

# IL-13 Regulates the Immune Response to Inhaled Antigens<sup>1</sup>

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The large inhibitory effect of IL-13 blockers on the asthma phenotype prompted us to ask whether IL-13 would play a role in regulating the allergic immune response in addition to its documented effects on structural pulmonary cells. Because IL-13 does not interact with murine T or B cells, but with monocytes, macrophages, and dendritic cells (DCs), we examined the role of IL-13 in the activation of pulmonary macrophages and DCs and in the priming of an immune response to a harmless, inhaled Ag. We found that a majority of cells called "alveolar or interstitial macrophages" express CD11c at high levels (CD11c<sup>high</sup>) and are a mixture of at least two cell types as follows: 1) cells of a mixed phenotype expressing DC and macrophage markers (CD11c, CD205, and F4/80) but little MHC class II (MHC II); and 2) DC-like cells expressing CD11c, CD205, MHC II, and costimulatory molecules. Endogenous IL-13 was necessary to induce and sustain the increase in MHC II and CD40 expression by pulmonary CD11c<sup>high</sup> cells, demonstrated by giving an IL-13 inhibitor as a measure of prevention or reversal to allergen-primed and -challenged mice. Conversely, IL-13 given by inhalation to naive mice increased the expression of MHC II and costimulatory molecules by CD11c<sup>high</sup> cells in an IL-4R $\alpha$ -dependent manner. We found that exogenous IL-13 exaggerated the immune and inflammatory responses to an inhaled, harmless Ag, whereas endogenous IL-13 was necessary for the priming of naive mice with an inhaled, harmless Ag. These data indicate that blockade of IL-13 may have therapeutic potential for controlling the immune response to inhaled Ags. *The Journal of Immunology*, 2005, 174: 8097–8105.

Interleukin-13 is a major effector cytokine in mouse models of inflammatory lung injury. IL-13 controls the development of airway hyperreactivity, mucous cell hyperplasia and airway inflammation (1–8), emphysema, and fibrosis (9). The evidence that IL-13 is also a critical mediator in human asthma is indirect. Increased levels of IL-13 in the asthmatic lungs (10), a correlation of polymorphisms in the *IL-13* gene with asthma and allergy (11, 12), and increased levels of IL-13 intermediaries, e.g., chitinases (13), in the asthmatic airways were demonstrated.

IL-13 induces these changes in the lungs by interacting directly with structural cells and by elaborating intermediary growth factors (4, 5, 9, 13). The puzzling finding is that a closely related cytokine, IL-4, is not as significant as IL-13 in mediating the asthma phenotype (6, 7), although IL-4 shares the same receptor with IL-13; and IL-4, but not IL-13, induces differentiation of Th2 cells and class switching to IgE, the immunological hallmarks of asthma (10).

We hypothesized that, in addition to the effects on structural pulmonary cells, IL-13 would also have effects on immune cells, providing IL-13-mediated control over the immune response to inhaled Ags. If IL-13, just like IL-4, would have the ability to control the allergic immune response as well as the production of harmful mediators, and responses of structural cells in the lungs, then even small differences in the bioavailability between IL-13 and IL-4 would determine the significance of each cytokine in inducing lung inflammation and remodeling. In the mouse, thus far, all available evidence has shown that the IL-13R $\alpha$ 1 and IL-4R $\alpha$  that make up the IL-13R (also known as IL-4 type II receptor) are expressed by myeloid cells but not by B cells or T cells. The effects of IL-4 on T and B cells are mediated by the IL-4 type I receptor comprised of the common  $\gamma$ -chain ( $\gamma_c$ )<sup>3</sup> and the IL-4R $\alpha$  (14).

IL-13 has been shown to affect the activation and maturation of dendritic cells (DCs), macrophages, and their precursors in the blood (monocytes) and bone marrow (BM) (14–17), as demonstrated by in vitro and in vivo experiments using human and mouse cells. IL-13, like IL-4, amplifies and directs the signals that myeloid DC precursors receive when exposed to GM-CSF (14). In the lungs, DCs have been shown to be critically important for the priming of immune responses to inhaled Ags, such as microbial Ags or allergens (18–22). Furthermore, Constant et al. (23) have shown that resident pulmonary APCs are sufficient for the priming and effector functions of an allergen-specific immune response. The lungs are populated by alveolar and interstitial macrophages (24). In naive animals, alveolar macrophages are the major cell type in the airspaces that is washed out by bronchoalveolar lavage (BAL). Unlike pulmonary DCs, resting alveolar macrophages are

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<sup>3</sup>Abbreviations used in this paper:  $\gamma_c$ , common  $\gamma$ -chain (common  $\gamma$  cytokine receptor); DC, dendritic cell; BM, bone marrow; BAL, bronchoalveolar lavage; MHC II, MHC class II; Asp. Ag, *Aspergillus fumigatus* Ag; DT, diphtheria toxin; i.n., intranasal; KO, knockout.

poor inducers of T cell proliferation and inhibit Ag-specific T cell proliferation (25–27). However, alveolar macrophages isolated from inflamed lungs can induce T cell responses (28–30).

We hypothesized that in the lungs, DCs and macrophages as well as their common precursor, monocytes, may be particularly well prepared to respond to IL-13 signals and that allergen-induced or exogenous IL-13 would induce in pulmonary DCs the expression of MHC class II (MHC II) and costimulatory molecules (e.g., CD40 and CD86), thereby promoting the development of immune responses to inhaled Ags. We planned to distinguish pulmonary myeloid DCs by their expression of high levels of CD11c. CD11c-expressing cells in mouse spleens and lymph nodes are DCs that have been shown to be necessary for the priming of protective CD8<sup>+</sup> cytotoxic T cell response to microbial Ags (31). We hypothesized that alveolar and interstitial macrophages could be identified by their expression of F4/80 (32). We chose to test our hypotheses using *Aspergillus fumigatus* Ag (Asp. Ag), which induces inflammatory responses upon inhalation in naive mice (33), and OVA, a harmless Ag that induces no or only very mild inflammation in naive mice. We chose to concentrate on Th2 immune responses, one component of the immune response to allergens in asthmatic individuals, because of the complexity of the commonly seen mixed Th1 and Th2 responses in asthmatic patients (reviewed in Ref. 34).

## Materials and Methods

### Reagents

Recombinant mouse IL-13 and the IL-13 inhibitor IL-13Rα2-Fc (35) (both LPS negative) were the generous gift of Dr. D. Donaldson and the group of Dr. S. J. Goldman at Wyeth Research Institute (Cambridge, MA); recombinant mouse IL-13 was also purchased from PeproTech. The Asp. Ag (LPS negative) was the generous gift of Dr. V. P. Kurup (Medical College of Wisconsin, Veterans Affairs Medical Center, Milwaukee, WI). Diphtheria toxin (DT) was from Calbiochem; collagenase type 4 was from Worthington Biochemical; DNase I and BSA (both low in LPS) was from Sigma-Aldrich; and the LPS detection kit (E-toxate) were from Sigma-Aldrich. All reagents were diluted in buffers that were low in LPS. OVA from Worthington Biochemical contained >0.06 U/ml LPS in an OVA solution of 0.3 mg/ml; therefore, the concentration used for the intranasal (i.n.) application (1 mg/ml) contained LPS. OVA from Seikugaku contained >0.06 U/mg LPS at an OVA concentration of 5 mg/ml; but LPS was undetectable at an OVA concentration of 1.8 or 0.6 mg/ml. Therefore, the concentration of OVA (1 mg/ml) used for i.n. application did not contain detectable levels of LPS.

