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George T. De Sanctis, ... , Francis H.Y. Green, Jeffrey M. Drazen

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Article

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Interleukin-8 receptor modulates IgE production and B-cell expansion and trafficking in allergen-induced pulmonary inflammation

George T. De Sanctis,¹ James A. MacLean,² Shixin Qin,³ Walter W. Wolyniec,⁴ Hartmut Grasemann,¹ Chandri N. Yandava,¹ Aiping Jiao,¹ Thomas Noonan,⁴ Joan Stein-Streilein,⁵ Francis H.Y. Green,⁶ and Jeffrey M. Drazen¹

¹Combined Program in Pulmonary and Critical Care Medicine, Brigham and Women's Hospital and Harvard Medical School, Boston, Massachusetts 02115, USA

²Clinical Immunology and Allergy Units, Massachusetts General Hospital, Boston, Massachusetts 02114, USA

³Leukosite Inc., Cambridge, Massachusetts 02142, USA

⁴Boehringer Ingelheim Pharmaceuticals Inc., Ridgefield, Connecticut 06877, USA

⁵Schepens Eye Research Institute, Boston, Massachusetts 02114, USA

⁶Respiratory Research Group, University of Calgary, Calgary, Alberta T2N-4N1, Canada

Address correspondence to: George T. De Sanctis, Combined Program in Pulmonary and Critical Care Medicine, Brigham and Women's Hospital and Harvard Medical School, Boston, Massachusetts 02115, USA. Phone: (617) 732-7420; Fax: (617) 732-7421; E-mail: desanctis@bustoff.bwh.harv.edu

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We examined the role of the interleukin-8 (IL-8) receptor in a murine model of allergen-induced pulmonary inflammation using mice with a targeted deletion of the murine IL-8 receptor homologue (IL-8r^{-/-}). Wild-type (Wt) and IL-8r^{-/-} mice were systemically immunized to ovalbumin (OVA) and were exposed with either single or multiple challenge of aerosolized phosphate-buffered saline (OVA/PBS) or OVA (OVA/OVA). Analysis of cells recovered from bronchoalveolar lavage (BAL) revealed a diminished recruitment of neutrophils to the airway lumen after single challenge in IL-8r^{-/-} mice compared with Wt mice, whereas multiply challenged IL-8r^{-/-} mice had increased B cells and fewer neutrophils compared with Wt mice. Both Wt and IL-8r^{-/-} OVA/OVA mice recruited similar numbers of eosinophils to the BAL fluid and exhibited comparable degrees of pulmonary inflammation histologically. Both total and OVA-specific IgE levels were greater in multiply challenged IL-8r^{-/-} OVA/OVA mice than in Wt mice. Both the IL-8r^{-/-} OVA/OVA and OVA/PBS mice were significantly less responsive to methacholine than their respective Wt groups, but both Wt and IL-8r^{-/-} mice showed similar degrees of enhancement after multiple allergen challenge. The data demonstrate that the IL-8 modulates IgE production, airway responsiveness, and the composition of the cells (B cells and neutrophils) recruited to the airway lumen in response to antigen.

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Introduction

Airway inflammation with eosinophils, lymphocytes, and neutrophils is a characteristic feature of human asthma (1-3). There is growing evidence to suggest that interleukin (IL)-8 is implicated in the pathobiology of asthma. Several studies have demonstrated the presence of IL-8 in the bronchoalveolar lavage fluid of patients with asthma (4-6). In addition, Shute *et al.* (7) demonstrated an upregulation of free and complexed IL-8 in the blood and bronchial mucosa in asthmatics and suggested that free IL-8 has a proinflammatory role by contributing to eosinophil activation. IL-8 is an eosinophil and neutrophil chemoattractant (8-11). IL-8 receptors (CXCR2) are induced on IL-5-primed eosinophils in humans (12), and IL-8 protein expression is elevated in the eosinophils of asthmatics (13). In animal models, administration of exogenous IL-8 has been shown to recruit neutrophils into the airway lumen and enhance airway reactivity to inhaled histamine in guinea pigs (14). With regard to its biologic effects on lymphocyte function, IL-8 has also been

shown to cause the release of T-lymphocyte chemoattractants from neutrophils *in vitro* and *in vivo* (10).

Two high-affinity IL-8 receptors have been cloned and characterized in humans (15). Cacalano *et al.* (16) cloned a murine gene from a genomic library with a high degree of homology to the two known human IL-8 receptors, and using homologous recombination in embryonic stem cells, mice carrying a targeted deletion of this IL-8 receptor homologue, IL-8r^{-/-}, were generated. These IL-8r^{-/-} mice demonstrated B-cell expansion and diminished capacity to recruit neutrophils to inflammatory sites (16).

Examination of murine models of human allergic asthma has provided novel insight into the pathobiologic events linking airway inflammation with airway dysfunction (17-27). In these models, systemic sensitization to ovalbumin (OVA) followed by repeated exposure to aerosolized OVA results in increased IgE production, pulmonary inflammatory cell infiltrates, and airway hyperresponsiveness (17-27). We reasoned that stimulation via the murine IL-8 receptor homologue could be an important signaling pathway in these models. To test this hypothesis,

Figure 1

Schematic representation of the sensitization and challenge protocols. For the acute challenge protocol (a), mice were sensitized and boosted on days 1 and 7 with OVA, received a single aerosol challenge of either OVA or PBS on day 14, and were studied on day 15. For the chronic challenge protocol (b), mice were sensitized and boosted on days 1 and 7, received repeated aerosol challenge of either OVA or PBS on days 14–20, and were studied on day 21. *IP*, intraperitoneally; *OVA*, ovalbumin.

we examined the response of *IL-8r^{-/-}* mice to systemic OVA sensitization and aerosol challenge with OVA.

