptor transgenic CD4⁺ T cells were injected intravenously. On days 0 and 7, mice were primed intratracheally with 1×10^6 bone marrow-derived dendritic cells (DCs)⁴⁰ with or without OVA pulsing, followed by intratracheal challenges with 100 μ g OVA on days 14, 16, 18 and 20. Lungs cells were harvested on day 21. Bronchoalveolar lavage cells were collected by flushing with 7 ml saline through a 22G catheter fastened to the trachea. Cells were counted, spun onto slides and stained with Diff-Quik (Dade Behring, Newark, DE, USA). Differentials of BAL cells were determined by counting 200 cells per mouse and 6–8 mice per group were used. Serum anti-OVA specific immunoglobulin G (IgG) isotypes and IgE subclass were determined by ELISA as described.³⁹ A pooled serum from OVA-immunized mice was used as a reference with the titer of anti-OVA subclass Abs defined as 1000 units.

Airway hyper-responsiveness measurements

AHR was measured by airway pressure time index (APTI)⁴¹ in mice anesthetized with intraperitoneal ketamine and xylazine. Following induction of deep anesthesia, the trachea was cannulated with a 19-gauge metal cannula, after which the mouse was ventilated with a small rodent ventilator (Harvard Apparatus model 55-7066; South Natick, MA, USA) with a tidal volume of 0.2 ml, a rate of 120 breaths/min, and 3 cm H₂O of positive end-expiratory pressure. After intraperitoneal administration of decamethonium bromide (25 mg per kg body weight) to abolish inspiratory or expiratory artifact on the pressure waveform, a venous cannula was inserted into the inferior vena cava through a midline laparotomy. A baseline airway pressure waveform was then recorded over 5 min, after which bronchial provocation was induced by intravascular injection of methacholine (100 μ g per kg body weight in saline) at a volume of 50 μ l per 20 g body weight. The airway pressure waveform was then measured for 5 min following methacholine injection. The APTI (cm H₂O-s) to methacholine was calculated by subtracting the area of the 5 min prechallenge airway waveform from the area of the 5 min waveform immediately following methacholine provocation as previously described.⁴²

Lung and spleen cell isolation

Lung single cell suspensions were prepared essentially as described.⁴³ Briefly, 8- to 12-week-old BALB/c mice were anesthetized by ketamine/xylazine solutions containing 200 U heparin. The aorta was excised and

the lungs were perfused gently with 0.5 ml of saline *via* the right ventricle. The lungs were excised, minced with scissors and digested for 45 min with 400 U/ml collagenase D and 30 µg/ml DNase I. The single cell suspensions were pelleted and the red blood cell lysed by hypotonic NaCl solutions. The debris was filtered on a cotton plug and cell populations were enriched by magnetic microbead selection. Spleen cells were similarly prepared. The excised spleen was squashed with the butt of a syringe plunger and the capsular material minced and digested for 15 min at 37 °C with 133 U/ml collagenase D and 30 µg/ml DNase I. After incubation the particulate material was disrupted by pipetting, and the cells were pelleted. Single cell suspensions were obtained after red blood lysis and filtering as for lung cells.

Magnetic microbead selection

Lung and spleen cells were blocked in 30% heat-inactivated (56 °C, 30 min) fetal bovine serum (FBS) and 50 µg/ml 2.4G2 anti-FcγRII/FcγRIII mAbs on ice for 15 min. For the selection of F4/80⁺ cells, the suspensions were stained with FITC-anti-F4/80 mAbs followed by anti-FITC IgG-magnetic microbead as described by the manufacturer (Miltenyi, Auburn, CA, USA). For CD11c⁺ cell enrichment, cell suspensions were directly incubated with anti-CD11c IgG-magnetic microbeads. Magnetic sorting was performed on a VarioMACS with LS columns. Positive cells were reloaded two additional times on the column after the first selection to improve purity.

Surface antigen or intracellular chemokine staining and flow cytometry analysis

Cells for surface staining were blocked with 2.4G2 mAbs and stained for 30 min with fluorochrome-conjugated mAbs at 4 °C. For intracellular chemokine staining, isolated cells were either put on ice or incubated at 37 °C in medium with or without stimulants for 4 h in the presence of 2 µM monensin and on Teflon petri dishes (Saville, Minnetonka, MN, USA) to prevent cellular adherence. The incubated cells were pipetted out, washed in cold phosphate-buffered saline, and stained with lineage-restricted surface markers. Intracellular chemokine staining was performed essentially as described.⁴⁴ Briefly, stained cells were fixed in 4% paraformaldehyde for 20 min, permeabilized with 0.1% saponin and blocked with 5% nonfat dry milk. The permeabilized cells were stained with antichemokine primary Abs followed by Alexa fluor 647-conjugated antirabbit Abs and analyzed on a FACSCalibur flow cytometer. Appropriate species-matched IgG or isotype-matched mAbs were used as controls. Data were analyzed with FlowJo software (Tree Star, Ashland, OR, USA).

Cell sorting for chemokine production measurements and RNA isolation

Fluorescence-activated cell sorting (FACS) purification of eosinophils was performed on a FACS Vantage SE flow cytometer with DIVA software. Lung eosinophils from naive mice were sorted from anti-F4/80⁺ magnetic bead-enriched populations by their negativity for I-A and CD11c and their positivity for Siglec-F and CD11b. Lung eosinophils from mice sensitized and challenged with OVA were sorted from lung cell populations by their high side-scatter and low forward-scatter, and their positivity for Siglec-F and CD11b and low MHC II expression. Sorted cells were >98% purity. For chemokine production measurements, eosinophils were incubated in medium at 0.5×10⁶ cells/ml for 8–10 h with 20 ng/ml phorbol myristate acetate and 1 µM ionomycin and the supernatants were assayed for chemokines by a sandwich ELISA using Abs from R&D (Minneapolis, MN, USA) or Peprotech. Standard curves were performed with chemokines

from Peprotech. The sensitivities of the assays ranged from 1 to 10 ng/ml. For total RNA isolation, cell pellets were homogenized in RNeasy lysis buffer.

In vitro stimulation of transgenic DO11.10⁺ T-cell proliferation by eosinophils and DCs

Splenic CD4⁺CD25[−]DO11.10⁺ T-cell antigen receptor transgenic T cells were isolated by negative selection using magnetic microbeads conjugated with mAbs against CD8, CD19, DX5, CD11c and CD25 (Miltenyi Biotec) and plated at 1×10⁵/well. Lung eosinophils were purified to 98% purity by anti-F4/80 magnetic microbead enrichment followed by FACS for Siglec-F⁺CD11b⁺CCR3⁺ cells. DCs were purified by selection of CD11c⁺ lung cells by magnetic beads followed by FACS for I-A⁺CD11c⁺ cells. Graded doses of eosinophils or DCs were added to CD4⁺CD25[−]DO11.10⁺ T cells and cells were cultured in round bottom wells in triplicates with 25 µM OVA_{323–339} peptide or 8 µg/ml anti-CD3 mAbs for 5 d. [³H]-thymidine (1 µCi/well) was added for the last 8 h and T-cell proliferation was assessed by scintillation counting.

Confocal and phase-contrast microscopy

Lungs were fix-inflated at 20 cm hydrostatic pressure with 0.7% paraformaldehyde for 30 min, fixed overnight in 0.7% paraformaldehyde, exchanged for 2–3 d in 30% sucrose, and immersed overnight followed by freezing in optimal cutting temperature compound. Tissue was used both in hematoxylin and eosin, and fluorescent Ab staining. Confocal microscopy was performed essentially as described.⁴³ Tissues were sectioned at 5 µm, extracted with 0.3% triton X-100, blocked with mouse, horse, donkey and chicken sera (5% each) in RPMI-1640, and stained with primary and secondary Abs. Sections were examined with a Zeiss LSM 510 confocal microscopy assembly. For single cell suspensions, sorted cells (0.5×10⁵–3×10⁵) were spun onto microscope slides in a Cytospin 2 centrifuge (Shandon, Pittsburgh, PA, USA) and stained by Diff-Quik. Images were captured on an Olympus BX51 fluorescence microscope equipped with a Q-color 3 digital camera.

Real-time PCR

Total RNA was obtained from FACS-purified cells by extraction with RNeasy (Qiagen, Valencia, CA, USA) and reverse-transcribed by the Advantage RT for PCR Kit (BD Clontech Palo Alto, CA, USA). Real-time PCR was performed with the chemokine primers reported previously⁴⁵ in a Bio-Rad i-Cycler Thermal Cycler as described. The PCR conditions were: 39 cycles (94 °C for 22 s, 59–62 °C for 30 s and 72 °C for 30 s); and 1 cycle (94 °C for 22 s, 59–62 °C for 30 s and 72 °C for 5 min). PCR products were verified by melting curves and by agarose (1.8%) gel electrophoresis.

Microarray analysis

Affymetrix microarray analysis of sorted CD103⁺ and CD11b^{high} DCs has been described.⁴⁵ The accession number for the data set in Gene Expression Omnibus is GSE17322.

Statistical analysis

The mean and SD of multiple trials were calculated by the program Excel (Microsoft, Redmond, WA, USA). Statistical significance using Student's *t*-test was determined by the programs SlideWrite plus (Advanced Graphics Software, Carlsbad, CA, USA) and InStat 3 (Graph Pad Software Inc., San Diego, CA, USA).

RESULTS

Eosinophil identification in normal lungs

Lung eosinophils and macrophages are difficult to distinguish because lung macrophages express the eosinophil marker Siglec-F,⁴³ and some macrophages express CCR3, another eosinophil marker (Figure 1C). In lung F4/80⁺ macrophage fractions isolated by magnetic microbeads, an I-A⁺CD11c⁺ population was identified as eosinophils based on their expression of CCR3 and Siglec-F,^{46,47} and their high side scatter and low forward scatter (Figure 1A and C). The F4/80⁺CD11c⁺ population contained no eosinophils, but contained macrophages and DCs (Figure 1B). This analysis showed that eosinophils in the resting state were present almost exclusively in the CD11c⁺ fraction. The flow-through F4/80⁺CD11c⁺ fraction also contained no eosinophils, indicating that all resting eosinophils were F4/80⁺. Besides F4/80, CCR3 and Siglec-F, lung eosinophils expressed integrin β_7 , CD11b, Ly-6G (Gr-1) and IL-7R α , but not Ly-6C (Figure 1C, top row). This staining pattern is similar to that of bone marrow-derived eosinophils.¹⁵ F4/80⁺ lung cells contained two additional populations besides eosinophils. They were the CD11c⁺ and high FL1 and FL2 autofluorescence macrophages and the CD11c⁺I-A⁺ DCs (Figure 1A). Lung macrophages were similar in staining to eosinophils in that they were positive for Siglec-F and F4/80, and a macrophage subpopulation is CCR3⁺ (Figure 1C, middle row). However, in contrast to eosinophils, they were negative for integrin β_7 , CD11b, Ly-6G and IL-7R.

The DCs in the F4/80⁺ population were CD11b^{high}, and contained no CD103⁺ DC (Figure 1A). They express similar surface Ag as the CD11b^{high} DC population in the CD11c⁺ lung cell population (Figure 1B). As can be seen in the staining of DCs in the CD11c⁺F4/80⁺ population (Figure 1C, bottom row), DCs were CCR3⁺Gr-1⁺IL-7⁺. In earlier studies, CD11b⁺ DCs have also been shown to express no integrin β_7 and Siglec-F.³⁹ Thus the combination of I-A, CCR3, Siglec-F, integrin β_7 , F4/80, Ly-6G and IL-7R stainings distinguished eosinophils from the macrophage and DC populations in lungs.