### Mice

Mice were kept under specific pathogen-free conditions in isolator cages and fed autoclaved water and sterilized standard mouse chow. Experiments were performed with approval of the institutional board. C57BL/6 and BALB/c wild-type, DO11.10 transgenic, and transgenic mice expressing GFP and the DT receptor under the control of the CD11c promoter

(C57BL/6) originally made by Dr. S. Jung (31) were from The Jackson Laboratory. The 129SvEv wild-type and RAG2-deficient mice were from Taconic Farms. IL-4Rα knockout (KO) mice (BALB/c) were the generous gift of Dr. M. Mohrs (Trudeau Institute, Saranac Lake, NY), and γ<sub>c</sub> KO mice on a RAG2-KO (γ<sub>c</sub> × RAG KO) were generously provided by Dr. J. P. Di Santo (Institute Pasteur, Paris, France) and backcrossed to 129SvEv in Dr. D. Rennick's (DNAX Research Institute, Los Altos, CA) laboratory. IL-13-KO mice (BALB/c) were made by Dr. A. N. J. McKenzie and generously provided by Dr. D. Umetsu (Stanford University, Stanford, CA). IL-5-KO mice (BALB/c) were backcrossed and generously provided by Dr. P. S. Foster (Australian National University, Canberra, Australia). CD205KO mice (C57BL/6) were made and generously provided by Dr. M. Nussenzweig (Rockefeller University, New York, NY).

### Phenotypic evaluation of BAL cells

BAL cells and single cells prepared by digestion of lung tissue were examined for the expression of CD11c, F4/80, MHC II, CD40, and CD86 by flow cytometry. In one study, peritoneal lavage cells were analyzed side by side with BAL cells.

**BAL and lung cell suspensions.** The mice were euthanized by an overdose of ketamine/xylazine. BAL was performed through a tracheal catheter by inserting and removing three aliquots of 1 ml of HBSS (33). The lungs were transferred into HBSS. Lungs were minced and incubated in HBSS containing collagenase type 4 (1 mg/ml), DNase I (0.13 mg/ml), and FCS (1%) on ice for 60 min (36). The tissues were passed through a 100-μm pore wire mesh and a 70-μm strainer using PBS containing EDTA (10 mM) and DNase I (0.18 mg/ml). BAL and lung cells were fixed in 4% formaldehyde for 20 min at room temperature, washed, stored in FACS buffer (PBS, 0.5% BSA, and 1 mM sodium azide) at 4°C, labeled within 24 h, and analyzed within the next 3 days (33).

**Peritoneal lavage cells.** Peritoneal lavage was performed by gently inserting 10 ml of HBSS solution into the peritoneal cavity using a syringe attached to a blunted catheter. The wash fluid was gently retrieved, and the cells were collected by centrifugation, fixed, and processed as described above.

**Flow cytometry.** The mAbs used for staining are shown in Table I. Non-specific labeling was quenched by preparing the appropriate dilutions of specific mAbs in FACS buffer containing anti-CD16/32 (BD Pharmingen) and 10% normal rat Ig (Jackson ImmunoResearch Laboratories). For the staining with the anti-CD205 mAb, 10% normal mouse Ig (Jackson ImmunoResearch Laboratories) was used instead of anti-CD16/32 and rat Ig. The cells were incubated with mAbs for 20 min at room temperature. The cells were analyzed with a FACSCalibur flow cytometer and CellQuest software (BD Biosciences).

### Evaluation of the Ag presentation ability of BAL cells

BAL cells were pulsed with Ag (OVA) in vivo or in vitro and then cultured together with OVA-specific T cells. The ability of the BAL cells to present Ag was evaluated by assessing T cell proliferation. Culture medium was RPMI 1640 supplemented with 10% FCS, HEPES (1 mM), pyruvate (1 mM), glutamine (2 mM), penicillin (50 U/ml), streptomycin (50 μg/ml), and 2-ME (10 μM).

**In vivo OVA delivery.** BALB/c mice were given 50 μg of OVA (Worthington Biochemical; LPS containing) in 50 μl of PBS i.n.; 18 h later, BAL was harvested. The BAL cell counts were between 15,000 and 40,000 cells per sample. The BAL cells from individual animals were transferred into

Table I. Abs used for the analysis of BAL and lung cells by flow cytometry<sup>a</sup>

| Specificity    | Clone      | Supplier      | Fluorochrome   | Secondary Reagent |
|----------------|------------|---------------|----------------|-------------------|
| CD11c          | HL3        | BD Pharmingen | FITC, PE, APCy |                   |
| F4/80          | CL:A3-1    | Serotec       | PE, biotin     | SAV-PerCP         |
| MHC II         | 25-9-17    | BD Pharmingen | FITC, biotin   | SAV-PerCP         |
|                | M5/14.15.2 | eBioscience   | FITC           |                   |
| CD40           | IC10       | eBioscience   | APCy           |                   |
| CD11b          | M1/70      | BD Pharmingen | FITC, PE       |                   |
| CD86           | GL1        | BD Pharmingen | PE             |                   |
| CD205          | NLDC-145   | Serotec       | Purified       | Anti-rat Ig       |
| Anti-rat Ig    | G28-5      | BD Pharmingen | Biotin         | SAV-PE            |
| Clonotypic TCR | KJ1-26     | BD Pharmingen | PE             |                   |
| CD19           | 1D3        | BD Pharmingen | PE, APCy       |                   |

<sup>a</sup> mAbs labeled with FITC, PE, biotin, or allophycocyanin (APCy) were purchased from BD Pharmingen (BD Biosciences), eBioscience, and Serotec. Biotinylated mAbs were detected with PerCP-labeled or PE-labeled streptavidin (SAV; BD Pharmingen).

single wells in 96-well plates (100  $\mu$ l of medium per well). A 1/10 dilution of each BAL sample was made by removing 10  $\mu$ l of the cell suspension to a duplicate well containing 90  $\mu$ l of medium. The culture volume was adjusted to 130  $\mu$ l/well.

**In vitro OVA delivery.** BAL cells were isolated from BALB/c mice, pooled, resuspended in medium, and transferred into a 96-well plate at 40,000 cells/well. A 1/10 dilution of the BAL cells was made by removing 10  $\mu$ l of the cell suspension to a duplicate well containing 90  $\mu$ l of medium. To each well, medium or OVA (Worthington Biochemical; 25  $\mu$ g/ml, LPS containing) or OVA and LPS (Sigma-Aldrich; 83 ng/ml) were added; the final culture volume was 200  $\mu$ l. After 18 h of culture, the cells were washed and resuspended in 130  $\mu$ l of medium.