Methods

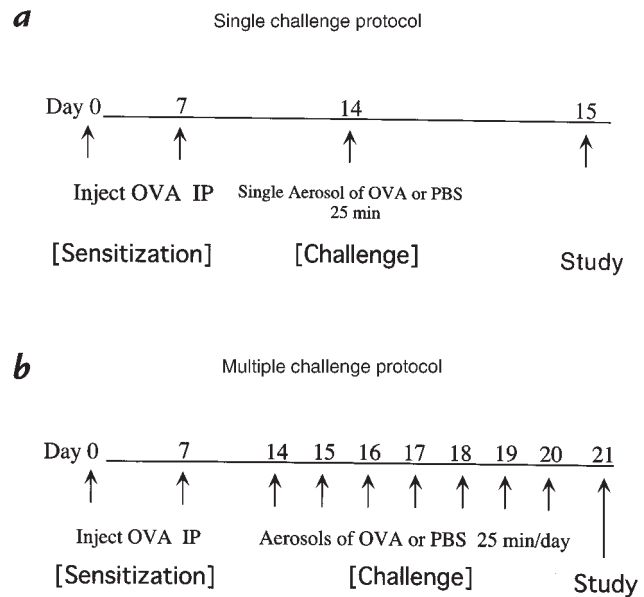
Animals. Four-week-old male viral antibody-free (VAF) BALB/cJ (Wt) mice and mice with a targeted deletion of the *IL-8r* homologue *IL-8r^{-/-}* (16) (*BALB/c-Cmkar2tm1Mwm*), backcrossed to BALB/cJ mice, were purchased from The Jackson Laboratory (Bar Harbor, Maine, USA). Mice were housed in isolation cages under VAF conditions.

Sensitization and challenge protocols. Two sensitization and challenge protocols were used in these studies (Fig. 1). The single challenge protocol involved the intraperitoneal immunization of mice on days 0 and 7 with 10 µg OVA (Grade III; Sigma Chemical Co., St. Louis, Missouri, USA) and 1 mg alum (J.T. Baker Chemical Co., Phillipsburg, New Jersey, USA) in 0.2 ml of PBS, followed by a single aerosol exposure to either PBS (OVA/PBS-1d) or 6% OVA (OVA/OVA-1d) for 25 min. Mice in protocol 1 were studied 18 h after the aerosol challenge. All single challenge experiments were performed two times with a minimum of five mice per group. The multiple challenge protocol used a similar immunization scheme with intraperitoneal OVA and alum on days 0 and 7, followed by repeated aerosol challenges of either PBS (OVA/PBS-7d) or OVA (OVA/OVA-7d) for 25 min/day for 7 consecutive days from day 14 to day 20. Mice were studied 24 h after the last aerosol challenge. All multiple challenge experiments were performed with a minimum of six mice per group on a minimum of three occasions.

Airway responsiveness. Airway responsiveness was measured as described previously (21, 28, 29). Methacholine dose–response curves were obtained by intravenous administration of sequentially increasing doses of methacholine (33 to 1,000 µg/kg) in a 20- to 35-µl volume. Each dose–response curve was log-transformed and then subjected to regression analysis to interpolate the dose required for a twofold increase in lung resistance (R_L) ($\log ED_{200} R_L$). This dose, referred to as the effective dose required to increase R_L to 200% of control values ($ED_{200} R_L$), was used as an index of airway responsiveness; numerically low values of $ED_{200} R_L$ indicate a high level of sensitivity to the administered agonist and are consistent with an asthma-like hyperresponsive phenotype (28, 29).

Bronchoalveolar lavage. For bronchoalveolar lavage (BAL), two aliquots of 1 ml PBS with 0.6 mM EDTA were used to lavage the lung. The lavagete was centrifuged at 2,000 g for 10 min. The supernatant was then separated from the cell pellet, and the cells were resuspended in HBSS (JRH Biosciences, Lenexa, Kansas, USA). Slides were prepared by spinning samples at 800 g for 10 min (Cytospin 2; Shandon Inc., Pittsburgh, Pennsylvania, USA). BAL specimens were stained with a Wright-Giemsa stain, and differentials were determined by counting approximately 250 cells for each sample. The investigator counting the cells was blinded to the treatment group assignment of each section.

Tissue sample collection. Animals were removed from the plethysmograph and sacrificed by cervical dislocation under surgical anesthesia. Blood was collected by cardiac puncture (for measurement of serum IgE levels), and the lungs were removed from



the thoracic cavity and inflated with pH-balanced formaldehyde fixative (pH 7.4). Tissue sections were embedded in paraffin, cut at 5 µm, stained with hematoxylin and eosin (H&E) and PAS/alcian blue (pH 2.5), and examined by light microscopy.

Genotype analysis by PCR. Mice were genotyped by PCR using primers for the *IL-8r* wild-type (Wt) gene and the *Neo* gene for the knockout mice. Primers used were forward primer 5'-GGT CGT ACT GCG TAT CCT GCC TCA G-3' (LMR453) and reverse primer 5'-TAG CCA TGA TCT TGA GAA GTC CAT-3' (LMR454), as well as forward primer 5'-CTT GGG TGG AGA GGC TAT TC-3' (IMR013) and reverse primer 5'-AGG TGA GAT GAC AGG AGA TC-3' (IMR014). LMR453 with LMR454 amplifies a 350-bp DNA product from the Wt gene, and IMR013 and IMR014 amplify a 280-bp product from the *Neo* gene. A PCR reaction mix containing 500 ng of genomic DNA; PCR buffer (Boehringer Mannheim GmbH, Mannheim, Germany) with 1.5 mM MgCl₂ (final concentration), 200 µM dATP, dCTP, dGTP, and dTTP, 10 pmol of each primer; and 1.5 U of Taq polymerase in a 25-µl reaction mixture was incubated in a thermal cycler under the following conditions: 6 min at 94°C, followed by 35 cycles at 94°C for 1 min, 59°C for 1 min, and 72°C for 1 min. Chain elongation at 72°C was continued for 5 min after the last cycle. After adding 2 µl of stop solution, 18 µl of the amplicon was resolved by electrophoresis on a 1.5% agarose gel and stained with ethidium bromide for analysis. The length of the PCR products was assessed by comparison with a 100-bp DNA ladder (GIBCO BRL, Rockville, Maryland, USA).

Measurement of BAL eosinophil peroxidase and total protein. BAL eosinophil peroxidase (EPO) and total protein were measured as described previously by Wolyniec *et al.* (20). BAL samples were read on a plate reader (Spectramax Model 340; Molecular Devices Corp., Sunnyvale, California, USA), and regression analysis was performed with a SOFTmax analysis software package included with the instrument.