Eosinophils in lungs and spleen were phenotypically similar

Whether the homing of resting eosinophils to lungs is a unique feature of mucosal tissues was tested by isolating splenic eosinophils from normal mice (Figure 2). To improve the analysis of control lung and splenic eosinophils, single cell suspensions were depleted of CD11c⁺ cells which have been shown to contain no detectable eosinophils. Enrichment with anti-F4/80 magnetic microbeads yielded highly enriched eosinophil fractions (Figure 2d). Analysis with a large panel of mAbs including lineage markers, costimulation molecules and adhesion molecules showed that lung and splenic eosinophils expressed similar surface Ag. Splenic eosinophils were also characterized by the expression of Siglec-F (Figure 2d), CCR3, Gr-1, F4/80 (Figure 2a), CD11b, integrin β_7 and LPAM-1 ($\alpha_4\beta_7$) (Figure 2b).

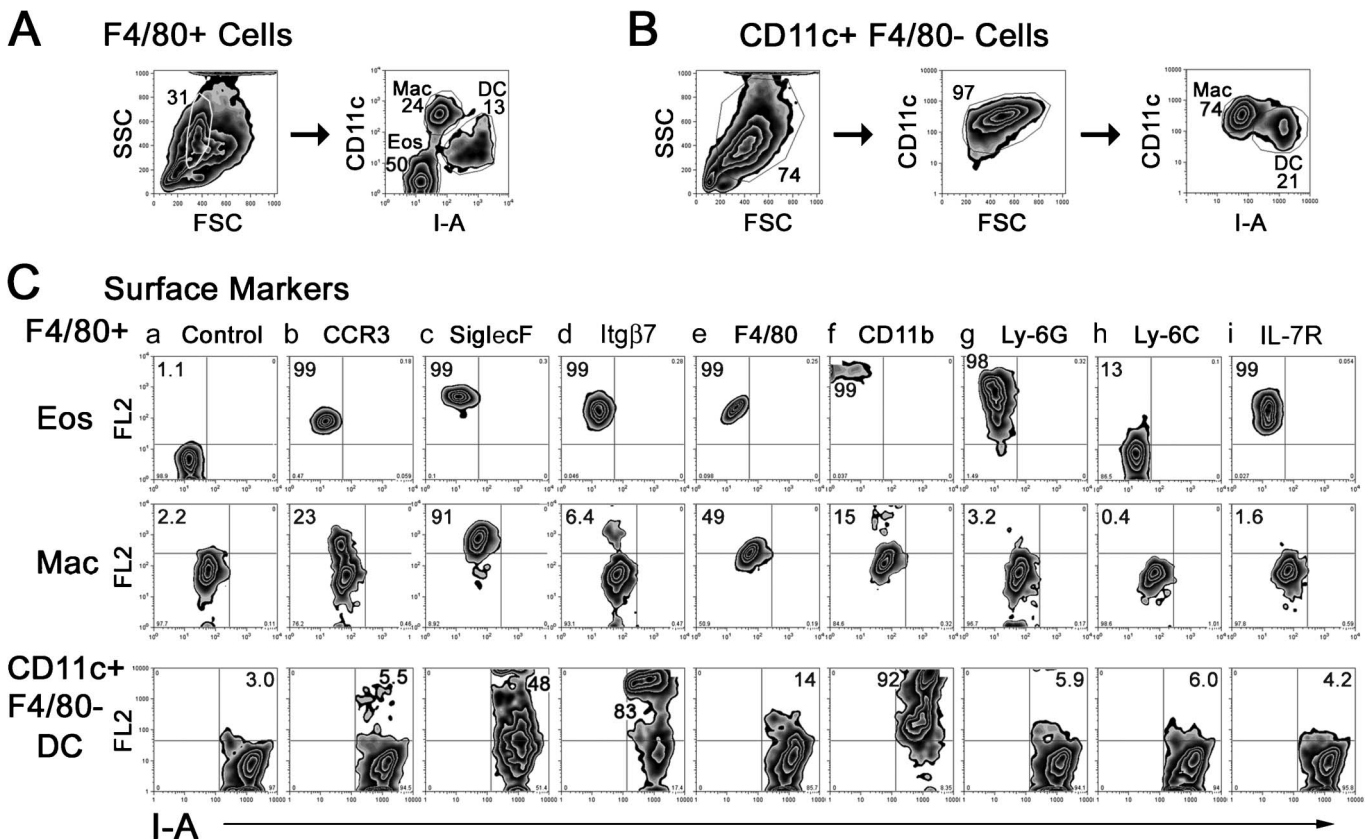


Figure 1 Comparison of lung eosinophil, macrophage and DC surface markers in naive mice. Lung digests were prepared from naive mice as described in the 'Materials and methods' section and stained with FITC-conjugated F4/80 for eosinophil enrichment by anti-FITC conjugated magnetic microbeads. (A) F4/80⁺ cells were resolved into three populations identified as Eos, Mac and DCs. The surface markers of the gated Eos and Mac populations are shown in (C). (B) The F4/80⁺ cells in the flow-through after anti-F4/80 magnetic bead selection were further selected by anti-CD11c-conjugated magnetic beads and stained with mAbs against I-A, CD11c and surface markers shown in (C). The surface markers of the gated DC population are shown in (C), bottom panel. The marker expression of F4/80⁺ and F4/80⁺ macrophages in panels A and B respectively was similar. The results are representative of four independent experiments. The percentages of marker positive cells are shown in the zebra plots. DC, dendritic cell; Eos, eosinophils; mAb, monoclonal antibody; Mac, macrophages.

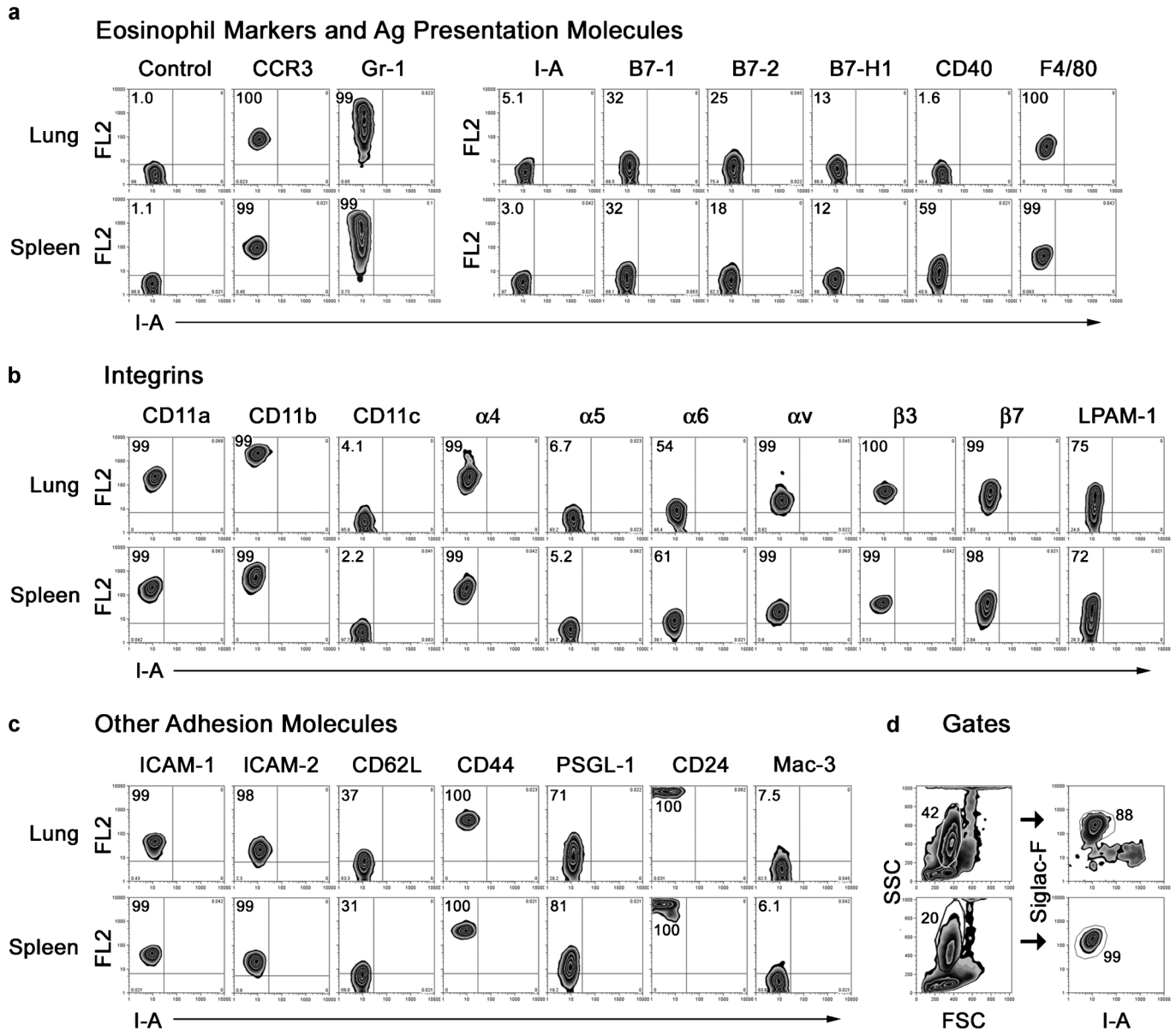


Figure 2 Lung and splenic eosinophil surface costimulation and adhesion molecules in naive mice. Lung and splenic single cell suspensions were cleared of CD11c⁺ DCs and macrophages by anti-CD11c-magnetic beads followed by F4/80 selection as described in Figure 1. The cleared CD11c⁺ cells contained no eosinophils. Live cells gated on eosinophils as shown in (d) were analyzed for costimulation and adhesion molecules shown in (a–c). The experiment has been repeated three times. DC, dendritic cell.

Eosinophils from both lung and spleen expressed low levels of MHC II and the accessory molecules B7-1, B7-2, B7-H1 and CD40 (Figure 2a) which participate in Ag presentation. The capacity to present Ag by these resting eosinophils was directly tested (Figure 3). Strong stimulation of DO11.10 transgenic CD4⁺ T cells by both anti-CD3 mAbs and the T epitope peptide OVA_{323–339} was observed in the presence of sorted lung DCs. However, when sorted eosinophils from normal lungs were used as antigen presenting cells, the stimulation was low. The results suggested that in normal lungs eosinophils played a very minor role as antigen presenting cells.