**T cell proliferation.** T cells were isolated from the spleens of DO11.10 mice using anti-CD4-labeled magnetic beads (Miltenyi Biotec) and labeled with CFSE (Molecular Probes) using standard procedures (22). The labeled T cells were washed and resuspended at  $0.5 \times 10^6$ /ml in medium; and 70  $\mu$ l was added to the wells containing BAL cells or to control wells. After coculture for 5 days, the cells were harvested and stained with a PE-labeled clonotypic anti-TCR Ab (KJ1-26).

### Protocols for allergen sensitization

**Effects of an IL-13 inhibitor on the stimulation of pulmonary CD11c<sup>high</sup> cells.** Naive mice were primed with Asp. Ag i.p. and then challenged i.n. This protocol has been shown to induce the full asthma phenotype (airway hyperreactivity, goblet cell hyperplasia, and eosinophilia) (8, 33). The mice were given the inhibitor as measure of prevention or reversal. BAL and lung CD11c<sup>high</sup> cells were examined for levels of expression of MHC II, CD40, and CD11c.

Asp. Ag exposure was as follows: Asp. Ag prepared free of living organisms and free of LPS (80  $\mu$ g in 200  $\mu$ l of PBS) (8, 33) was used to prime mice i.p. for four times, at 3- to 4-day intervals. Three to 4 days later, mice were anesthetized with isoflurane (BALB/c or 129SvEv mice) or with a combination of 0.6 mg of ketamine and 0.125 mg of xylazine given i.p., and isoflurane was given as a vapor (C57BL/6 mice) and challenged i.n. with 80  $\mu$ g of Asp. Ag in 50  $\mu$ l of PBS. Groups of mice were challenged i.n. once or twice (4 days apart).

IL-13 inhibition was as follows: The IL-13 inhibitor (IL-13R $\alpha$ 2-Fc; 0.4 mg in 150  $\mu$ l of PBS) or control protein (human Ig) (35) was given a few hours before the first i.n. Asp. Ag challenge and daily for the next 3 days (prevention); or at the time of the second i.n. Asp. Ag challenge and for the next 3 days (reversal). The mice were analyzed 18 h after the last dose of IL-13R $\alpha$ 2-Fc.

**Effects of IL-13 on the responses to inhaled OVA.** Naive wild-type mice were given OVA (preparation devoid of LPS) i.n. together with IL-13 or the control protein BSA. IL-13-KO mice were given OVA and control protein. The mice were then rested and challenged once with OVA containing LPS and twice with OVA devoid of LPS. The mice were examined for lung function, BAL eosinophils, lung inflammation, goblet cell hyperplasia, and serum Ab titers.

OVA exposure was as follows: Wild-type mice (BALB/c or C57BL/6) were anesthetized as outlined above and given 50  $\mu$ l of BSA (1 mg/ml) or IL-13 (5  $\mu$ g in PBS/BSA) i.n. The IL-13-KO mice were given 50  $\mu$ l of BSA (1 mg/ml) i.n. Twenty minutes later, the mice were given OVA (Seikugaku; 50  $\mu$ g in 50  $\mu$ l of PBS) or PBS i.n. Priming was repeated every other day for a total of three times. The mice were rested for 2 wk and then challenged i.n. once with OVA (50  $\mu$ g/50  $\mu$ l of PBS; Worthington Biochemical) and twice with OVA (50  $\mu$ g/50  $\mu$ l of PBS; Seikugaku) every other day. The mice were analyzed 1 day after the last OVA challenge for airway obstruction by measuring forced expiratory volume in 50 ms, using a commercially available system (Buxco Electronics) similar to the ones described previously (37) that apply forced breathing maneuvers to anesthetized and ventilated mice. BAL was harvested, and numbers and percentages of eosinophils were determined as we have published previously (2, 8, 33). The lungs were removed and processed by the laboratory at St. Luke's Roosevelt Hospital using standard technology. Histological sections were stained with H&E for detection of inflammation or with periodic acid-Schiff for detection of mucous cell hyperplasia. Serum was harvested and stored frozen ( $-80^\circ\text{C}$ ) until analysis.

### Determinations of Ab titers

Total serum IgE levels were determined by ELISA (33), using a capture Ab from Southern Biotechnology Associates and biotinylated detection Ab, IgE standard, and HRP-conjugated streptavidin from BD Pharmingen. OVA-specific IgG1 and IgG2a serum Ab titers were determined by coating the ELISA plates with OVA followed by addition of serum titrated in 2-fold dilutions, from 1/20 to 1/64,000. Each plate contained rows of a standard serum to ensure similar color development. The wells were then

incubated with HRP-conjugated anti-IgG1 and IgG2a Abs (Southern Biotechnology Associates). The color was developed using *o*-phenylenediamine substrate (Roche); OD<sub>490</sub> were read (reference filter at 600 nm). The OVA-specific Ab titer was determined by multiplying the last serum dilution that resulted in an OD reading of 0.1 or greater with the OD value measured. All serum samples were assayed in one experiment.

### Response of pulmonary CD11c<sup>high</sup> cells to IL-13 challenge

IL-13 (5  $\mu$ g in 50  $\mu$ l of PBS/BSA (1 mg/ml)) was administered i.n. to naive mice anesthetized as described above; control mice were given PBS/BSA (2, 8). The proteins were given once daily every second day for up to three times. Pulmonary CD11c<sup>high</sup> cells were analyzed for expression of MHC II, costimulatory molecules, and CD11c.

### Depletion of pulmonary CD11c<sup>high</sup> cells of transgenic mice expressing the DT receptor under a CD11c promoter

Intact lungs or lung cells from naive mice given control protein or IL-13 or primed mice challenged with Asp. Ag were examined by confocal microscopy or flow cytometry for the presence of CD11c<sup>high</sup> cells.

**DT treatment.** DT was given simultaneously i.p. (4 ng/g body weight in 150  $\mu$ l of PBS) (31) and i.n. (4–8 ng/g body weight in 50  $\mu$ l of PBS) to mice anesthetized with a combination of injectable and inhaled anesthetics as described above.

**Confocal microscopy of intact lungs.** Lungs excised from anesthetized mice were air inflated through a tracheal cannula. The airway pressure was maintained constant at 5 cm of H<sub>2</sub>O (MLT844, Powerlab; ADInstruments) during the experimental period. The lung surface was kept moist with saline. The mouse lung preparation was positioned on a vibration-free air table. Confocal images of the lung were obtained using a LSM 510 Meta (Carl Zeiss MicroImaging) confocal imaging system mounted on an Axioskop 2 microscope (Zeiss). During imaging, GFP was excited at 488 nm using an argon laser (30 mW; Zeiss). Emitted fluorescence was collected using a  $\times 40$  objective lens (Acroplan  $\times 40/0.8$  W; Zeiss), passed through appropriate interference filters (BP505-530; Zeiss), and captured by photo multiplier tubes. Images were recorded at 1024  $\times$  1024 resolution and analyzed using Zeiss LSM software.