OVA-specific IgE, total serum IgE, and OVA-specific IgG subclass analysis. OVA-specific IgE levels were measured by capture-ELISA as described previously (22). ELISA microtiter plates (Nunc Immuno-Plates; Nalge Nunc International, Naperville, Illinois, USA) were coated with a purified anti-mouse IgE monoclonal antibody (PharMingen, San Diego, California, USA) at a concentration of 2 µg/ml and blocked with PBS/10% FCS. Serum samples were diluted in PBS/3% FCS and incubated in the wells for 2 h. After washing with PBS/0.05% Tween-20,

biotinylated OVA was added to the wells and incubated for 1 h. The plates were washed with PBS/Tween-20 followed by the addition of avidin alkaline phosphatase (Sigma Chemical Co.) for 1 h. The plates were washed with PBS/Tween-20 and distilled water before the addition of the phosphatase substrate (KPL Laboratories, Gaithersburg, Maryland, USA). The plates were allowed to develop for 30 min. The plates were read in an ELISA plate reader at an OD of 405 nm.

Total serum IgE was measured by capture ELISA in a manner similar to the detection of OVA-specific IgE. A biotinylated rat anti-mouse IgE (PharMingen) was used to detect captured IgE in place of the biotinylated OVA. A standard curve for total IgE was generated using a purified mouse IgE standard (PharMingen). The limit of detection of the mouse IgE ELISA is ~5 ng/ml.

IgG1, IgG2a, IgG2b, and IgG3 OVA-specific antibodies were measured by standard ELISA. Microtiter plates (96-well) were coated with OVA (100 mg/ml) in 0.1 M NaHCO₃ at 4°C overnight. The plates were washed with PBS/0.05% Tween-20 and blocked with PBS/10% FCS. Serum samples were diluted in PBS/3% FCS and incubated in the wells for 2 h. Serial dilutions of pooled serum from OVA/OVA-7d mice were used as a reference standard (30). After washing with PBS/Tween-20, the biotinylated anti-IgG subclass antibodies (PharMingen) were added to the well at a concentration of 2 µg/ml and incubated for 1 h. The plates were washed with PBS/Tween-20 followed by the addition of avidin alkaline phosphatase (Sigma Chemical Co.) for 1 h. The plates were washed with PBS/Tween-20 and distilled water before the addition of the phosphatase substrate. The plates were allowed to develop for 30 min. The plates were read in an ELISA plate reader at an OD of 405 nm.

Flow cytometry. Immunofluorescence staining of BAL cells was carried out using directly conjugated monoclonal antibodies. The following anti-murine monoclonal antibodies were purchased from PharMingen: CD3-FITC (clone 145-2C11), CD25-PE (clone 3C7), and B220-PE (clone RA3-6B2). The staining was detected on a FACScan flow cytometer (Becton Dickinson Immunocytometry Systems, San Jose, California, USA).

Histological grading. A semiquantitative grading system was used to score the extent of the inflammation in the histological sections. The inflammatory infiltrate was analyzed for its cellular components, *i.e.*, granulocytes and mononuclear cells. The inflammation was graded in each of three compartments: perivenular, peribronchial, and intra-alveolar. In addition, intra-alveolar giant cells and mucous cell metaplasia was also graded. Inflammation was graded as 0 for absent, 1 for mild, 2 for moderate, and 3 for severe. The histological sections were graded in a blinded fashion.

Statistical analysis. Computations were performed with the JMP 3.15 statistical package (SAS Institute Inc., Cary, North Carolina, USA). For parametric data, differences between groups were analyzed with Student's *t* test; for nonparametric data, differences between groups were analyzed with the Wilcoxon rank sum test. Results were expressed as means ± SEM, and unless otherwise stated, were considered statistically significant at *P* < 0.05.

Results

Cell recruitment to BAL of Wt and IL-8r^{-/-} mice. BAL cell counts and differentials were determined in mice undergoing both single and multiple aerosol challenge (Fig. 2). Total cell counts in the BAL of singly challenged (OVA/OVA-1d) Wt were significantly increased compared with Wt OVA/PBS-1d controls (*P* = 0.004) (Fig. 2a). In IL-8r^{-/-} mice, total cell counts were greater in the OVA/OVA-1d-challenged mice compared with the OVA/PBS-1d controls but did not reach statistical significance. In multiply challenged mice, both OVA/OVA-7d Wt and IL-8r^{-/-} groups showed significant increases in total cell counts compared with their respective phosphate-buffered saline (PBS) controls (both *P* < 0.05) (Fig. 2c). Interestingly, after multiple challenges with OVA, similar numbers of total cells were recruited to the airway lumen of both Wt and IL-8r^{-/-} mice, *i.e.*, not significantly different (Fig. 2c).