Lung eosinophils expressed a large number of adhesion molecules including CD11a, integrin- α_4 , - α_6 , - α_v and - β_3 (Figure 2b), and other integrin- and selectin-related molecules ICAM-1, ICAM-2, CD62L, CD44, PSGL-1 and CD24 (Figure 2c). These adhesion molecules are

important for their emigration into lungs and spleen, and for their interactions with the extracellular matrix and with other cell types. Mac-3 (LAMP-2), a myeloid marker, was also expressed by these eosinophils. Among the prominent markers not expressed on normal lung eosinophils were CD11c (Figure 1A), CD103, CXCR4, CCR5, CCR6, CCR7, TLR2, TLR4, MD-2, RP105 and MD-1 (not shown). Small percentages of eosinophils expressed CCR1 (27%) and CXCR3 (11%). Quantitation with calibration beads yielded similar eosinophil numbers in lung and spleen. In three determinations, the mean eosinophils were $(2.03 \pm 0.13) \times 10^5/\text{lung}$ and $(2.05 \pm 0.22) \times 10^5/\text{spleen}$. There were some eosinophils in peripheral blood. Blood eosinophil could be identified easily by staining for CD11b, Siglec-F and CCR3. They were also I-A⁺ (Figure 4A). The average number from four determinations was $(4.2 \pm 0.77) \times 10^4/\text{ml}$ blood. This low number

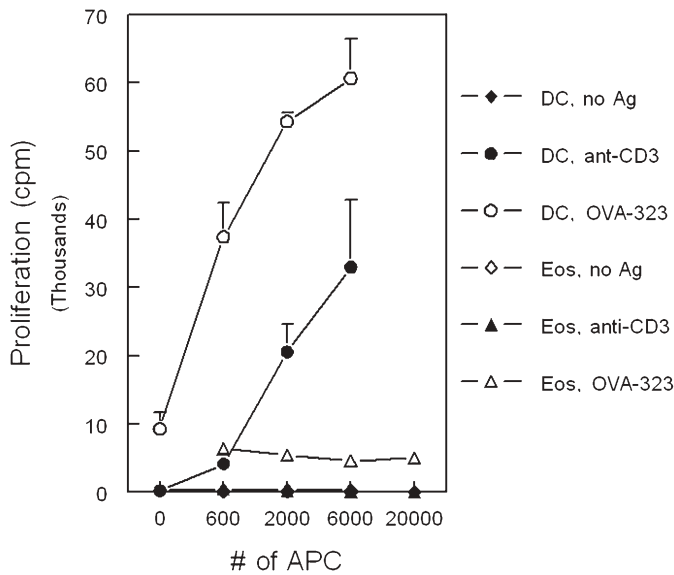


Figure 3 Stimulation of T-cell proliferation by lung eosinophils and DCs. Lung eosinophils and DCs from naive mice were sorted to 98% purity as described in the 'Materials and methods' section and added to cell cultures in the numbers per well as indicated. $CD4^+CD25^-$ splenic T cells were obtained from DO11.10 mice by magnetic microbead depletion as described in the 'Materials and methods' section and used at 1×10^5 /well. OVA₃₂₃₋₃₃₉ peptide and anti-CD3 mAbs were added to a final concentration of 25 μ M and 8 μ g/ml respectively and incubated for 5 d. [^{3} H]-thymidine was added for the last 8 h incubation. The experiment was repeated twice with similar results. APC, antigen presenting cell; DC, dendritic cell; Eos, eosinophils; mAb, monoclonal antibody; OVA, ovalbumin.

indicated that eosinophils from residual blood contributed little to the total resident lung and spleen eosinophil numbers.

Eosinophilic granule staining of lung eosinophils

To further confirm that $I-A^-CD11c^-F4/80^+Siglec-F^+$ cells were eosinophils, total lung cells from naive mice were enriched for eosinophils by anti-F4/80 magnetic microbeads and further purified by FACS to 98% purity by sorting $Siglec-F^+CD11b^{high}I-A^-CD11c^-$ cells. Geimsa–Wright staining of cytospin preparations showed that all cells exhibited the characteristic lobed nuclei and contained eosinophilic granules (Figure 4B-b), thus confirming the $F4/80^+Siglec-F^+I-A^-$ population as eosinophils. Eosinophils from inflamed lungs, induced by an allergic lung inflammation and AHR protocol (Figure 5A), exhibited a similar morphology of lobed nuclei and eosinophilic granule staining (Figure 4B-d). The percentages of eosinophils in naive lungs were small and no eosinophils could easily be found in the total lung single cell suspensions (Figure 4B-a). In inflamed lungs, on the other hand, large percentages of eosinophils could be found in lung digests (Figure 4B-c).

Upregulation of eosinophil surface Ag in inflamed lungs

The studies on eosinophils in uninflamed lungs provided background surface Ag and secretory product expression levels against which increases in gene and protein expression by activated eosinophils could be compared. Activated lung eosinophils were isolated from mice immunized by intratracheal DC priming followed by intratracheal challenges with OVA as Ag.³⁹ This immunization protocol (Figure 5A) induced significant increases in airway resistance (Figure 5B), a critical piece of information not previously shown. Marked eosinophilia (Figure 5C and D) and lung inflammation

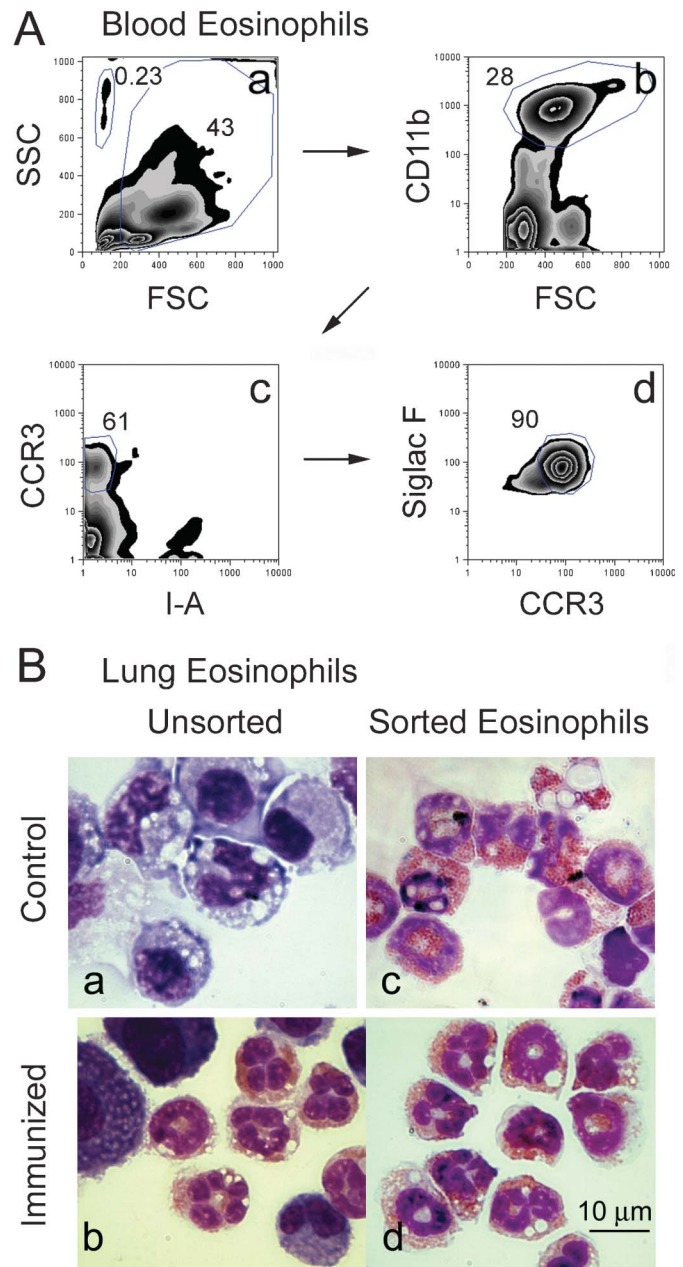


Figure 4 Blood and lung eosinophils. (A) Quantitation of blood eosinophils. Blood leukocytes after red blood cell lysis were stained by 7-amino-actinomycin D (7-AAD) and mAbs against I-A, CCR3, Siglec-F and CD11b and live cells were successively gated and analyzed as shown in the figure. Eosinophil numbers in (d) were quantitated with Caltag calibration beads. Four such determinations of blood cells from multiple mice in each experiment have been performed. (B) Lung eosinophil staining. Cells from total lung digests of naive mice (a) were selected by anti-F4/80 followed by magnetic microbeads, stained with fluorochrome-conjugated mAbs against I-A, Siglec-F, CD11b, CCR3 and GR-1, and sorted (c). Total lung cells from mice immunized with OVA (b) were similarly stained and sorted (d) without preselection with F4/80 magnetic microbead. Cytospin preparations of total lung cells (a, b) and sorted eosinophils (c, d) were stained with Diff-Quik. This experiment has been repeated twice. mAb, monoclonal antibody; OVA, ovalbumin.

(Figure 5F), and increased serum Ag-specific IgE (Figure 5E) were also observed. Eosinophils from inflamed lungs expressed the same markers as those from control unimmunized mice, and were positive for

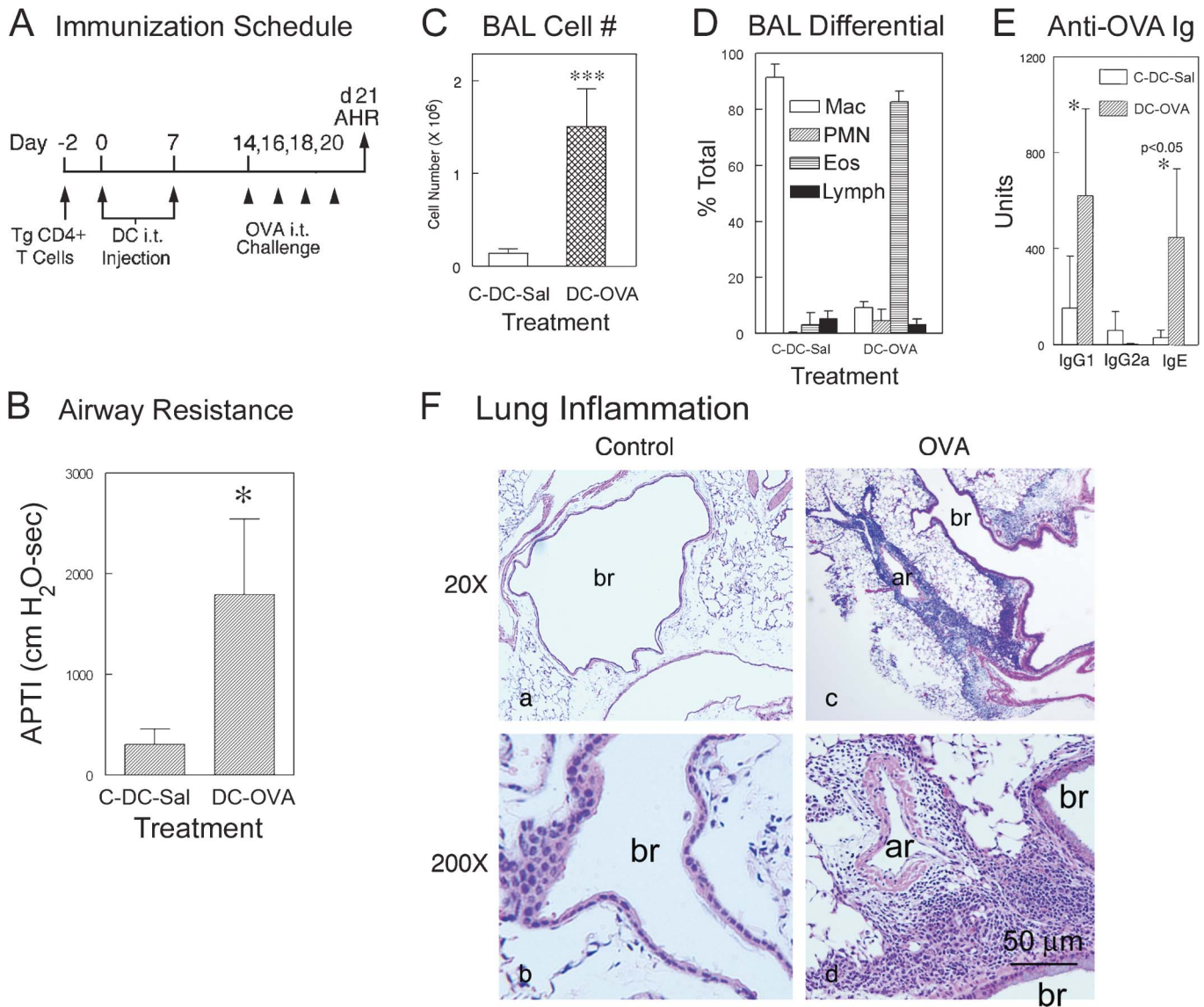


Figure 5 Allergic airway inflammation in mice. BALB/c mice were immunized as described in the 'Materials and methods' section and in (A). (B) Airway resistance was measured and expressed as APTI. A total of eight mice were used per group. Bronchoalveolar lavage cell number (C) and differential (D) were determined as described.³⁹ OVA-specific IgG were determined by ELISA and expressed as units against a reference standard (E). In (F), hematoxylin and eosin staining of control and immunized lung sections are shown in $\times 2$ magnification. The experiments have been repeated three times. Bars show SD and significance of differences between groups are shown as: * $P < 0.05$; *** $P < 0.005$. AHR, airway hyper-responsiveness; APTI, airway pressure time index; ar, arterioles; br, bronchioles; Eos, eosinophils; Ig, immunoglobulin; Mac, macrophages; OVA, ovalbumin; PMN, neutrophils.