### Statistical analysis

Pairwise comparisons were performed using the two-tailed, unpaired Mann-Whitney *U* test. Multiple comparisons were performed using the unpaired Kruskal-Wallis *H* test. When significant differences were detected, the Mann-Whitney *U* test was used for comparisons of two groups. A confidence level of  $p < 0.05$  was used.

## Results

### CD11c-expressing pulmonary cells from control mice

To our surprise, we found that BAL cells and cell suspensions prepared from the whole lung contained a large percentage of cells expressing high levels of CD11c (CD11c<sup>high</sup>) (Fig. 1). Most of the cells coexpressed the macrophage marker, F4/80 at low levels and MHC II, mostly at low levels. These cells are traditionally known as alveolar macrophages and interstitial macrophages. A very small percentage of CD11c<sup>high</sup> cells expressed no F4/80 and high levels of MHC II; these cells are likely pulmonary DCs. For comparison, we analyzed peritoneal lavage cells and BAL cells side by side (Fig. 1). The comparison demonstrates the similarity in the microscopic appearance of the BAL and peritoneal lavage macrophage-like cells (Fig. 1, A and D) and the striking difference in the expression of CD11c, F4/80, and MHC II (Fig. 1, B, C, E, and F). In contrast to BAL cells, peritoneal lavage cells contain clearly distinct cell populations of DCs (expressing CD11c and MHC II) and macrophages (expressing F4/80 but not CD11c). Peritoneal lavage cells also contain MHC II-expressing B cells. Our data supported recently published work showing that murine alveolar macrophages express CD11c (38–41). The expression of both DC and macrophage markers raised the question whether BAL cells could present an exogenous Ag to CD4<sup>+</sup> T cells.

We reasoned that BAL cells would function differently when the Ag was delivered to the natural growth factor and cellular environment (in vivo, Ag delivered i.n.) or when given in overnight

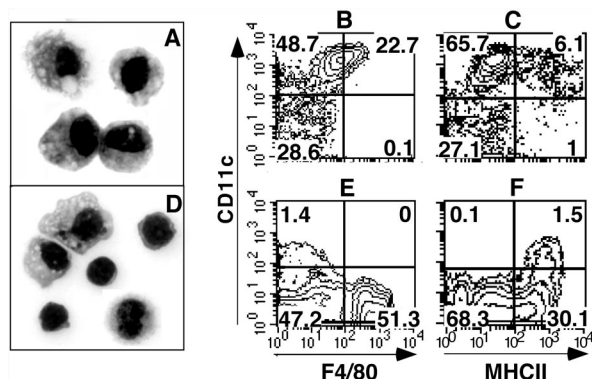


**FIGURE 1.** Comparative analysis of BAL cells and peritoneal lavage cells from naive mice. BAL cells (A–C) and peritoneal lavage cells (D–F) were examined. Photomicrographs (A and D) show the appearance of macrophage-like cells and lymphocytes (magnification,  $\times 80$ ). The cells were then analyzed by flow cytometry for expression of CD11c, F4/80, and MHC II (B, C, E, and F). The percentage of cells in each quadrant is indicated. The plots are representative of at least 10 individual mice.

cell culture (in vitro). Our data supported this hypothesis (Fig. 2) because BAL cells, isolated from naive mice that had been given OVA 18 h earlier by inhalation, readily induced T cell proliferation. In contrast, BAL cells that were isolated from naive mice and then put into culture for 18 h with OVA, or with OVA and LPS, were poor inducers of T cell proliferation as expected (25–27). These data indicate that in vivo conditions for Ag presentation in the airspaces would not be reproduced in vitro. Therefore, additional experiments to explore the role of IL-13 on pulmonary APCs were performed in vivo. The whole population of pulmonary CD11c<sup>high</sup> cells was studied because of the overlap in CD11c and MHC II expression (Fig. 1) by the alveolar macrophages expressing DC and macrophage markers and DCs.

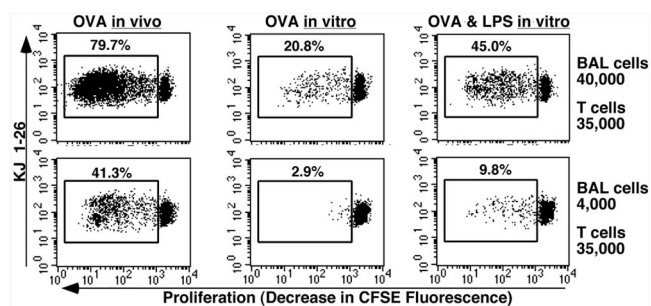


**FIGURE 2.** BAL cells induce T cell proliferation. Naive mice were given OVA i.n., and 18 h later, BAL cells were harvested (OVA in vivo). Alternatively, BAL cells were isolated from naive mice and incubated with OVA or with OVA and LPS for 18 h (OVA in vitro). The BAL cells were harvested from mice that were given inhaled OVA 18 h previously were similar in composition when compared with cells from mice that received nothing or PBS. MHC II expression remained restricted to CD11c<sup>high</sup> cells, and the numbers of CD11c<sup>high</sup> cells per sample were statistically not different (mean  $\pm$  SEM,  $3.3 \pm 0.78 \times 10^4$  vs  $6.39 \pm 2.89 \times 10^4$ ; PBS vs OVA;  $p = 0.63$ ; 9–10 mice per group). The BAL of OVA exposed mice showed a trend to an increase in the numbers of neutrophils ( $0.44 \pm 0.14 \times 10^4$  vs  $1.15 \pm 0.39 \times 10^4$ ; PBS vs OVA;  $p = 0.055$ ) and expression levels of MHC II (mean fluorescence intensity,  $110.4 \pm 11.2$  vs  $215.3 \pm 45.8$ ; PBS vs OVA;  $p = 0.066$ ). BAL cells were then cocultured for 5 days with OVA-specific DO11.10 T cells labeled with CFSE. Proliferation of OVA-specific T cells (KJ1-26-positive cells) was determined by assessing loss of CFSE fluorescence (percentages indicated). The plots are representative of 10 individual BAL samples or three pools of five BAL samples each.

### Role of IL-13 in the activation of CD11c<sup>high</sup> cells in the course of allergen-induced pulmonary inflammation

Wild-type mice were primed and challenged with Asp. Ag (8, 33) and given an IL-13 inhibitor (IL-13R $\alpha$ 2-Fc) (1, 2, 8, 35) as a measure of prevention or reversal. We found that CD11c<sup>high</sup> cells present in the BAL or in lung cell suspensions prepared from allergen-primed and -challenged mice expressed 3- to 10-fold increased levels of MHC II and CD40 (Fig. 3). When the mice were given an IL-13 inhibitor, the CD11c<sup>high</sup> cells expressed lower levels of MHC II and CD40 (Fig. 3). These data demonstrated that endogenous IL-13 is necessary for the allergen-induced relative increase in the population of CD11c<sup>high</sup> cells that express high levels of MHC II and CD40.