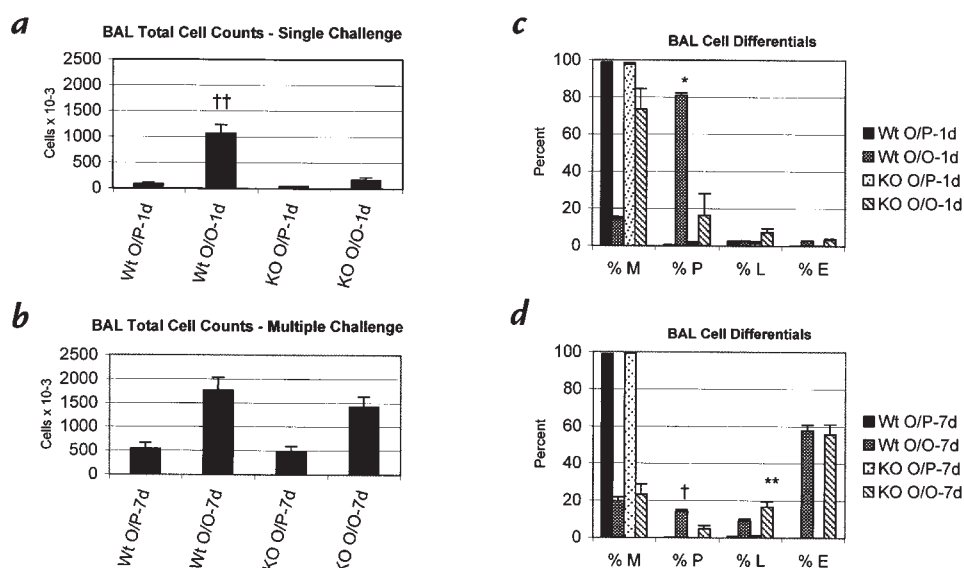


Figure 2

BAL total and differential cell counts from singly and multiply challenged mice. BAL total cell counts were determined in singly (a) and multiply (b) challenged Wt and IL-8r^{-/-} mice. Likewise, BAL cell differentials were enumerated from singly (c) and multiply (d) challenged Wt and IL-8r^{-/-} mice. Data are expressed as mean cell counts or percents ± SEM and are from a representative experiment. Minimum of five mice per group; range of 5–11 mice per group. **P* = 0.003 comparing IL-8r^{-/-} OVA/OVA-1d vs. Wt OVA/OVA-1d; *P* = 0.004 comparing IL-8r^{-/-} OVA/OVA-1d vs. Wt OVA/OVA-1d; ***P* = 0.02 comparing IL-8r^{-/-} OVA/OVA-7d vs. Wt OVA/OVA-7d; *P* = 0.002 comparing IL-8r^{-/-} OVA/OVA-7d vs. Wt OVA/OVA-7d. BAL, bronchoalveolar lavage.

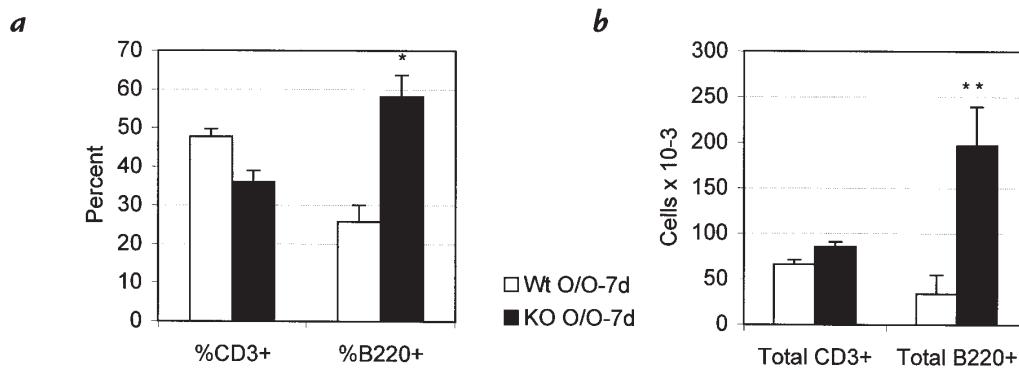


Figure 3

Proportion and total number of BAL CD3⁺ and B220⁺ lymphocytes in multiply challenged mice. (a) The proportion of CD3⁺ and B220⁺ cells from the BAL of OVA/OVA Wt and IL-8r^{-/-} mice as assessed by flow cytometry. (b) The calculated total number of CD3⁺ and B220⁺ cells from the BAL of OVA/OVA Wt and IL-8r KO^{-/-} mice. Data in a represent the percentage of positive CD3⁺ and B220⁺ cells in the lymphocyte gate of stained BAL cells. Data in b represent the product of the total BAL cell count, the percentage of lymphocytes on differential, and the percentage of CD3⁺ or B220⁺ cells in the lymphocyte gate. Data are expressed as mean ± SEM from a representative experiment. Minimum of eight mice per group. *P = 0.01 comparing IL-8r^{-/-} OVA/OVA-7d vs. Wt OVA/OVA-7d; **P = 0.0002 comparing IL-8r^{-/-} OVA/OVA-7d vs. Wt OVA/OVA-7d.

BAL cell count differential analysis revealed that all OVA/PBS-treated mice, whether undergoing a single or multiple challenges, showed a predominance of macrophages in their BAL (Fig. 2, b and d). The BAL cell differentials from OVA/OVA-1d Wt mice showed a predominance of neutrophils that was significantly greater than that seen in similarly treated IL-8r^{-/-} mice (P = 0.003) (Fig. 2b). These findings suggested a diminished early neutrophil recruitment to the airway of IL-8r^{-/-} mice after a single challenge with OVA. Neither the percentage nor the total number of recruited eosinophils in OVA/OVA-1d Wt and IL-8r^{-/-} mice was different (P = 0.06 for total eosinophil) (Fig. 2b).

BAL cell differentials in multiply challenged Wt and IL-8r^{-/-} mice (OVA/OVA-7d) differed in the percentages of lymphocytes (greater in IL-8r^{-/-} mice) and neutrophils (greater in Wt mice) (Fig. 2d). There was a 5.8-fold higher (P = 0.002) percentage of neutrophils in the Wt OVA/OVA-7d group than in similarly treated IL-8r^{-/-} mice, and a 1.4-fold greater (P = 0.02) percentage of lymphocytes in the IL-8r^{-/-} mice compared with Wt mice. The percentage and total number of eosinophils and macrophages was not significantly different between the OVA/OVA-7d Wt and IL-8r^{-/-} mice.

Flow cytometric analysis of BAL lymphocytes. Analysis of

the proportion and total number of BAL cells gated for lymphocytes is presented in Fig. 3. In OVA/OVA-7d mice, there was a significant increase in the proportion (Fig. 3a) of CD3⁺ lymphocytes in the BAL of the Wt mice compared with IL-8r^{-/-} mice (P = 0.01); however, the total number of CD3⁺ cells in the BAL of OVA/OVA-7d Wt and IL-8r^{-/-} mice was not significantly different (Fig. 3b). In contrast, both the proportion (P = 0.01) (Fig. 3a) and absolute number (P = 0.0002) (Fig. 3b) of B220⁺ lymphocytes were greater in IL-8r^{-/-} mice compared with the OVA/OVA-7d Wt group (P < 0.05). There were insufficient numbers of lymphocytes in the BAL of OVA/OVA-1d and single/multiple challenged OVA/PBS mice for meaningful fluorescence-activated cell sorter analysis, and thus such data are not reported.