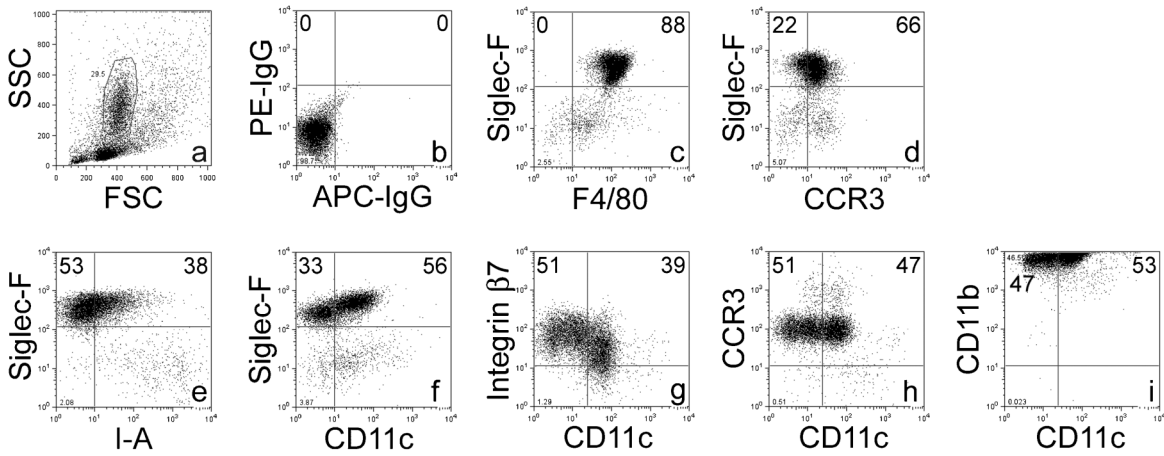
F4/80, Siglec-F, CCR3, integrin β_7 and CD11b (Figure 6A). In contrast to resting eosinophils which were I-A⁻, a substantial percentage of eosinophils in inflamed lungs expressed low to intermediate amounts of I-A (Figure 6A-e), which explains the reported Ag presentation capability of these eosinophils.³ A large subpopulation of eosinophils also upregulated its surface CD11c expression (Figure 6A-f). The CD11c⁺ and CD11c⁻ populations were similar in their expression of the eosinophil markers CCR3 and CD11b (Figure 6A-h, i), but their Siglec-F and integrin β_7 expression levels were different (Figure 6A-f, g), with a higher Siglec-F and a lower integrin β_7 expression for the CD11c⁺ population. Compared to eosinophils in homeostasis, eosinophils from inflamed lungs acquired an activated phenotype by expressing higher B7-1 (38% versus 86% positive; 3–5 experiments; $P < 0.001$). Two eosinophil populations from inflamed lungs expressing different

levels of CD11c, integrin β_7 , LPAM-1 and PSGL-1 were found (Figure 6B-j, p, q, v). The difference in LPAM-1 (integrin $\alpha_4\beta_7$) expression in the two populations was likely due to differences in their integrin β_7 expression. The expression levels of other eosinophil markers, costimulation molecules, and adhesion molecules were similar between lung eosinophils in homeostasis and in inflammation.

Lung eosinophil chemokine mRNA expression in homeostasis and inflammation

Murine eosinophil production of chemokines has not been explored systemically. In this study, eosinophil chemokine production was first studied by measuring chemokine mRNA expression in FACS-purified eosinophils by a panel of chemokine primers used in myeloid cell studies.⁴⁵ Eosinophils from both naive and immunized mice expressed

A I-A and CD11c Expression



B Costimulation and Adhesion Molecules

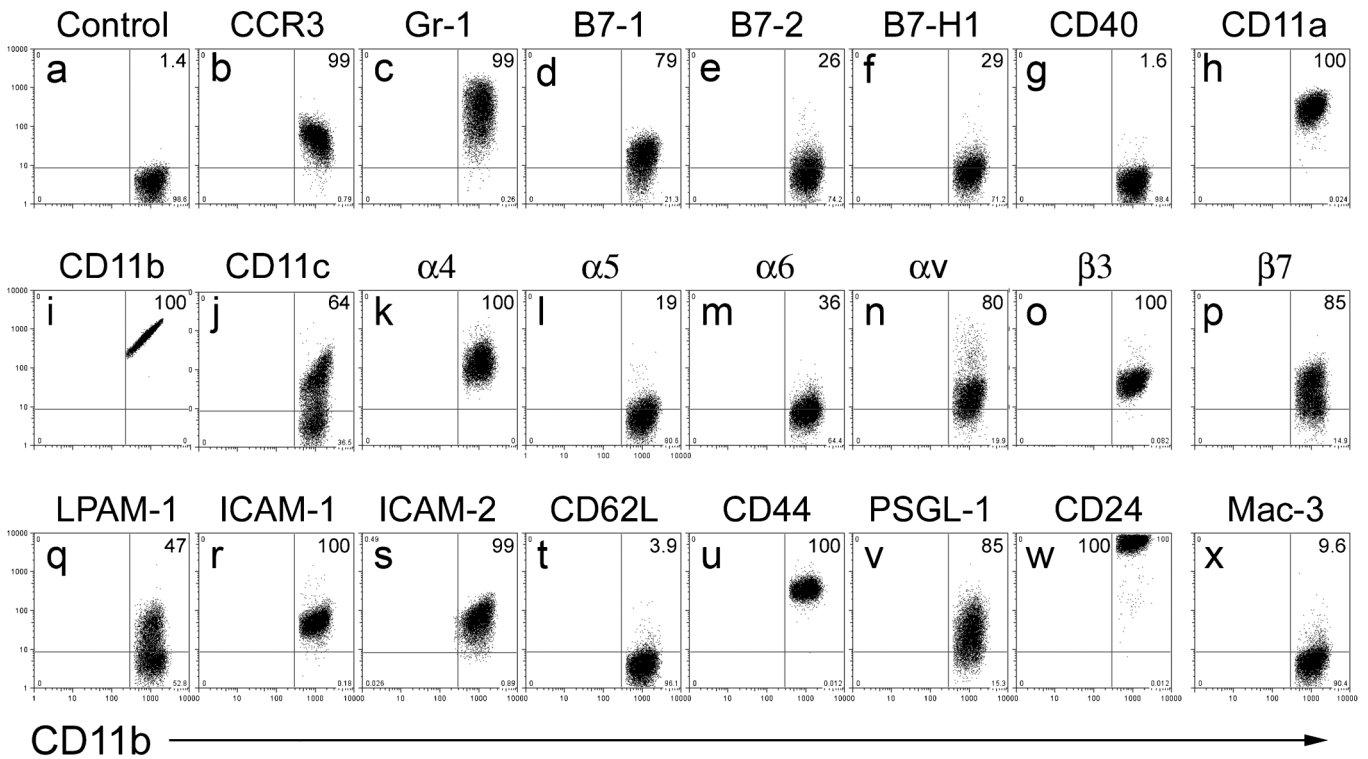


Figure 6 Surface marker, costimulation molecule, and adhesion molecule expression on eosinophils from immunized mice. Mice were immunized as in Figure 5A and eosinophils gated as in (A-a) and 7-AAD⁻ live cells were analyzed for marker expression shown in (A). These cells were further gated on Siglec-F⁺ and CD11b^{high} cells and assessed for their expression of costimulation and adhesion molecules in (B). A representative experiment of three trials is shown. APC, antigen presenting cell; IgG, immunoglobulin G.

a large number of chemokine mRNA species ranging in relative intensities from 0.01×10^{-2} to 56×10^{-2} (Table 1). The highest levels of chemokine mRNA expression in both resting and inflammatory eosinophils were C10, MIP-2, MIP-1 α , MIP-1 β , MIP-1 γ and Epstein-Barr virus-induced molecule-1 ligand chemokine (ELC) (Figure 7A; Table 1). There were only slight changes in relative mRNA levels of these latter chemokines between the eosinophils in the resting and

activated states. However, mRNA levels of a number of C-X-C and C-C chemokines including KC, IP-10, TCA-3, MCP-1, RANTES, MCP-2, MCP-3, eotaxin-2 and mucosa-associated epithelial chemokine were increased in activated eosinophils from inflamed lungs (Figure 7A; Table 1). Though significant changes were found in other chemokine mRNA, their absolute levels were low and thus were less likely to contribute significantly to the cytokine milieu.

Table 1 Chemokine mRNA expression in lung eosinophils during homeostasis and inflammation^a

Chemokine	mRNA expression		Inflammatory/Control
	(chemokine/ β -actin $\times 10^2$)		
	Control eosinophils	Inflammatory eosinophils	
CXCL1/KC	0.67	2.2	3.3
CXCL2/MIP-2	37.2	12.7	0.34
CXCL4/PF-4	0.051	0.69	13.5
CXCL5/LIX	0.02	0.24	12.0
CXCL8/IL-8	0.012	0.70	58.3
CXCL10/IP-10	0.15	0.64	4.4
CXCL12/SDF-1 α	0.002	0.007	3.5
CXCL12/SDF-1 β	0.001	0.004	4.0
CCL1/TCA-3	0.031	0.12	3.9
CCL2/MCP-1	0.11	1.14	10.4
CCL3/MIP-1 α	2.69	2.25	0.84
CCL4/MIP-1 β	4.5	7.3	1.6
CCL5/RANTES	0.18	1.39	7.5
CCL6/C10	55.7	32.3	0.58
CCL7/MCP-3	0.11	0.96	8.7
CCL8/MCP-2	0.21	3.04	14.8
CCL9/10 /MIP-1 γ	1.05	0.93	0.88
CCL11/Eotaxin	0.04	0.16	4.6
CCL12/MCP-5	0.095	0.41	4.4
CCL17/TARC	0.18	0.41	2.3
CCL19/ELC	0.55	0.88	1.6
CCL24/Eotaxin-2	0.01	0.25	22
CCL28/MEC	0.052	0.13	2.4

^a Total RNA were extracted from FACS-purified lung eosinophils as described⁴⁵ and used in real-time PCR determination of mRNA levels. The values were normalized against β -actin mRNA. The experiments were performed 2–3 times and representative values of triplicates are shown. The SDs were within 5% of the mean and not shown. High expression levels are shown in bold.