### Role of exogenous IL-13 in the activation of pulmonary CD11c<sup>high</sup> cells

Exogenous IL-13 changed the population of pulmonary CD11c<sup>high</sup> cells (Fig. 4). Compared with cells from mice given control protein (BSA), CD11c<sup>high</sup> cells from naive mice given IL-13 had increased expression of CD11c and CD11b, and decreased expression of F4/80. Pulmonary CD11c<sup>high</sup> cells from mice challenged with IL-13 had a large percentage of cells expressing high levels of MHC II, CD40, and CD86 (Fig. 4). CD40 and CD86 were expressed at higher levels in cells that also expressed MHC II,

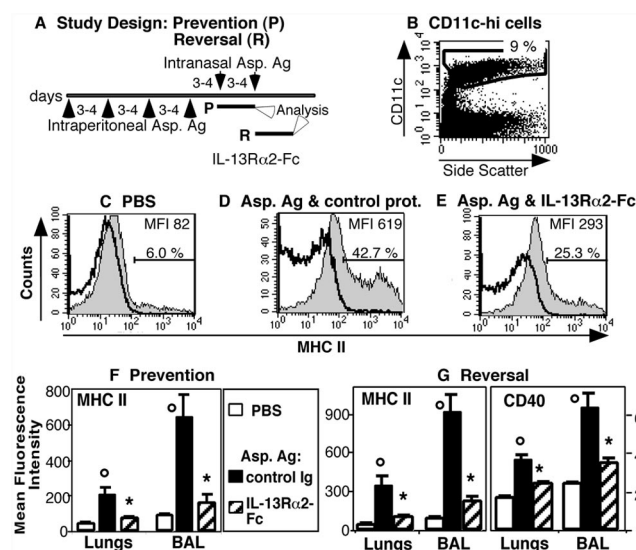
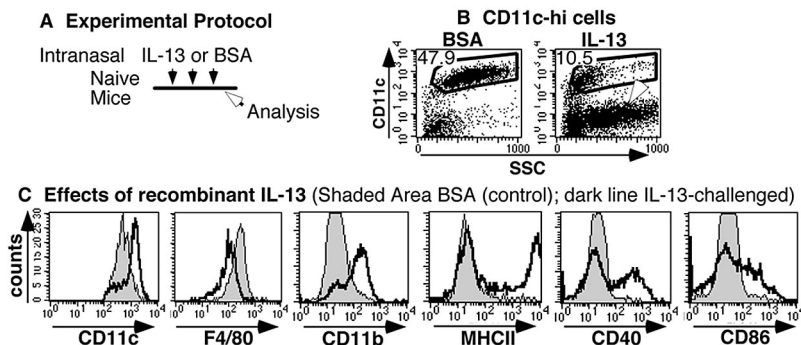


**FIGURE 3.** MHC II and CD40 expression by pulmonary CD11c<sup>high</sup> cells from allergen-primed and -challenged mice and effects of an IL-13 inhibitor. A, Experimental protocol. B, Gating strategy for the analysis of CD11c<sup>high</sup> cells in lung cell suspensions; the percentage of CD11c<sup>high</sup> cells is indicated. C–E, MHC II expression by pulmonary CD11c<sup>high</sup> cells from mice given PBS (C), Asp. Ag and control protein (D), or Asp. Ag and IL-13 inhibitor (IL-13R $\alpha$ 2-Fc) (E). The traces produced by the fluorescently labeled anti-MHC II Ab are indicated by the shaded areas, and the traces recorded with isotype control Ab are indicated by the thick lines. In each plot, the mean fluorescence intensity (MFI) of staining with the MHC II Ab is indicated, as well as the percentage of cells that express MHC II above isotype-control baseline levels. F and G, Mean fluorescence intensity of MHC II or CD40 expressed by pulmonary CD11c<sup>high</sup> cells from control and Asp. Ag-primed and -challenged mice. The IL-13 inhibitor (IL-13R $\alpha$ 2-Fc) was given as a measure of prevention (F), or reversal (G). The data are presented as means and SEM of results pooled from two to three independent experiments; 5–10 mice per group. The statistical analysis was performed using the Mann-Whitney *U* test. °, Differences ( $p < 0.05$ ) from mice primed and challenged with PBS; \*, differences ( $p < 0.05$ ) between mice primed and challenged with Asp. Ag and given control protein or IL-13 inhibitor.

**FIGURE 4.** Phenotypic analysis of pulmonary CD11c<sup>high</sup> cells from mice challenged with IL-13. **A**, Experimental protocol. **B**, Gating strategy for the analysis of CD11c<sup>high</sup> cells; the percentages of CD11c<sup>high</sup> cells are indicated. The open arrow points to a cell population containing eosinophils. **C**, Effects of challenge with IL-13. The plots are representative of 5–10 mice per group. The dark lines represent the traces of CD11c<sup>high</sup> cells from mice challenged with IL-13, the shaded areas represent the traces of CD11c<sup>high</sup> cells from mice given control protein (BSA). The plots shown in this figure are from BAL samples; similar data were obtained analyzing CD11c<sup>high</sup> cells from lung cell suspensions.



whereas no expression of F4/80 was seen in cells that expressed high levels of MHC II (data not shown). The increased expression of MHC II and costimulatory molecules by pulmonary CD11c<sup>high</sup> cells was seen in mice that were challenged with three doses of IL-13, spanning a total of 5 days. We found that the phenotypic change in the pulmonary CD11c<sup>high</sup> cells, except for the increase in the expression of CD11c, was not evident after a single challenge with IL-13 and incomplete after two challenges with IL-13 (data not shown).

#### DC phenotype in CD11c<sup>high</sup> cells from IL-13-challenged mice

Transcriptional activity of the *CD11c* gene in vivo has been used to distinguish murine splenic and lymph node myeloid DCs phenotypically and functionally from other cell types (31). Therefore, we studied mice that are transgenic for a CD11c promoter-driven reporter comprised of GFP linked to the DT receptor (Ref. 31 and Fig. 5), to test the possibility that pulmonary CD11c<sup>high</sup> cells would be DCs. Mouse cells do not express the DT receptor; therefore, only cells expressing the transgene are DT sensitive. Treatment of transgenic mice with DT led to the deletion of all GFP-positive cells in the lung parenchyma as demonstrated by confocal microscopy (Fig. 5B). These GFP-positive cells were most likely pulmonary DCs due to their typical parenchymal location (42, 43). By flow cytometry, a distinct GFP-positive cell population among the pulmonary CD11c<sup>high</sup> cells could not be defined because of the high levels of green autofluorescence in this cell type. However, the depletion of CD11c<sup>high</sup> cells in mice treated with DT could be quantified using flow cytometry by the shift to the left in intensity of green fluorescence. The loss of green fluorescence was statistically significant in pulmonary CD11c<sup>high</sup> cells isolated from mice

challenged with allergen or IL-13 but not in CD11c<sup>high</sup> cells isolated from control mice. Splenic CD11c<sup>high</sup> cells were deleted in all animals (Fig. 5C), as expected (31). Our data also clearly showed that DT treatment depleted pulmonary CD11c<sup>high</sup> cells that expressed high levels of MHC II (Fig. 5D), but due to the autofluorescence of the CD11c<sup>high</sup> cells, we were not able to determine whether CD11c<sup>high</sup> cells that expressed low levels of MHC II were depleted as well (Fig. 5D).