BAL protein and EPO levels in multiply challenged mice. BAL protein levels were measured in multiply challenged mice as a measure of increased lung permeability. Wt OVA/OVA-7d (571.9 ± 58.8 ng/ml) mice had significantly greater protein levels than their OVA/PBS-7d (262.6 ± 72.3 ng/ml) controls (P < 0.05). IL-8r^{-/-} OVA/OVA-7d (670.4 ± 115 ng/ml) mice had a similar significant increase compared with their respective OVA/PBS-7d (199.3 ± 41.1 ng/ml) controls (P < 0.05). There were no significant differences in BAL protein concentrations

Table 1

Grading of lung histology for single allergen challenge

Mouse type and treatment group	n	Venules: eosinophils	Venules: lymphocytes	Bronchi: eosinophils	Bronchi: lymphocytes	Alveoli: eosinophils	Alveoli: lymphocytes
Wt OVA/PBS-1d	3	0	0.7 ± 0.3	0	0.3 ± 0.3	0	0
Wt OVA/OVA-1d	4	2.8 ± 0.3 ^A	2.8 ± 0.3 ^A	1.5 ± 0.3 ^A	1.8 ± 0.3	0.8 ± 0.3	1.3 ± 0.3 ^A
IL-8r ^{-/-} OVA/PBS-1d	3	0	0	0	0	0	0
IL-8r ^{-/-} OVA/OVA-1d	3	2.7 ± 0.3 ^B	2.7 ± 0.3 ^B	1.7 ± 0.3 ^B	2.0 ± 0.0 ^B	0.7 ± 0.3	1.3 ± 0.3 ^B

There was no significant difference between similarly treated Wt and IL-8r^{-/-} mice. Data are expressed as mean severity scores ± SEM. ^AP < 0.05 comparing Wt OVA/OVA-1d vs. Wt OVA/PBS-1d. ^BP < 0.05 comparing IL-8r^{-/-} OVA/OVA-1d vs. IL-8r^{-/-} OVA/PBS-1d. OVA, ovalbumin.

Table 2

Grading of lung histology for multiple allergen challenge

Mouse type and treatment group	<i>n</i>	Venules: eosinophils	Venules: lymphocytes	Bronchi: eosinophils	Bronchi: lymphocytes	Alveoli: eosinophils	Alveoli: lymphocytes
Wt OVA/PBS-7d	4	0	1 ± 0.0	0.5 ± 0.3	1.5 ± 0.3	0	0
Wt OVA/OVA-7d	11	3 ± 0.0 ^A	3 ± 0.0 ^A	2.6 ± 0.2 ^A	2.7 ± 0.1 ^A	2.3 ± 0.2 ^A	2.3 ± 0.2 ^A
IL-8r ^{-/-} OVA/PBS-7d	4	0	0.5 ± 0.3	0	0.5 ± 0.3	0	0
IL-8r ^{-/-} OVA/OVA-7d	12	3 ± 0.0 ^B	3 ± 0.0 ^B	2.9 ± 0.1 ^B	2.9 ± 0.1 ^B	2.8 ± 0.1 ^B	2.8 ± 0.1 ^B

There was no significant difference between similarly treated Wt and IL-8r^{-/-} mice. Data are expressed as mean severity scores ± SEM. ^A*P* < 0.05 comparing Wt OVA/OVA-7d vs. Wt OVA/PBS-7d. ^B*P* < 0.05 comparing IL-8r^{-/-} OVA/OVA-7d vs. IL-8r^{-/-} OVA/PBS-7d.

between the Wt and IL-8r^{-/-} OVA/OVA-7d mice or between the Wt and IL-8r^{-/-} OVA/PBS-7d groups.

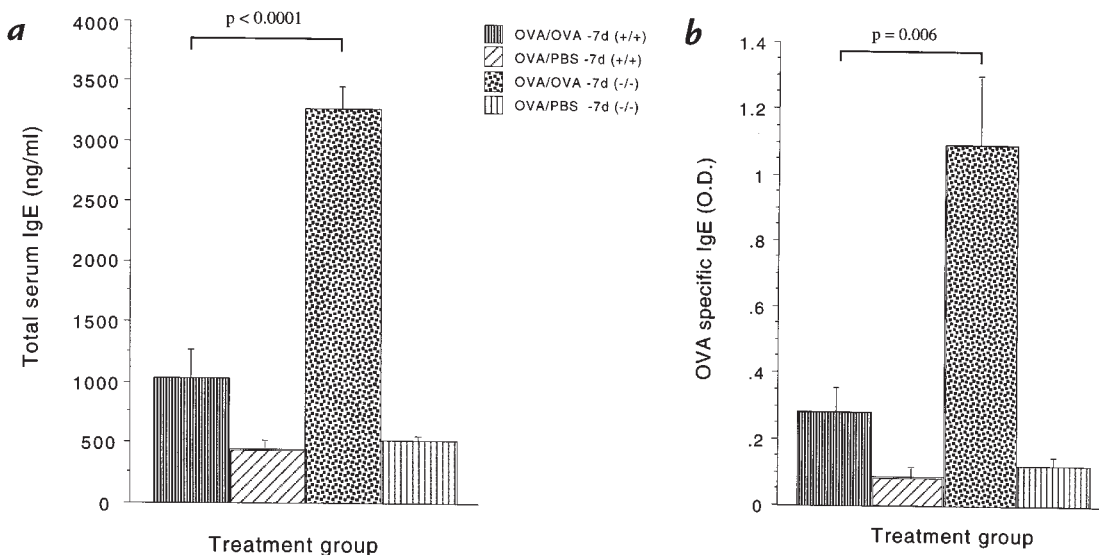
The levels of EPO, an eosinophil activation marker, were significantly higher in the BAL of both the Wt (26.8 ± 5.58 vs. 5.56 ± 0.90 ng/ml; *P* < 0.05) and IL-8r^{-/-} (33.1 ± 6.24 vs. 10.3 ± 2.15 ng/ml; *P* < 0.05) OVA/OVA-7d groups than in the respective OVA/PBS-7d groups. There were no significant differences in BAL EPO concentrations between the Wt and IL-8r^{-/-} OVA/OVA-7d mice.

Blood total cell counts. Total white blood cell counts were not significantly different between the Wt and IL-8r^{-/-} treatment groups, OVA/OVA-7d or OVA/PBS-7d (data not shown).