Chemokine production by lung eosinophils

Eosinophil chemokine production was determined first by intracellular chemokine staining. Significant chemokine staining in resting or inflammatory eosinophils was found by staining with a panel of anti-chemokine Abs (Figure 7B and C). In lungs, the mean fluorescence intensities (MFI) of chemokine-producing eosinophils correlated with the chemokine mRNA levels with a ranking of C10>MIP-2>eotaxin>MIP-1 α >MIP-1 γ >MCP5. Only eotaxin production was higher than predicted based on mRNA measurements (Table 1; Figure 7B). In activated eosinophils, C10 and MIP-1 γ were produced at the highest levels by resting eosinophils (Figure 7C-d,e) and lower levels of MIP-2, MIP-1 α , eotaxin and MCP-5 were also detected (Figure 7C-f–7C-i). Again, the MFI of chemokine-producing eosinophils correlated with chemokine mRNA levels in general. However, MIP-1 γ production was much higher and MIP-2 production was much lower than the mRNA level would indicate. The lower chemokine MFI in eosinophils from inflamed lung compared to unimmunized lungs was due to the higher gain adjustment for the inflamed eosinophil staining (Figure 7C-c). A small population of eosinophils was also found to have high autofluorescence in inflamed lung eosinophils (Figure 7C-c).

The amounts of changes in eosinophil intracellular chemokines upon *in vitro* culture were also examined by flow cytometry. When incubated in medium for 4 h, the intracellular chemokine staining for C10, MIP-1 γ and other chemokines were much reduced (cf. eosinophil chemokine MFI between upper and lower panels in Figure 7B

and C). This could be due to the eosinophil release of chemokines by piecemeal degranulation.⁴⁸ The intensities were also scattered over a wide range after *in vitro* incubation. However, MIP-2 in activated eosinophils was an exception to this decrease in intracellular chemokine (Figure 7C-f). The slight MFI increase in MIP-2 may represent the equilibrium between chemokine release and *de novo* chemokine synthesis by eosinophils in culture. It should be noted that very high levels of MIP-2 mRNA (relative mRNA level = 12.7×10^{-2}) was detected in activated eosinophils and thus rapid protein synthesis could occur in culture.

Without *in vitro* incubation, eosinophils in inflamed lungs could be resolved into two populations based on Siglec-F expression (Figure 7C-b). There were small differences in the amounts of intracellular chemokines between the two populations (Figure 7C-c–7C-i, upper panels). The Siglec-F-high population was also found to be CD11c⁺ (Figure 6A-f) and thus represented a more activated population. After *in vitro* incubation for 4 h, the percentage of eosinophils in population I with higher Siglec-F and I-A staining (Figure 7C-b,d,e) was reduced, as was the intracellular chemokine (Figure 7C-b,d,e,i; cf. upper and lower panels). Population II was also reduced and a new population with low Siglec-F staining was found (population III, Figure 7D-b). Populations II and III exhibited very high autofluorescence and intracellular chemokines were difficult to detect (not shown). These data suggested that freshly explanted eosinophils released intracellular chemokines concomitant with significant phenotypic changes including downregulation of surface markers and changes in autofluorescence properties when incubated in medium.

Chemokine production by eosinophils was further quantitated by ELISA. C10 in the supernatants of sorted eosinophils was the highest in concentration, and there was a twofold increase in C10 production by eosinophils in inflamed lungs (Figure 7D). There were lower levels of MIP-1 α , MIP-1 γ and MIP-2 compared to C10 produced by eosinophils and significant increases were found in supernatant of lung eosinophils from OVA-immunized compared to naive mice. Eotaxin and MCP-5 found by intracellular chemokine staining were not detected in the supernatants (not shown) and part of the reason may be the utilization of the released chemokines by the eosinophils.

Eosinophils are the major producers of C10 and MIP-1 γ in allergic lung inflammation

In allergic airway inflammation, lung CD11b^{high} DCs and macrophages have been shown to be significant producers of C10, MIP-1 α and other chemokines.⁴⁵ Because eosinophils were found to produce high levels of C10 and MIP-1 γ *in vitro*, their contribution of chemokine production compared to other lung cell type in inflammation was examined by confocal microscopy (Figure 8). The results indicated that eosinophils shown as MBP⁺ cells were the major producers of C10 and MIP-1 γ (Figure 8a and b; arrows). There were small numbers of I-A⁺ macrophage-like and I-A⁺ DC-like C10 and MIP-1 γ producers (Figure 8a and b, arrowheads). Lower amounts of MIP-1 α , eotaxin and MCP-5 were produced by eosinophils (Figure 8c–e; arrows) and there were other MBP⁺ cell types that stained strongly for the three chemokines (Figure 8c–e, arrowheads). Though found to express mRNA for ELC, eosinophils stained very weakly for the chemokine protein (Figure 8f, arrows; Table 1). The predominant ELC-staining cells were CD11b⁺ MBP⁺, and were likely to be macrophages. The results showed that eosinophils play significant roles in mediating the influx of CCR1-, CCR2-, CCR3- and CCR5-bearing cells during allergic airway inflammation.

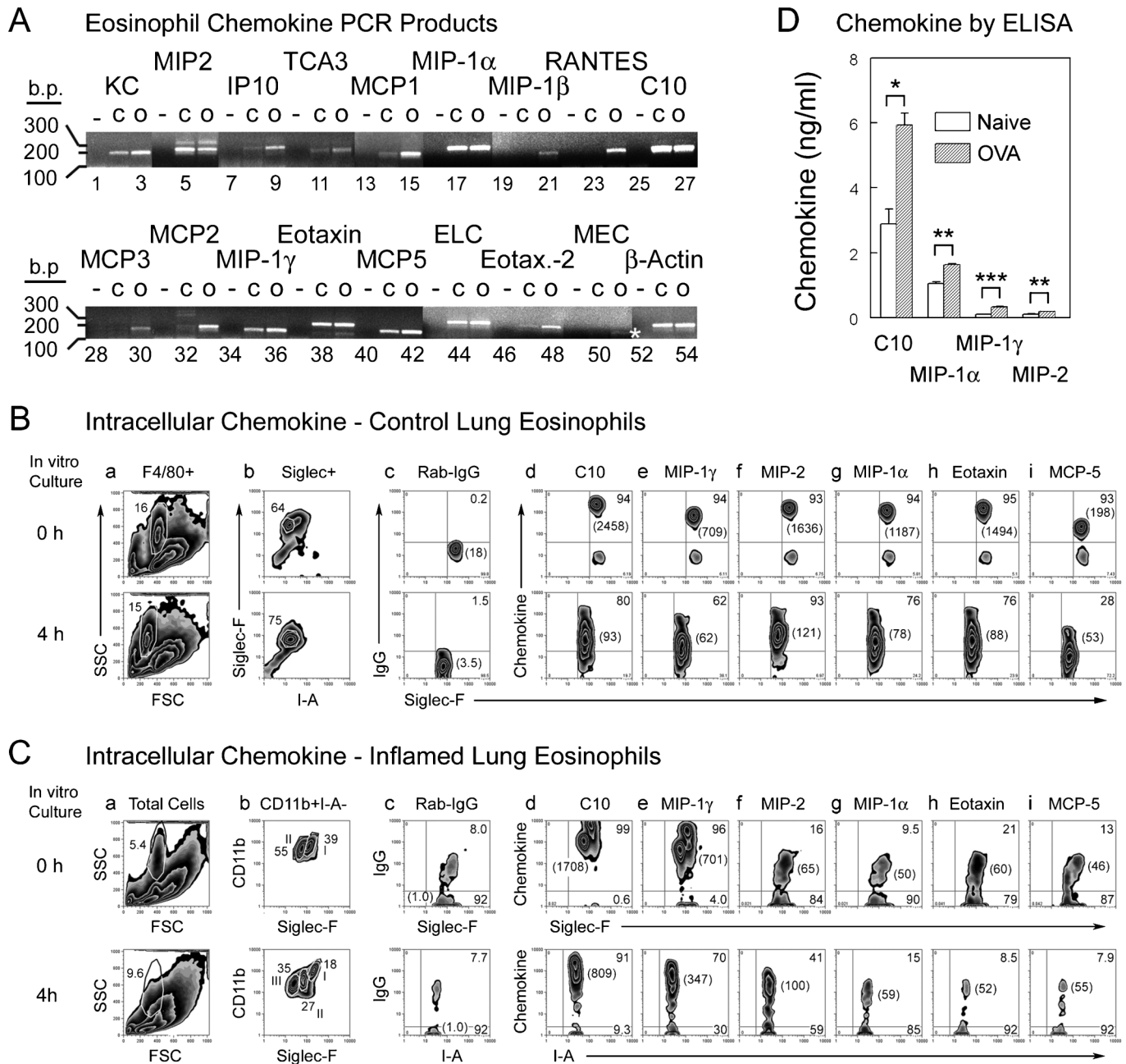


Figure 7 Expression of chemokine mRNA and protein by eosinophils from naive and OVA-immunized mice. **(A)** Eosinophils from naive or OVA-immunized mice were sorted to 98% purity as described in the 'Materials and methods' section and lysed in RNeasy for total RNA preparation. cDNA were prepared and amplified by real-time PCR with primers described previously.⁴⁵ The PCR products at the end of 40 cycles were run on a 1.8% agarose gel to confirm amplification of the correct product. The lanes were: -, no cDNA; C, eosinophil cDNA from control naive mice; O, eosinophil cDNA from OVA-immunized mice. **(B, C)** Intracellular chemokine expression in eosinophils from naive mice **(B)** or OVA-immunized mice **(C)**. Intracellular chemokine staining was performed as described in the 'Materials and methods' section. Eosinophils were enriched by anti-F4/80 mAbs plus magnetic microbeads for control lung cells **(B)** but not for lung cells from OVA-immunized mice **(C)**. Cells were either stained for eosinophil markers followed by fixation immediately after cell isolation (0 h) or incubated in medium with monensin for 4 h (4 h) before staining and fixation. **(D)** Chemokine production by sorted eosinophils from naive mice or OVA-immunized mice. Eosinophils were sorted as described in **(A)** and cultured at $0.5 \times 10^6/\text{ml}$ as described in Materials and methods. Supernatants were assayed for chemokines by ELISA. Error bars show the standard deviation of triplicates. Significance in the differences of chemokine production are shown as: * $P < 0.05$; ** $P < 0.01$; *** $P < 0.005$. **(A–C)** were repeated three times and **(D)** twice. b.p., base pairs; ELC, Epstein–Barr virus-induced molecule-1 ligand chemokine; IgG, immunoglobulin G; mAb, monoclonal antibody; MCP, monocyte chemoattractant protein; MEC, mucosa-associated epithelial chemokine; MIP, macrophage inflammatory protein; OVA, ovalbumin; TCA, thymus-derived chemotactic agent.