These data suggested that the pulmonary CD11c<sup>high</sup> cells expressing high levels of MHC II and costimulatory molecules could be DCs. This notion was confirmed by the expression of high levels of the DC marker CD205 (44) by the majority of CD11c<sup>high</sup> cells expressing high levels of MHC II from mice challenged with IL-13 (Fig. 6). Mice deficient in CD205 (Fig. 6) or isotype control Abs demonstrated the specificity of the staining with the anti-CD205 Ab. It is of note that pulmonary CD11c<sup>high</sup> cells were inhomogeneous for expression of CD205 in mice challenged with IL-13, whereas pulmonary CD11c<sup>high</sup> cells from control mice expressed uniformly high levels of CD205, further confirming the dual macrophage-DC marker expression of the majority of CD11c<sup>high</sup> cells.

#### Signaling requirements and potential secondary mediators for the IL-13-induced changes in pulmonary CD11c<sup>high</sup> cells

The effects of IL-13 likely involved secondary mediators because of the long time period (5 days) and repeated stimulation required to shift the pulmonary CD11c<sup>high</sup> cell population toward a DC phenotype. Therefore, a number of gene-deficient mice were examined to determine the signaling requirements and to test for

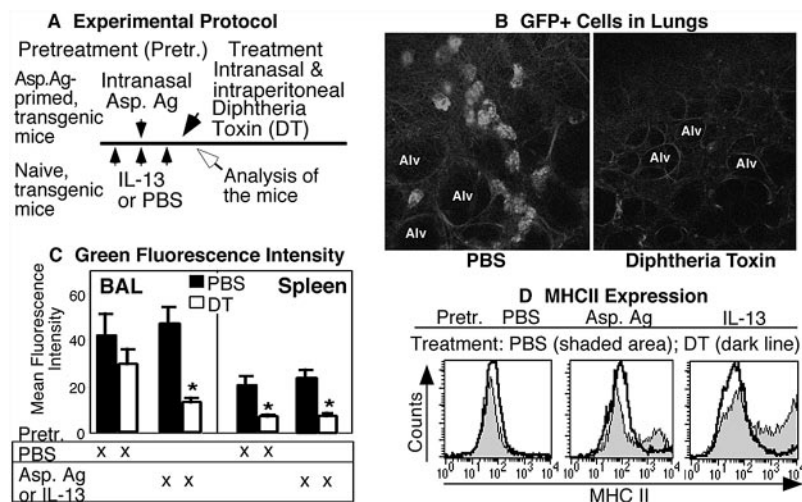


**FIGURE 5.** CD11c promoter-driven DT receptor and GFP expression in pulmonary CD11c<sup>high</sup> cells. **A**, Experimental protocol. Mice that express GFP and DT receptor under the control of the CD11c promoter (31) were examined. **B**, Detection of green fluorescent cells in intact lungs examined by confocal microscopy. **C**, Green fluorescence intensity of CD11c<sup>high</sup> cells from BAL or spleen cell suspensions examined by flow cytometry. The data are represented as means and SEMs of results pooled from three independent experiments; five to eight mice per group. Statistical significance ( $p < 0.05$ ) was determined by the two-tailed Mann-Whitney *U* test, comparing to saline (PBS)-treated mice. **D**, MHC II expression by CD11c<sup>high</sup> cells from the BAL of transgenic mice pretreated with PBS, or Asp. Ag, or IL-13. The mice were treated with DT (dark lines) or saline (PBS; shaded areas). The plots are representative of five to eight mice per group.

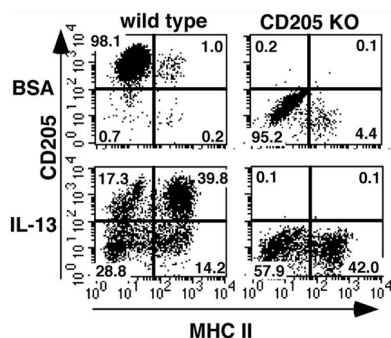


**FIGURE 6.** CD205 expression in pulmonary CD11c<sup>high</sup> cells. Naive wild-type and CD205 KO mice were given BSA or IL-13 as shown in Fig. 4A, and pulmonary CD11c<sup>high</sup> cells were gated as shown in Fig. 4B. Representative FACS plots (3–10 mice per group) show expression of MHC II (abscissa) vs expression of CD205 (ordinate). The percentage of cells in each quadrant is indicated. BAL cells and cells from lung cell suspensions demonstrated similar expression patterns.

candidate intermediary cytokines and cells (Fig. 7). IL-13 challenge of IL-4R $\alpha$ -KO mice induced no shift in the phenotype of the pulmonary CD11c<sup>high</sup> cell (Fig. 7). IL-4 is not likely to be an intermediary of the IL-13-induced changes in the CD11c<sup>high</sup> population because IL-13 challenge of mice deficient in the  $\gamma_c$  (an essential component of the IL-4 type I receptor) and RAG2 ( $\gamma_c \times$  RAG KO) demonstrated more variable but statistically indistinguishable changes in the pulmonary CD11c<sup>high</sup> cells relative to wild-type mice (Fig. 7). To exclude the possibility that endogenous IL-13 was required for the differentiation of CD11c<sup>high</sup> cells, IL-13-KO mice were examined. The phenotype of pulmonary CD11c<sup>high</sup> cells from IL-13-KO given BSA or IL-13 was similar to wild-type pulmonary CD11c<sup>high</sup> cells (Fig. 7).

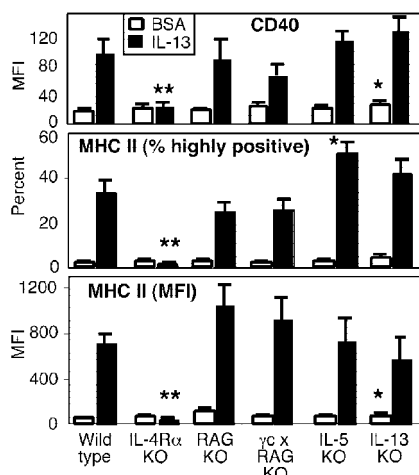


**FIGURE 7.** Effects of inhaled IL-13 on pulmonary CD11c<sup>high</sup> cells from different mouse strains. Naive wild-type and gene-deficient mice were given BSA or IL-13 as shown in Fig. 4A, and CD11c<sup>high</sup> cells from the BAL were gated as shown in Fig. 4B. Expression levels of MHC II and CD40 were determined by mean fluorescence intensity (MFI) of MHC II or CD40 staining of the CD11c<sup>high</sup> cells. The percentage of CD11c<sup>high</sup> cells that were highly positive for MHC II (Fig. 4C) was also determined. Data are shown as means and SEMs of 4–16 mice per group, and altogether the data of 112 mice are depicted. Asterisks denote significant differences to the appropriate group of wild-type mice (\*\*,  $p < 0.003$ ; \*,  $p < 0.05$ ). Note that in all groups of mice (except the IL-4R $\alpha$ -KO mice), IL-13 induced increased MHC II and CD40 expression in pulmonary CD11c<sup>high</sup> cells ( $p$  values between 0.034 and 0.000).