Antibody titers. OVA sensitization and challenge were associated with a significant increase in total and OVA-specific IgE in both Wt and IL-8r^{-/-} mice (Fig. 4, *a* and *b*). Although OVA sensitization and challenge resulted in a significant (*P* < 0.05) increase in IgE titers in both groups, there was a 3.1-fold and 3.9-fold greater increase in total and OVA-specific IgE in the IL-8r^{-/-} OVA/OVA-7d mice, respectively, than in the Wt OVA/OVA-7d group. There were no significant differences in either total or OVA specific IgE between the Wt and IL-8r^{-/-} mice from the OVA/PBS-7d exposure groups. When the differences in total and OVA-specific IgE levels were

examined within the IL-8r^{-/-} and Wt groups (OVA/OVA-7d vs. OVA/PBS-7d for each genotype), there were 6.3-fold (IL-8r^{-/-}) (*P* < 0.05) vs. 2.4-fold (Wt) (*P* < 0.05) differences in total IgE, and 9.1-fold (IL-8r^{-/-}) (*P* < 0.05) and 3.5-fold (Wt) (*P* < 0.05) increases in OVA-specific IgE, respectively. OVA-specific IgG subclasses (IgG1, IgG2a, IgG2b, and IgG3) were also measured in OVA/OVA-7d mice, and there were no significant differences between Wt and IL-8r^{-/-} mice (data not shown).

Lung histology. The extent of the inflammatory infiltrate was graded in both singly (Table 1) and multiply (Table 2) challenged mice. Representative photomicrographs of OVA-PBS-7d and OVA/OVA-7d lungs are shown in Fig 5. Wt and IL-8r^{-/-} mice exposed to PBS with either a single (not shown) or multiple (Fig. 5, *a* and *b*) challenge protocol showed normal lung architecture, with only rare mononuclear cells seen in a perivascular and peribronchial distribution in some mice (Table 1). Both Wt and IL-8r^{-/-} mice receiving single challenge with OVA demonstrated a significant perivascular and peribronchial inflammation with both mononuclear cells and granulocytes compared with their respective PBS controls (Table 1). There was no significant difference in the extent of the inflammation between OVA/OVA-1d Wt and IL-8r^{-/-} mice (Table 1).

**Figure 4**

Total and OVA-specific IgE levels in Wt and IL-8r^{-/-} mice. Total (*a*) and OVA-specific (*b*) IgE were measured in OVA/OVA-7d and OVA/PBS-7d Wt and IL-8r^{-/-} mice. Data are expressed as mean ± SEM from the four groups of mice (minimum of five mice per group) from a representative experiment.

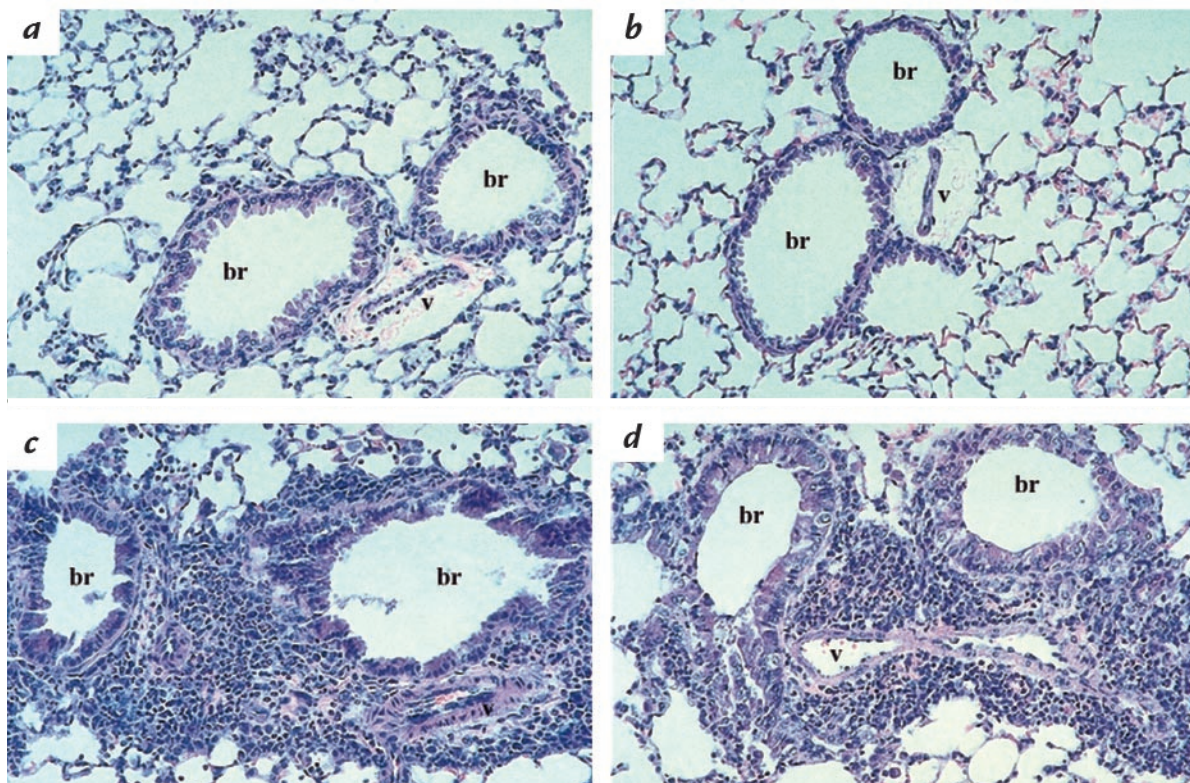


Figure 5 Lung histology from multiply challenged Wt and IL-8^{r-/-} mice. Formalin-fixed lung tissue from OVA/PBS-7d Wt (**a**) and IL-8^{r-/-} (**b**) mice showed normal lung architecture. Lung tissue from OVA/OVA-7d Wt (**c**) and IL-8^{r-/-} (**d**) mice showed a peribronchial and perivascular accumulation of inflammatory cells, including eosinophils and mononuclear cells. Photomicrographs are representative sections stained with hematoxylin and eosin. $\times 180$. br, bronchus; v, vessel.