Preferential CD11b^{high} DC accumulation in allergic airway inflammation and CCR1 expression

The functional consequence of the high CCR1 ligand production by eosinophils during allergic airway inflammation was examined by

assaying for chemokine receptor expression by major infiltration cell populations. A major infiltrating leukocyte type was identified as the CD11b^{high} DC, one of the two major lung DC types. Its accumulation in inflamed lungs increased by 17-fold compared to control lungs

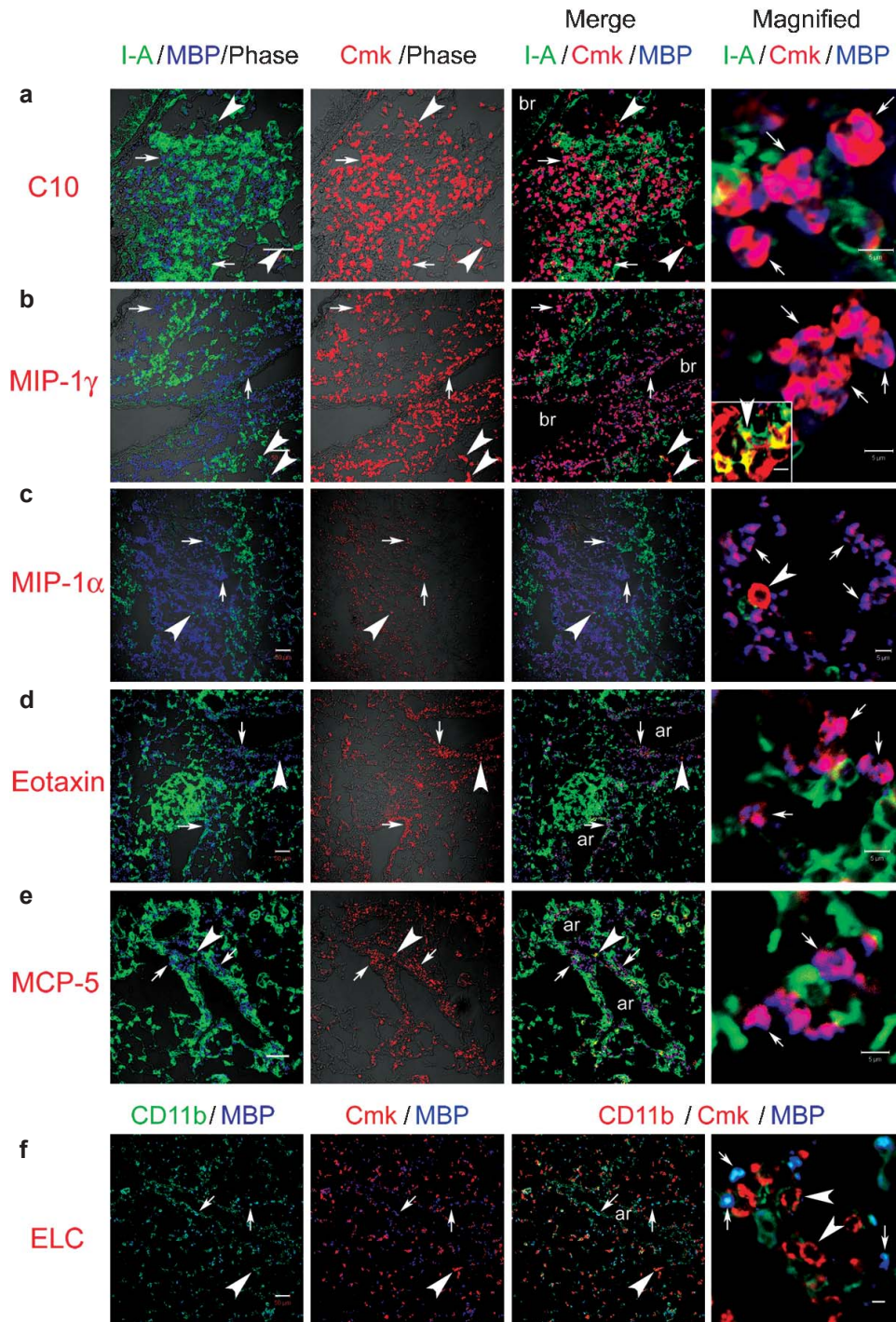


Figure 8 Eosinophil production of chemokines in mice with allergic lung inflammation. Mice were immunized as shown in Figure 5A, fixed in paraformaldehyde, equilibrated in sucrose and sectioned. In (a–e), sections were stained with mouse anti-I-A-FITC mAbs, rabbit antichemokine Abs, and rat anti-mMBP mAbs followed by anti-FITC-Alexa dye 488, antirabbit IgG-Alexa dye 555, and antirat IgG-Alexa dye 647. In (f), anti-CD11b-FITC instead of anti-I-A-FITC was used. Confocal microscopy was performed as described.⁴³ Arrows indicate eosinophils and arrowheads show non-eosinophil cells. Bars in the left column represent 50 μ m and those in the right column are 5 μ m. The staining has been repeated three times on different tissue blocks. Ab, antibody; ar, arterioles; br, bronchioles; ELC, Epstein–Barr virus-induced molecule-1 ligand chemokine; IgG, immunoglobulin G; mAb, monoclonal antibody; MCP, monocyte chemoattractant protein; MIP, macrophage inflammatory protein; mMBP, murine major basic protein.

(Figure 9a). On the other hand, lung CD103⁺ DC accumulation increased by less than twofold. Lung DC mRNA expression profile of chemokine receptors which respond to the major chemokines produced by eosinophils was examined. Affymetrix microarray analysis

showed that lung CD11b^{high} and CD103⁺ DCs expressed significant levels of CCR1, CCR2 and CCR5 mRNA (Figure 9b). CCR2 expression did not correlate with DC influx during inflammation because despite expressing higher CCR2 mRNA by CD103⁺ DCs compared with

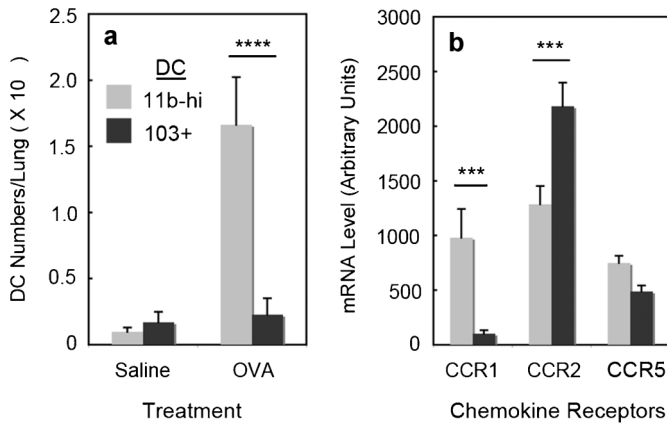


Figure 9 Lung DC accumulation during allergic airway inflammation and chemokine receptor expression. **(a)** Lung CD11b^{high} DCs (gray bars) and CD103⁺ DCs (black bars) numbers from control saline-injected mice and OVA-immunized mice were determined by flow cytometry as described in the 'Materials and methods' section. The mean and SD from 11 mice in each group are shown. **(b)** Chemokine receptor mRNA levels of CD11b^{high} DCs and CD103⁺ DCs were determined by Affymetrix microarrays and the mean and SD of three determinations are shown. ****P*<0.005; *****P*<0.001. DC, dendritic cell; OVA, ovalbumin.

CD11b^{high} DCs, the increase in CD103⁺ DC numbers was small. CCR1 expression, however, correlated well with CD11b^{high} DC influx which increased markedly during lung inflammation and CD11b^{high} DC CCR1 mRNA expression is almost 10 times higher than that of CD103⁺ DCs. These results implicate that the potent production of the CCR1 ligands C10 and MIP-1 γ by eosinophils may be critical for CD11b^{high} DC lung infiltration during allergic airway inflammation.

DISCUSSION

Mouse tissue eosinophils in the homeostatic state are difficult to study because of their low numbers in normal tissues. However, it is important to establish a phenotypic and functional baseline for these cells for further understanding eosinophil activation during allergic inflammation and infections. In addition, eosinophils interact with other tissue cell types to maintain cellular functions including maturation, activation and migration.^{1,49} In this report, resting eosinophils were enriched by magnetic bead selection which renders them amenable to phenotypic and functional studies. By a combination of surface markers, eosinophils can be readily distinguished from other myeloid cell types in lungs. Lung macrophages have an unusual phenotype in that they express the eosinophil marker Siglec-F (Figure 1C⁴³), but are distinguishable from eosinophils by their high autofluorescence in the green and red channels, and their lack of CD11b and GR-1 expression (Figure 1C). Eosinophils from lungs and spleen expressed similar adhesion molecules, suggesting that they interacted with similar ligands for homing and emigration in the two organs. Resting eosinophils express a wide variety of chemokine mRNA and produce large amounts of chemokines. Though they occur in small numbers in normal tissues, eosinophil production may have significant local functions.

Compared to eosinophils from naive mice, lung eosinophils in OVA-immunized mice exhibited an activated phenotype. The latter upregulated MHC class II, B7-1 and CD11c surface expression (Figures 2 and 6), increased KC, PF-4, LIX, IL-8, IP-10, MCP-1, MIP-1 β , RANTES, MCP-3, eotaxin, MCP-5, eotaxin-2 and mucosa-associated epithelial chemokine mRNA expression (Figure 7A; Table 1), and produced more C10, MIP-1 α , MIP-1 γ and MIP-2 protein

(Figure 7D). Because eosinophils studied in mice are predominantly induced by inflammation, cytokines or transgenes,^{3,15,34–39} the eosinophil phenotypes reported in the literature are predominantly those of activated eosinophils.

Lung and spleen eosinophils express the costimulation molecules B7-1 and B7-2 but few MHC class II molecules in homeostasis (Figure 2). The failure of homeostatic eosinophils to induce appreciably DO11.10 T-cell antigen receptor transgenic T-cell proliferation (Figure 3) and the small numbers of eosinophils in naive mice suggest that eosinophils are an insignificant antigen presenting cell type for T-cell priming, as shown earlier.⁵⁰ However, eosinophils from inflamed lungs upregulate MHC class II substantially (Figure 6A-e) along with B7-1 upregulation. That may partly account for their ability to present Ag in an immunized mouse.³ Because of the large number of eosinophils in mouse lungs after repeated antigenic challenges (Figure 5D), and their ability to migrate to the lung-draining lymph nodes, the contribution of eosinophils in Ag presentation can be highly significant. The emergence of a CD11c⁺ lung eosinophil population with slightly different Siglec-F and integrin β_7 expression levels (Figure 6A-f,g) in inflamed mouse lung is of interest. This CD11c⁺ population produced more C10 and MIP-1 γ (Figure 7D). However, as a CD11c⁺ population, eosinophils are readily distinguishable from lung DCs and macrophages because of their much lower CD11c expression.⁴³ CD11c⁺ eosinophils with an activated phenotype have been found in the corticomedullary region of the thymus in young mice.⁵¹ Similar to their thymic counterparts, lung CD11c⁺ eosinophils in inflamed lungs represent an activated population as well.

Eosinophils are important producers of chemokines. Human eosinophils have been shown to produce a number of CXC and CC chemokines including CXCL1/GRO- α , CXCL5/ENA-78, CXCL9/MIG, CXCL10/IP-10, CCL2/MCP-1, CCL5/RANTES and CCL18/PARC.^{33,52–57} In mice, eosinophils produce CCL3/MIP-1 α and CCL5/RANTES in response to virus infections⁵⁸ and elicit CCR4 ligands CCL17/TARC and CCL22/MDC which play key roles in allergic airway inflammation.²⁷ In this report, we showed that eosinophils are proficient in chemokine production (Figure 7A; Table 1). The most prominent chemokine mRNA in both resting and activated eosinophils were KC, MIP-2, IP-10, MCP-1, MIP-1 α , MIP-1 β , RANTES, MCP-3, MCP-2, C10, MIP-1 γ and MCP-5. However, chemokine production seemed to be translationally controlled. Intracellular staining of eosinophils could only confirm the high protein production of several chemokines, especially in activated eosinophils (Figure 7B and C). In control mice, C10, MIP-2, MIP-1 α , eotaxin and MIP-1 γ were produced at the highest levels. However, after immunization, C10 and MIP-1 γ production was found to be the highest in activated eosinophils, followed by MIP-2, MIP-1 α , eotaxin and MCP-5. ELISA assays confirmed the high production of C10 and MIP-1 α production, but detected less MIP-1 γ and MIP-2. The discrepancy between intracellular staining and ELISA results may be due to the reutilization of chemokines by eosinophils, or the lability of the chemokine during the overnight culture. It is also noteworthy that more chemokines were produced by activated eosinophils, although their mRNA levels of several chemokines were the same or lower than those in resting eosinophils (cf. Table 1 and Figure 7). A caveat in the comparison of chemokine mRNA expression between resting and activated eosinophils is that the level of the house-keeping gene β -actin may be increased in activated cells, which will result in an underestimation of chemokine mRNA levels in activated cells. This possibility can also explain the decrease in mRNA levels of some chemokine species such as MIP-2 and C10 in activated eosinophils.