B cells express MHC II and costimulatory molecules. Eosinophils (45, 46) can express CD11c, MHC II, and CD40. Our studies indicated that pulmonary CD19-positive cells (B cells) did not express CD11c (data not shown), whereas eosinophils expressed low levels of CD11c (Fig. 4B). To formally exclude the possibility that IL-13-activated eosinophils or B cells would be gated with the CD11c<sup>high</sup> cells, we analyzed IL-5-KO and RAG KO mice, respectively. The phenotype of BAL CD11c<sup>high</sup> cells from IL-5-KO and RAG KO mice challenged with IL-13 (which lack eosinophils or B cells in the BAL, respectively) was similar to wild-type BAL CD11c<sup>high</sup> cells (Fig. 7).

#### *Role of exogenous and endogenous IL-13 for the development of immune responses to inhaled Ag that has no proinflammatory effects*

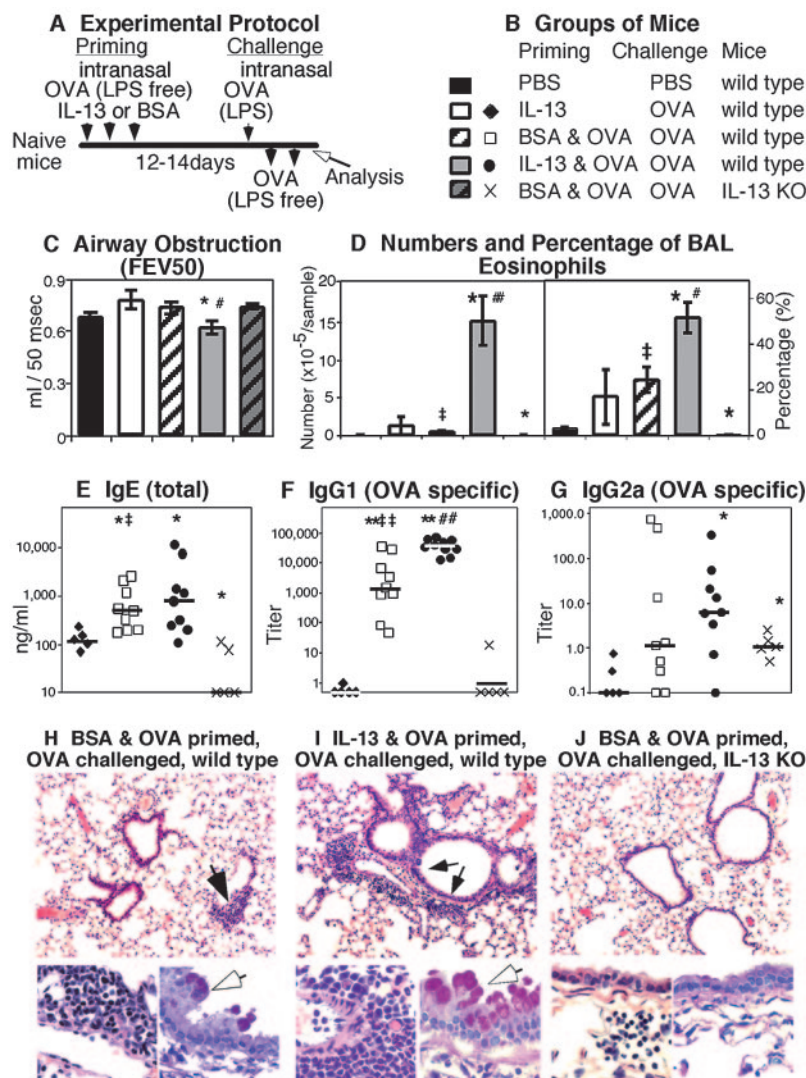
To test the hypothesis that the IL-13 induced increased expression of MHC II and costimulatory molecules by pulmonary CD11c<sup>high</sup> cells would increase the efficiency of priming via the inhalational route, mice were studied that were given i.n. an Ag that has no proinflammatory effects (LPS-free OVA). Groups of wild-type mice were coadministered IL-13 or control protein (LPS-free BSA), and a group of IL-13-KO mice was given control protein. In the current as well as in previously published studies, we have not noted proinflammatory effects of inhaled BSA (Figs. 3, 5, and 6, and Refs. 2 and 8). The recall challenge was given once with an LPS-containing OVA preparation and then with LPS-free OVA. The first recall challenge was with LPS-containing OVA because it induces a slight degree of inflammation, which is sufficient for recruiting Ag-specific T cells into the lungs of primed mice with the subsequent development of airway obstruction and inflammation (47). Our data (Fig. 8) showed that exogenous IL-13 increased the immune and inflammatory responses (airway obstruction, BAL eosinophils, OVA-specific IgG1, IgG2a titers, and total IgE, perivascular and peribronchial infiltration with mononuclear cells and eosinophils, and mucous cell hyperplasia) induced by priming and challenge with inhaled OVA. The responses of wild-type BALB/c and C57BL/6 mice were indistinguishable (data not shown). Furthermore, our data demonstrated that IL-13-KO mice primed with inhaled, harmless OVA did not develop BAL eosinophils, increases in total IgE and OVA-specific IgG1 titers, and perivascular inflammation seen in wild-type mice (Fig. 8).

## Discussion

To the best of our knowledge, we are the first to describe the critical significance of endogenous and exogenous IL-13 for the development of an immune response to inhaled Ags and for the increased expression of MHC II and costimulatory molecules by CD11c<sup>high</sup> cells in the lungs in allergen-challenged mice.

Our data are supported by the finding that endogenous IL-13 is necessary for the priming of a Th2 immune response to OVA administered epicutaneously (48) and by the studies of McKenzie et al. (49), demonstrating that mice, lacking IL-13, develop blunted Th2 and IgE Ab responses, whereas mice overexpressing IL-13 have increased IgE Ab levels (50). Our data are reminiscent of the model of murine asthma, described by Gelfand and coworkers (51), induced by priming of the immune response through repeated inhalation of OVA. In contrast to our data, there is a large body of literature that describes that inhaled OVA induces immune tolerance to the induction of IgE responses with intact IgG1 and IgG2a responses (52, 53) and tolerance to inducing eosinophilic inflammation (54). There are two explanations for these discrepancies: 1) we describe the same phenomenon (52–54) but examine the mice at different time points of the study protocol and with different levels of sensitivity of the assays; and 2) our data are different from