In multiply challenged Wt and IL-8^{r-/-} mice, the extent of the inflammation was more pronounced compared with their respective OVA/PBS-7d control mice (Table 2 and Fig. 5). As in the singly challenged mice, there was no significant difference in the extent of the inflammation between OVA/OVA-7d Wt and IL-8^{r-/-} mice. Multiply challenged Wt and IL-8^{r-/-} also showed a marked increase in mucus-containing cells as identified in the PAS/alcian blue-stained sections (not shown). The mucous cell metaplasia/hyperplasia was seen in both large and small airways and was similar in Wt and IL-8^{r-/-} mice.

Assessment of lung resistance after methacholine challenge. All physiological assessments were performed 24 hours after the last aerosol challenge. Lung resistance (R_L) was measured in relation to the dose of intravenously infused methacholine in both singly and multiply challenged mice. There was no significant difference in the log ED₂₀₀ R_L values in OVA/OVA-1d Wt mice compared with the PBS-challenged Wt controls (Fig. 6a). Likewise, there was no significant difference in lung resistance in OVA/OVA-1d IL-8^{r-/-} compared with their PBS controls (Fig. 6a). Interestingly, both OVA/OVA-1d and OVA/PBS-1d IL-8^{r-/-} mice were significantly less responsive to methacholine than their respective Wt treatment groups ($P = 0.008$ and $P = 0.031$, respectively) (Fig. 6a). In contrast, OVA/OVA-7d Wt and IL-8^{r-/-} mice exhibited significant increases in lung resistance (assessed by the log ED₂₀₀ values) after methacholine challenge compared with their

respective OVA/PBS-7d controls (1.3-fold increase for both groups, $P < 0.05$) (Fig. 6b). When the log ED₂₀₀ R_L values were compared between the OVA/OVA-7d groups, the IL-8^{r-/-} mice were 2.3-fold less responsive ($P = 0.0001$) than the Wt OVA/OVA-7d group. Examination of the Wt and IL-8^{r-/-} OVA/PBS-7d groups revealed, however, that the IL-8^{r-/-} OVA/PBS-7d mice were 3.3-fold less responsive than the Wt OVA/PBS-7d group ($P = 0.0006$).

Discussion

The murine model of allergen-induced airway inflammation and airway hyperresponsiveness recapitulates many of the features of human asthma, including production of allergen-specific IgE (18–20, 31), airway hyperreactivity (32, 33), and abundant eosinophilic and lymphocytic infiltration (18–20, 31). The chemokine IL-8 has been implicated in a number of inflammatory lung diseases including asthma (9, 34). IL-8 is upregulated in serum and tissues of asthmatics (6, 7) and is a potent neutrophil (16), eosinophil (9), and T-lymphocyte chemotactic factor (10, 35). We studied the function of an IL-8 receptor homologue in mediating B-cell expansion, IgE production, tissue inflammation, and an increase in airway reactivity by examining mice with a targeted deletion of the IL-8 receptor homologue. We report the first *in vivo* evidence that IL-8^{r-/-} mice recruit significantly fewer total cells to the airway lumen after a single antigen challenge, mainly owing to an attenuated

early neutrophil recruitment. In multiply challenged IL-8^{-/-} mice, the total number of recruited cells to the airway lumen is not different than that of similarly treated Wt mice; however, a significantly greater number of B cells, and a significantly smaller number of neutrophils, are recruited to the airway lumen. In addition, there was a significantly greater production of total serum IgE and OVA-specific IgE in OVA/OVA IL-8^{-/-} mice than in similarly treated Wt mice. Finally, IL-8r OVA/PBS mice (single or multiple challenge) demonstrated a diminished airway responsiveness to methacholine compared with similarly treated Wt mice. However, in response to multiple OVA challenge, IL-8^{-/-} mice developed an increase in airway responsiveness that was comparable to that observed in similarly treated Wt mice.

Because IL-8 is thought to be an important proinflammatory mediator that may contribute to the development of airway hyperresponsiveness (14), we reasoned that IL-8 receptor homologue knockout mice IL-8^{-/-} would exhibit a diminished capacity to manifest airway hyperresponsiveness when sensitized and challenged with OVA antigen. Furthermore, because IL-8 is a strong chemoattractant for T lymphocytes (10, 11, 35), we hypothesized that in the absence of the IL-8 receptor homologue, fewer T cells would migrate into the airways, thus resulting in a lesser degree of airway responsiveness. Several studies have demonstrated the importance of T cells on the induction of airway responsiveness (29, 36–38). We have previously investigated the role of hematopoietic cells in general, and T cells specifically, in the genetic modulation of native airway responsiveness in mice. In that study (29), we demonstrated that intrinsic nonatopic airways hyperresponsiveness is mediated by T lymphocytes. A primary role for T cells in allergen-induced airway hyperresponsiveness in the mouse has been reported by other investigators examining T cell-deficient mice (36) and in studies examining the effect of T-cell elimination. The induction of airways hyperresponsiveness has also been shown by the passive transfer of lymphocytes from OVA-sensitized BALB/c mice into syngeneic nonimmune mice (37), and by the transfer of Vβ 8.1/8.2 T cells from sensitized mice into naive syngeneic recipients (38). Although these studies indicate that T cells are important in the induction of airways responsiveness, they do not explain the observations in our study. Specifically, although the IL-8^{-/-} OVA/OVA-7d-treated mice were significantly less responsive to methacholine challenge than the Wt OVA/OVA treatment group, there was a similar decrease in responsiveness between the OVA/PBS-7d control groups. This finding suggests that there are IL-8 receptor homologue-responsive elements that contribute to airway responsiveness, in the absence of antigen challenge, through a yet unknown mechanism.

When the OVA/OVA-7d groups for either genotype were compared with their respective OVA/PBS-7d controls, there was a significant increase in airway responsiveness in both groups, indicating that the ability of the IL-8^{-/-} homologue mice to mount an increase in airway hyperreactivity after OVA sensitization and challenge was similar to that of the Wt mice. This occurred despite a significantly greater levels of total and OVA-specific IgE and B cells in the BAL of IL-8^{-/-} mice. The similarity in airway responsiveness is surprising given that several

studies have reported a close relationship between airway responsiveness and serum IgE levels in humans (39, 40) and mice (41). On the basis of these findings, one might have predicted a greater increase in airway responsiveness in the IL-8^{-/-} mice, which presented with significantly higher levels of both total and antigen-specific IgE.