Our earlier report showed that lung macrophages and CD11b^{high} DCs were high producers of C10 and MIP-1 γ in mice with allergic airway inflammation.⁴⁵ It is remarkable that eosinophils were found to be a much higher producer of these two chemokines in inflamed lungs than macrophages and DCs (Figure 8a and b). The high intensities of chemokine staining in eosinophils combined with the high eosinophil numbers in allergic diseases and certain pathogen infections indicated that very high levels of C10 and MIP-1 γ were produced in inflamed lungs. It is thus not surprising that C10 or MIP-1 γ exert dominant effects as a CCR1 ligand in diseases with severe eosinophilia.^{59,60} In addition, the data showed that eosinophils are the principal cell type in C10 and MIP-1 γ production in allergic airway inflammation. The CCR1-binding function of C10 and MIP-1 γ are also interesting. These two chemokines along with human CCL15/MIP-1 δ /HCC-2 and CCL23/CK β 8 belong to the NC6 chemokine subfamily with a unique extended inhibitory domain upstream of the chemokine body. This full-length chemokine form binds weakly to CCR1. However, after proteolytic cleavage of this upstream domain by inflammatory proteases, CCR1 stimulatory potencies by NC6 chemokines increased by 10- to 100-fold.⁶¹ The high production of the CCR1 ligands C10, MIP-1 γ and MIP-1 α by eosinophils indicates that eosinophils are a major cell type in attracting CCR1⁺ cells to the inflammatory site. CCR1⁺ cells have been shown to play crucial roles in exacerbating pulmonary inflammatory responses induced by respiratory syncytial virus (RSV), pneumonia virus of mice and *Aspergillus fumigatus* infections,^{62–64} by allergens,⁶⁵ and by transgenic IL-13 expression.⁶⁰ C10 among the many CCR1 ligands plays a particularly important role in pulmonary *Aspergillus* infection and in transgenic IL-13-induced inflammation as shown by the marked abrogation of induced lung pathologies and malfunctions by anti-C10 Abs.^{59,60} Because CCR1 is expressed by many cell types including natural killer cells, T cells, macrophages, DCs, basophils, eosinophils and neutrophils,⁶⁶ lung eosinophil production of high levels of C10, MIP-1 γ and MIP-1 α is highly proinflammatory and will in all likelihood contribute significantly to the infiltration of a wide variety of inflammatory cells. C10 and MIP-1 γ may be responsible for the selective influx of CD11b^{high} DCs into lungs during allergic lung inflammation. CD11b^{high} DCs, but not CD103⁺ DCs, were found to increase up to 17-fold in inflamed lungs⁴³ and their CCR1 mRNA expression is 10 times higher than that of CD103⁺ DCs (Figure 9).

Eosinophils are important in producing CCR5 ligands. They produce large amounts of MIP-1 α protein (Figures 7B–D and 8c) and express high levels of mRNA for MIP-1 β , RANTES and MCP-2. Thus eosinophils are important in the recruitment of CCR5⁺ cells during lung inflammation with eosinophil involvement. CCR5⁺ cells which comprise lymphocytes, DCs and macrophages⁶⁶ are important in lung inflammation. In CCR5^{–/–} mice, lung inflammation induced by *A. fumigatus* infection and IL-13 transgenic expression is markedly reduced compared to wild-type mice.^{67,68} Blockade or deletion of MIP-1 α which binds both CCR1 and CCR5, however, yielded complex result in RSV infection. Though the overall lung infiltrating T-cell numbers are reduced, more RSV-specific proinflammatory T cells were recruited to the lungs along with increased weight loss and illness.⁶⁹ CCR5 function is also linked to childhood asthma.⁷⁰ Individuals homozygous for a non-functioning CCR5 deletion allele (CCR5 Δ 32) exhibit a reduced risk of childhood asthma. It will be of interest to examine the importance of eosinophil chemokine production in asthma and pathogenic infections. High levels of eotaxin, and MCP-5 have also been detected in eosinophils during allergic lung inflammation (Figure 8). The production of eotaxin by eosinophils

is interesting since it is one of the chemokines whose mRNA levels are elevated in eosinophils during lung inflammation and it is the major chemokine for inducing eosinophil influx by binding to CCR3. Thus eotaxin acts as an autocrine in allergic lung inflammation. Eotaxin, along with the eosinophil-derived CCR4 ligands TARC and MDC,²⁷ is also important for the recruitment of Th2 cells in allergic airway inflammation.⁷¹ The production of some chemokines by eosinophils does not correspond to their mRNA levels in the cells. An example is CCL19/ELC (Figure 8f). Very low levels of ELC were found in eosinophils in inflamed lungs although eosinophil ELC mRNA levels were relatively high (Table 1). The major ELC producer bears the phenotype of lung macrophages. This discrepancy in mRNA level and chemokine production can be due to either translational regulation or rapid secretion of the chemokine.

This report described the phenotype and function of tissue eosinophils in homeostasis. Using these eosinophils for baseline mRNA and protein measurements, the induction of chemokine mRNA expression and increases in chemokine production and surface activation marker expression during allergic airway inflammation in eosinophils were shown. Furthermore, real-time PCR and immunofluorescence staining have identified eosinophils as a major cell type in chemokine production during allergic airway inflammation. These studies indicate that a major function of eosinophils is the recruitment of leukocytes of various lineages to the inflammatory site.

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- 1 Blanchard C, Rothenberg ME. Biology of the eosinophil. *Adv Immunol* 2009; **101**: 81–121.
- 2 Rothenberg ME, Hogan SP. The eosinophil. *Annu Rev Immunol* 2006; **24**: 147–174.
- 3 Shi HZ, Humbles A, Gerard C, Jin Z, Weller PF. Lymph node trafficking and antigen presentation by endobronchial eosinophils. *J Clin Invest* 2000; **105**: 945–953.
- 4 Kay AB. Allergy and allergic diseases. First of two parts. *N Engl J Med* 2001; **344**: 30–37.
- 5 Kay AB. Allergy and allergic diseases. Second of two parts. *N Engl J Med* 2001; **344**: 109–113.
- 6 Ellis A. The pathological anatomy of bronchial asthma. *Am J Med Sci* 1908; **136**: 407–429.
- 7 Bousquet J, Chanez P, Lacoste JY, Barneon G, Ghavanian N, Enander I et al. Eosinophilic inflammation in asthma. *N Engl J Med* 1990; **323**: 1033–1039.
- 8 Jacobsen EA, Ochkur SI, Lee NA, Lee JJ. Eosinophils and asthma. *Curr Allergy Asthma Rep* 2007; **7**: 18–26.
- 9 Kay AB. The role of eosinophils in the pathogenesis of asthma. *Trends Mol Med* 2005; **11**: 148–152.
- 10 Zarogiannis S, Gourgoulis KI, Kostikas K, Szczeklik A, Gleich GJ, Pavord ID et al. Anti-interleukin-5 therapy and severe asthma. *N Engl J Med* 2009; **360**: 2576–2578.
- 11 Garrett JK, Jameson SC, Thomson B, Collins MH, Wagoner LE, Freese DK et al. Anti-interleukin-5 (mepolizumab) therapy for hypereosinophilic syndromes. *J Allergy Clin Immunol* 2004; **113**: 115–119.
- 12 Plotz SG, Simon HU, Darsow U, Simon D, Vassina E, Yousefi S et al. Use of an anti-interleukin-5 antibody in the hypereosinophilic syndrome with eosinophilic dermatitis. *N Engl J Med* 2003; **349**: 2334–2339.
- 13 Straumann A, Conus S, Grzonka P, Kita H, Kephart G, Bussmann C et al. Anti-interleukin-5 antibody treatment (mepolizumab) in active eosinophilic oesophagitis: a randomised, placebo-controlled, double-blind trial. *Gut* 2010; **59**: 21–30.
- 14 Wierenga EA, Snoek M, de Groot C, Chretien I, Bos JD, Jansen HM et al. Evidence for compartmentalization of functional subsets of CD2⁺ T lymphocytes in atopic patients. *J Immunol* 1990; **144**: 4651–4656.
- 15 Hamaguchi-Tsuru E, Nobumoto A, Hirose N, Kataoka S, Fujikawa-Adachi K, Furuya M et al. Development and functional analysis of eosinophils from murine embryonic stem cells. *Br J Haematol* 2004; **124**: 819–827.
- 16 Foster PS, Hogan SP, Ramsay AJ, Matthaei KI, Young IG. Interleukin 5 deficiency abolishes eosinophilia, airways hyperreactivity, and lung damage in a mouse asthma model. *J Exp Med* 1996; **183**: 195–201.