**FIGURE 8.** Effects of exogenous and endogenous IL-13 on the priming of an immune and inflammatory response to a harmless, inhaled, protein Ag. **A**, Experimental protocol. **B**, Groups of mice examined. **C**, Airway obstruction (forced expiratory volume in 50 ms (FEV<sub>50</sub>)). **D**, BAL eosinophils (numbers per BAL sample, or percentage of eosinophils). **E–G**, Serum Ab levels of total IgE (**E**), OVA-specific IgG1 (**F**), and OVA-specific IgG2a (**G**); each dot represents the data of an individual animal; medians are indicated by horizontal lines. **H–J**, Photomicrographs of histological sections stained with H&E for detection of inflammation or periodic acid-Schiff for detection of mucous cell hyperplasia. The photomicrographs are representative of five to nine mice per group. Dark arrows point to inflammatory infiltrates (**H** and **I**), and open arrows point to mucus-containing cells (**H** and **I**). **C–G**, Groups of mice were a mixture of BALB/c and C57BL/6, five to nine mice per group; the data are pooled from two experiments with similar outcome. Statistical analysis was performed using the unpaired Kruskal-Wallis *H* test to determine differences among multiple groups. When differences were detected, pairwise comparisons were performed using the two-tailed, unpaired Mann-Whitney *U* test. \*, *p* < 0.05, and \*\*, *p* < 0.005; differences compared with the group of mice primed with IL-13 (no OVA) and challenged with OVA. #, *p* < 0.05, and ##, *p* < 0.005; differences compared with the group of mice primed with inhaled OVA (BSA & OVA). †, *p* < 0.05, and ‡, *p* < 0.005; differences between wild-type and IL-13-KO mice primed with inhaled OVA (BSA & OVA).



the published literature on tolerance induced by inhaled OVA (52–54). Only a direct experiment comparing the different protocols would provide us with the information necessary to distinguish between the two possibilities.

Our data showed that approximately one-half of the pulmonary CD11c<sup>high</sup> cells from mice primed and challenged with allergen or from naive mice challenged with IL-13 had the phenotype of activated DCs. These cells expressed high levels of MHC II and costimulatory molecules such as CD40 and CD86, no F4/80, and used the CD11c promoter. Our data are supported by several reports. Cells isolated by BAL from human patients with inflammatory lung disease that were cytologically classified as alveolar macrophages have been shown to function similar to DCs because the cells were capable of inducing T cell responses (28, 29). Inflammatory stimuli have been shown to induce activation of alveolar macrophages (38, 39) in mice and inhibit their immunosuppressive activity (30). In a recent study, van Rijt et al. (40) suggested that the “alveolar macrophages” from allergen-challenged mice were DCs due to the increase in expression of CD11c and MHC II when compared with the cells from control mice. In our hands, only approximately one-half of the pulmonary CD11c<sup>high</sup> cells isolated from allergen-challenged or IL-13-challenged mice exhibited an activated phenotype; the other CD11c<sup>high</sup> cells were similar to the cells in control mice, with respect to the low levels of expression of MHC II and costimulatory molecules.

The heterogeneity of the pulmonary CD11c<sup>high</sup> cells from allergen-challenged and from IL-13-challenged mice was further underscored by the heterogeneous expression of CD205. We do not know the functional significance of the different populations of pulmonary CD11c<sup>high</sup> cells. Recent studies have shown that the DCs that induce Th2 responses to parasite Ags are quite different with respect to the expression levels of MHC II and costimulatory molecules when compared with DCs pulsed with Ags that induce Th1 responses (55, 56). Therefore, it is possible that the different populations of pulmonary CD11c<sup>high</sup> cells in allergen-challenged mice have a distinct but significant role in inducing and maintaining allergic lung inflammation.

The expression of CD11b by CD11c<sup>high</sup> pulmonary cells expressing high levels of MHC II and costimulatory molecules does not argue against the possibility that these cells are DCs. CD11b expression by pulmonary DCs isolated from allergen-primed and -challenged mice isolated by differential adherence and cell sorting has been reported before (36). CD11b and CD11c-positive BAL cells from allergen-challenged mice have been shown to retain Ag for presentation (57). Pulmonary CD11c<sup>high</sup> cells from Ag-challenged or IL-13-challenged mice did not express CD4 or CD8α (G. Grunig, unpublished data), demonstrating that these cells differ from major subpopulations of splenic or lymph node DCs that express either of these markers (58).

Our data suggest that the pulmonary CD11c<sup>high</sup> cells expressing low levels of MHC II and costimulatory molecules are unlikely to be macrophages because these cells expressed homogeneously DC markers, only low levels of F4/80, and were capable of Ag presentation. The pulmonary CD11c<sup>high</sup> cells in naive, unchallenged mice could be an intermediate between macrophages and DCs supported by the specific cytokine milieu in the lungs. This possibility is strengthened by our data that show the absence of this cell type from the peritoneal cavity (Fig. 1) or lymph nodes or spleens (G. Grunig, unpublished data). Further studies that account for the mobility and turnover of the pulmonary CD11c<sup>high</sup> cells and their precursors are needed to define this cell type.

IL-13 challenge of IL-4R $\alpha$ -KO mice induced no shift in the phenotype of pulmonary CD11c<sup>high</sup> cells, confirming the following: 1) the role of the IL-4R $\alpha$  as an essential part of the IL-13R; and 2) the absence of inadvertent contamination with substances such as LPS that rapidly and potently induce homogeneous up-regulation of the expression of MHC II and costimulatory molecules in pulmonary CD11c<sup>high</sup> cells (G. Grunig, unpublished data) and pulmonary DCs (59). IL-13 has been shown to be equally potent to IL-4 in inducing the differentiation of DCs from BM precursors grown with GM-CSF. GM-CSF is a constitutive and biologically active component of the lung's growth factor environment because the lack of GM-CSF has the most prominent effects in the lungs, causing abnormalities in alveolar macrophages and alveolar proteinosis in both man and mice (reviewed in Ref. 60). GM-CSF as part of the constitutive cytokine milieu of the lungs may be the basis for the IL-13-dependent control of the activation of pulmonary CD11c<sup>high</sup> cells and the immune responses to inhaled, harmless Ags.

We do not know why IL-4 did not substitute for IL-13 in allergen-challenged mice given IL-13 inhibitor. In a similar manner, others and we have found that IL-13, but not IL-4, is the critical mediator of the asthma phenotype in allergen-challenged mice (1, 2, 4, 6, 7). It is possible that IL-4 is not made at sufficient quantities and for the duration of time necessary for the optimal stimulation of CD11c<sup>high</sup> cells. Alternatively, it is possible that IL-4 signaling has different effects on CD11c<sup>high</sup> cells when compared with IL-13 signaling. This possibility would be analogous to the reported data, showing that IL-4, but not IL-13, induces the expression of IL-12 by BM-derived DCs by signals through the  $\gamma_c$  (14).

In conclusion, our data show that IL-13 was sufficient and necessary for the induction and maintenance of allergen-induced activation of pulmonary CD11c<sup>high</sup> cells. Endogenous IL-13 was required, and exogenous IL-13 enhanced the priming of an immune and inflammatory response to an inhaled, harmless Ag. Ag, presented by DCs that are minimally or not activated, induces T cell unresponsiveness instead of effector T cell responses (61). This raises the possibility that the blockade of IL-13 in a therapeutic setting may decrease the activation levels of pulmonary APCs, thereby altering the course of asthma by changing the immune responses to inhaled allergens.

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## Disclosures

D. D. Donaldson was an employee of Wyeth Research during the time most of the study was performed.

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