In view of the observation that IL-8 has been reported to inhibit IgE production *in vitro* (42, 43), we hypothesized that in the absence of the IL-8 receptor homologue, IgE antibody titers would be increased. Indeed, significantly higher serum concentrations of total and OVA-specific IgE were observed in IL-8^{-/-} mice sensitized and challenged with aerosolized OVA than in the Wt OVA/OVA group.

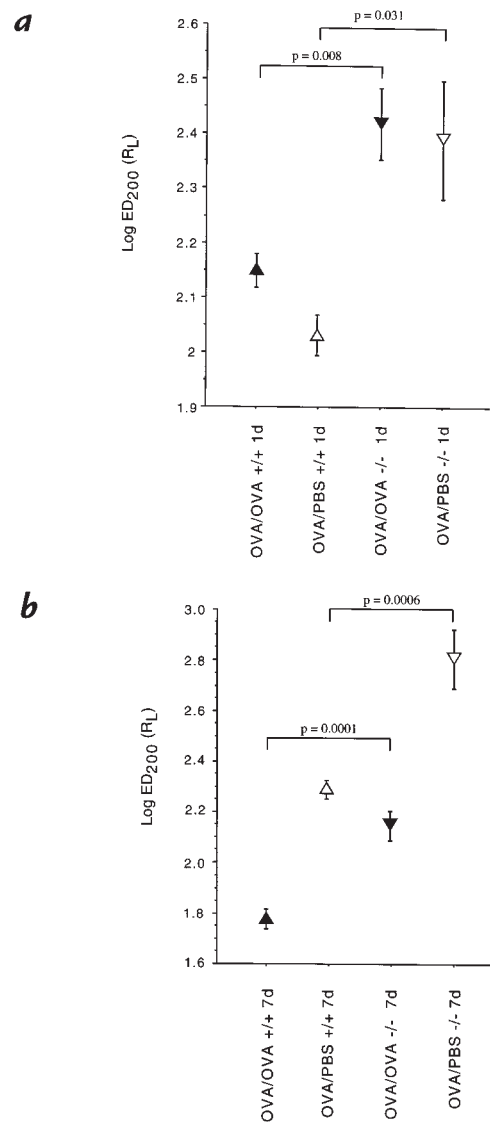


Figure 6 Airway responsiveness to methacholine. Airway responsiveness to methacholine was measured in singly (a) and multiply (b) challenged Wt and IL-8^{-/-} mice. Data are expressed as the log ED₂₀₀ R_L, calculated by linear interpolation of the methacholine dose vs. pulmonary resistance dose-response curves. Data represent mean ± SEM of the log ED₂₀₀ R_L from the eight groups of mice (minimum of six mice per group) from a representative experiment. R_L, lung resistance.

The higher *in vivo* increase in total and OVA-specific IgE in the IL-8^{-/-} OVA/OVA mice is consistent with the findings by two recent *in vitro* studies in which IL-8 was shown to selectively inhibit IL-4-induced IgE production in human B cells (43) and spontaneous IgE and IgG4 production in cultured B cells from atopic patients (42). The absence of the IL-8-mediated inhibition of IgE production in these IL-8^{-/-} mice most likely accounts for the significantly greater increase in serum IgE.

The elevation in total and OVA-specific IgE in OVA/OVA-7d IL-8^{-/-} mice compared with similarly treated Wt mice was not associated with pathological differences on lung histology. The accumulating evidence suggests that in murine models of asthma, the end points of airway hyperresponsiveness and pulmonary eosinophilia can develop by at least two distinct pathways: a mast cell-dependent pathway and a CD4⁺ T cell-dependent pathway (44, 45). The mast cell-dependent pathway is believed to result from the release of inflammatory mediators (e.g., tryptase, leukotrienes) from mast cells, which act in the airway microenvironment to increase responsiveness. Mast cell-dependent airway hyperresponsiveness has been demonstrated both in the presence (46) or absence (47) of a sensitizing antigen or after the passive transfer of antigen-specific antibodies (46, 48). Other studies (23, 49–51), however, clearly demonstrate that airway hyperresponsiveness can occur independently of IgE or mast cells. In these studies, it is presumed that airway hyperresponsiveness results from the recruitment of eosinophils to the lung by antigen-specific T cells, with the subsequent release of eosinophil-derived inflammatory and cytotoxic mediators (e.g., major basic protein, eosinophil cationic protein, and leukotrienes) in the bronchial mucosa. The lack of pathological differences in multiply challenged mice in the current study, despite a marked increase in IgE, may be due to the fact that the maximal recruitment of eosinophils to the lung had already been achieved, and, therefore, additional synergistic activity of IgE could not be demonstrated.

The significant increase in B cells in the BAL fluid of the IL-8^{-/-} mice is surprising, given that IL-8 has been shown *in vitro* to be a potent B-cell chemoattractant (8). In the absence of the IL-8 receptor, one might have anticipated a decrease, not an increase, in the numbers of infiltrating B cells. In a previous study investigating the proportion of B220⁺ lymphocytes in cervical lymph nodes in untreated IL-8^{-/-} homologue mice, Cacalano *et al.* (16) demonstrated a 1.9-fold greater proportion in B cells by flow cytometry than in Wt controls. Both Wt and knockout OVA/OVA groups exhibited similar degrees of lung inflammation and comparable percentages of eosinophils and eosinophil peroxidase in BAL. The similarity in the percentages and numbers of eosinophils in the BAL fluid and the similar change in enhanced airway reactivity in both OVA/OVA groups are consistent with the findings of a significant correlation between airway eosinophilia and airway hyperresponsiveness reported by others (52, 53).

In conclusion, these findings suggest an important role for the murine homologue of the IL-8 receptor and/or its ligands in modulating baseline airway responsiveness. In addition, the absence of the IL-8 homologue

significantly increases antigen-specific IgE production and modulates cellular recruitment to the airway lumen in the OVA sensitization and aerosol challenge model. These novel findings suggest an inhibitory role for the IL-8 receptor homologue in regulating B-cell expansion, B-cell migration into the airspaces, and IgE production in this murine model of asthma.

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