- 17 Hogan SP, Koskinen A, Foster PS. Interleukin-5 and eosinophils induce airway damage and bronchial hyperreactivity during allergic airway inflammation in BALB/c mice. *Immunol Cell Biol* 1997; **75**: 284–288.
- 18 Hamelmann E, Oshiba A, Loader J, Larsen GL, Gleich G, Lee J et al. Antiinterleukin-5 antibody prevents airway hyperresponsiveness in a murine model of airway sensitization. *Am J Respir Crit Care Med* 1997; **155**: 819–825.
- 19 Flood-Page P, Swenson C, Faierman I, Matthews J, Williams M, Brannick L et al. A study to evaluate safety and efficacy of mepolizumab in patients with moderate persistent asthma. *Am J Respir Crit Care Med* 2007; **176**: 1062–1071.
- 20 Kips JC, O'Connor BJ, Langley SJ, Woodcock A, Kerstjens HA, Postma DS et al. Effect of SCH55700, a humanized anti-human interleukin-5 antibody, in severe persistent asthma: a pilot study. *Am J Respir Crit Care Med* 2003; **167**: 1655–1659.
- 21 Leckie MJ, ten Brinke A, Khan J, Diamant Z, O'Connor BJ, Walls CM et al. Effects of an interleukin-5 blocking monoclonal antibody on eosinophils, airway hyperresponsiveness, and the late asthmatic response. *Lancet* 2000; **356**: 2144–2148.
- 22 Flood-Page PT, Menzies-Gow AN, Kay AB, Robinson DS. Eosinophil's role remains uncertain as anti-interleukin-5 only partially depletes numbers in asthmatic airway. *Am J Respir Crit Care Med* 2003; **167**: 199–204.
- 23 Haldar P, Brightling CE, Hargadon B, Gupta S, Monteiro W, Sousa A et al. Mepolizumab and exacerbations of refractory eosinophilic asthma. *N Engl J Med* 2009; **360**: 973–984.
- 24 Nair P, Pizzichini MM, Kjarsgaard M, Inman MD, Efthimiadis A, Pizzichini E et al. Mepolizumab for prednisone-dependent asthma with sputum eosinophilia. *N Engl J Med* 2009; **360**: 985–993.
- 25 Lee JJ, Dimina D, Macias MP, Ochkur SI, McGarry MP, O'Neill KR et al. Defining a link with asthma in mice congenitally deficient in eosinophils. *Science* 2004; **305**: 1773–1776.
- 26 Walsh ER, Sahu N, Kearley J, Benjamin E, Kang BH, Humbles A et al. Strain-specific requirement for eosinophils in the recruitment of T cells to the lung during the development of allergic asthma. *J Exp Med* 2008; **205**: 1285–1292.
- 27 Jacobsen EA, Ochkur SI, Pero RS, Taranova AG, Protheroe CA, Colbert DC et al. Allergic pulmonary inflammation in mice is dependent on eosinophil-induced recruitment of effector T cells. *J Exp Med* 2008; **205**: 699–710.
- 28 Corry DB, Folkesson HG, Warnock ML, Erle DJ, Matthay MA, Wiener-Kronish JP et al. Interleukin 4, but not interleukin 5 or eosinophils, is required in a murine model of acute airway hyperreactivity. *J Exp Med* 1996; **183**: 109–117.
- 29 Humbles AA, Lloyd CM, McMillan SJ, Friend DS, Xanthou G, McKenna EE et al. A critical role for eosinophils in allergic airways remodeling. *Science* 2004; **305**: 1776–1779.
- 30 Mattes J, Yang M, Mahalingam S, Kuehr J, Webb DC, Simson L et al. Intrinsic defect in T cell production of eosinophil (IL)-13 in the absence of both IL-5 and eotaxin precludes the development of eosinophilia and airways hyperreactivity in experimental asthma. *J Exp Med* 2002; **195**: 1433–1444.
- 31 Wills-Karp M, Luyimbazi J, Xu X, Schofield B, Neben TY, Karp CL et al. Interleukin-13: central mediator of allergic asthma. *Science* 1998; **282**: 2258–2261.
- 32 Nakajima T, Yamada H, Iikura M, Hirai K. Intracellular localization and release of eotaxin from normal eosinophils. *FEBS Lett* 1998; **434**: 226–230.
- 33 Ying S, Meng Q, Taborda-Barata L, Corrigan CJ, Barkans J, Assoufi B et al. Human eosinophils express messenger RNA encoding RANTES and store and release biologically active RANTES protein. *Eur J Immunol* 1996; **26**: 70–76.
- 34 Culley FJ, Brown A, Girod N, Pritchard DI, Williams TJ. Innate and cognate mechanisms of pulmonary eosinophilia in helminth infection. *Eur J Immunol* 2002; **32**: 1376–1385.
- 35 Dixon H, Blanchard C, Deschoolmeester ML, Yuill NC, Christie JW, Rothenberg ME et al. The role of Th2 cytokines, chemokines and parasite products in eosinophil recruitment to the gastrointestinal mucosa during helminth infection. *Eur J Immunol* 2006; **36**: 1753–1763.
- 36 Dombrowicz D, Capron M. Eosinophils, allergy and parasites. *Curr Opin Immunol* 2001; **13**: 716–720.
- 37 Gunther C, Wozel G, Dressler J, Meurer M, Pfeiffer C. Tissue eosinophilia in pemphigoid gestationis: association with eotaxin and upregulated activation markers on transmigrated eosinophils. *Am J Reprod Immunol* 2004; **51**: 32–39.
- 38 Ochkur SI, Jacobsen EA, Protheroe CA, Biechele TL, Pero RS, McGarry MP et al. Coexpression of IL-5 and eotaxin-2 in mice creates an eosinophil-dependent model of respiratory inflammation with characteristics of severe asthma. *J Immunol* 2007; **178**: 7879–7889.
- 39 Sung S, Rose CE, Fu SM. Intratracheal priming with ovalbumin- and ovalbumin 323–339 peptide-pulsed dendritic cells induces airway hyperresponsiveness, lung eosinophilia, goblet cell hyperplasia, and inflammation. *J Immunol* 2001; **166**: 1261–1271.
- 40 Inaba K, Inaba M, Romani N, Aya H, Deguchi M, Ikehara S et al. Generation of large numbers of dendritic cells from mouse bone marrow cultures supplemented with granulocyte/macrophage colony-stimulating factor. *J Exp Med* 1992; **176**: 1693–1702.
- 41 Finotto S, Neurath MF, Glickman JN, Qin S, Lehr HA, Green FH et al. Development of spontaneous airway changes consistent with human asthma in mice lacking T-bet. *Science* 2002; **295**: 336–338.
- 42 Levitt RC, Ewart SL. Genetic susceptibility to atracurium-induced bronchoconstriction. *Am J Respir Crit Care Med* 1995; **151**: 1537–1542.
- 43 Sung SS, Fu SM, Rose CE Jr, Gaskin F, Ju ST, Beaty SR. A major lung CD103 (alphaE)-beta7 integrin-positive epithelial dendritic cell population expressing Langerin and tight junction proteins. *J Immunol* 2006; **176**: 2161–2172.
- 44 Nishikomori R, Ehrhardt RO, Strober W. T helper type 2 cell differentiation occurs in the presence of interleukin 12 receptor beta2 chain expression and signaling. *J Exp Med* 2000; **191**: 847–858.
- 45 Beaty SR, Rose CE Jr, Sung SS. Diverse and potent chemokine production by lung CD11b^{high} dendritic cells in homeostasis and in allergic lung inflammation. *J Immunol* 2007; **178**: 1882–1895.
- 46 Prussin C, Metcalfe DD. 4. IgE, mast cells, basophils, and eosinophils. *J Allergy Clin Immunol* 2003; **111**: S486–S494.
- 47 Zhang JQ, Biedermann B, Nitschke L, Crocker PR. The murine inhibitory receptor mSiegE-E is expressed broadly on cells of the innate immune system whereas mSiegE-F is restricted to eosinophils. *Eur J Immunol* 2004; **34**: 1175–1184.
- 48 Dvorak AM, Furits T, Letourneau L, Ishizaka T, Ackerman SJ. Mature eosinophils stimulated to develop in human cord blood mononuclear cell cultures supplemented with recombinant human interleukin-5. Part I. Piecemeal degranulation of specific granules and distribution of Charcot-Leyden crystal protein. *Am J Pathol* 1991; **138**: 69–82.
- 49 Jacobsen EA, Taranova AG, Lee NA, Lee JJ. Eosinophils: singularly destructive effector cells or purveyors of immunoregulation? *J Allergy Clin Immunol* 2007; **119**: 1313–1320.
- 50 van Rijt LS, Vos N, Hijdra D, de Vries VC, Hoogsteden HC, Lambrecht BN. Airway eosinophils accumulate in the mediastinal lymph nodes but lack antigen-presenting potential for naive T cells. *J Immunol* 2003; **171**: 3372–3378.
- 51 Throsby M, Herbelin A, Pleau JM, Dardenne M. CD11c⁺ eosinophils in the murine thymus: developmental regulation and recruitment upon MHC class I-restricted thymocyte deletion. *J Immunol* 2000; **165**: 1965–1975.
- 52 Dajotoy T, Andersson P, Bjartell A, Lofdahl CG, Tapper H, Egesten A. Human eosinophils produce the T cell-attracting chemokines MIG and IP-10 upon stimulation with IFN-gamma. *J Leukoc Biol* 2004; **76**: 685–691.
- 53 Izumi S, Hirai K, Miyamasu M, Takahashi Y, Misaki Y, Takaiishi T et al. Expression and regulation of monocyte chemoattractant protein-1 by human eosinophils. *Eur J Immunol* 1997; **27**: 816–824.
- 54 Moqbel R, Levi-Schaffer F, Kay AB. Cytokine generation by eosinophils. *J Allergy Clin Immunol* 1994; **94**: 1183–1188.
- 55 Persson T, Monsef N, Andersson P, Bjartell A, Malm J, Calafat J et al. Expression of the neutrophil-activating CXC chemokine ENA-78/CXCL5 by human eosinophils. *Clin Exp Allergy* 2003; **33**: 531–537.
- 56 Persson-Dajotoy T, Andersson P, Bjartell A, Calafat J, Egesten A. Expression and production of the CXC chemokine growth-related oncogene-alpha by human eosinophils. *J Immunol* 2003; **170**: 5309–5316.
- 57 Schraufstatter I, Takamori H, Sikora L, Sriramarao P, DiScipio RG. Eosinophils and monocytes produce pulmonary and activation-regulated chemokine, which activates cultured monocytes/macrophages. *Am J Physiol Lung Cell Mol Physiol* 2004; **286**: L494–L501.
- 58 Olszewska-Pazdrak B, Casola A, Saito T, Alam R, Crowe SE, Mei F et al. Cell-specific expression of RANTES, MCP-1, and MIP-1alpha by lower airway epithelial cells and eosinophils infected with respiratory syncytial virus. *J Virol* 1998; **72**: 4756–4764.
- 59 Hogaboam CM, Gallinat CS, Taub DD, Strieter RM, Kunkel SL, Lukacs NW. Immunomodulatory role of C10 chemokine in a murine model of allergic bronchopulmonary aspergillosis. *J Immunol* 1999; **162**: 6071–6079.
- 60 Ma B, Zhu Z, Homer RJ, Gerard C, Strieter R, Elias JA. The C10/CCL6 chemokine and CCR1 play critical roles in the pathogenesis of IL-13-induced inflammation and remodeling. *J Immunol* 2004; **172**: 1872–1881.
- 61 Berahovich RD, Miao Z, Wang Y, Premack B, Howard MC, Schall TJ. Proteolytic activation of alternative CCR1 ligands in inflammation. *J Immunol* 2005; **174**: 7341–7351.
- 62 Blease K, Mehrad B, Standiford TJ, Lukacs NW, Kunkel SL, Chensue SW et al. Airway remodeling is absent in CCR1^{-/-} mice during chronic fungal allergic airway disease. *J Immunol* 2000; **165**: 1564–1572.
- 63 Domachowski JB, Bonville CA, Gao JL, Murphy PM, Easton AJ, Rosenberg HF. The chemokine macrophage-inflammatory protein-1 alpha and its receptor CCR1 control pulmonary inflammation and antiviral host defense in paramyxovirus infection. *J Immunol* 2000; **165**: 2677–2682.
- 64 John AE, Gerard CJ, Schaller M, Miller AL, Berlin AA, Humbles AA et al. Respiratory syncytial virus-induced exaggeration of allergic airway disease is dependent upon CCR1-associated immune responses. *Eur J Immunol* 2005; **35**: 108–116.
- 65 Schaller MA, Kallal LE, Lukacs NW. A key role for CC chemokine receptor 1 in T-cell-mediated respiratory inflammation. *Am J Pathol* 2008; **172**: 386–394.
- 66 Murphy PM, Baggiolini M, Charo IF, Hebert CA, Horuk R, Matsushima K et al. International union of pharmacology. XXII. Nomenclature for chemokine receptors. *Pharmacol Rev* 2000; **52**: 145–176.
- 67 Ma B, Liu W, Homer RJ, Lee PJ, Coyle AJ, Lora JM et al. Role of CCR5 in the pathogenesis of IL-13-induced inflammation and remodeling. *J Immunol* 2006; **176**: 4968–4978.
- 68 Schuh JM, Blease K, Hogaboam CM. The role of CC chemokine receptor 5 (CCR5) and RANTES/CCL5 during chronic fungal asthma in mice. *FASEB J* 2002; **16**: 228–230.
- 69 Rengeling JS, Pribul PK, Pennycook AM, Hussell T, Wang B, Lukacs N et al. The chemokine MIP1alpha/CCL3 determines pathology in primary RSV infection by regulating the balance of T cell populations in the murine lung. *PLoS one* 2010; **5**: e9381.
- 70 Srivastava P, Helms PJ, Stewart D, Main M, Russell G. Association of CCR5Delta32 with reduced risk of childhood but not adult asthma. *Thorax* 2003; **58**: 222–226.
- 71 Gutierrez-Ramos JC, Lloyd C, Kapsenberg ML, Gonzalo JA, Coyle AJ. Non-redundant functional groups of chemokines operate in a coordinate manner during the inflammatory response in the lung. *Immunol Rev* 2000; **177**: 31–42.