

Comparative Roles of IL-4, IL-13, and IL-4R α in Dendritic Cell Maturation and CD4⁺ Th2 Cell Function¹

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IL-4 and IL-13 play key roles in Th2 immunity and asthma pathogenesis. Although the function of these cytokines is partially linked through their shared use of IL-4R α for signaling, the interplay between these cytokines in the development of memory Th2 responses is not well delineated. In this investigation, we show that both IL-4 and IL-13 influence the maturation of dendritic cells (DC) in the lung and their ability to regulate secretion of IFN- γ and Th2 cytokines by memory CD4⁺ T cells. Cocultures of wild-type T cells with pulmonary DC from allergic, cytokine-deficient mice demonstrated that IL-4 enhanced the capacity of DC to stimulate T cell secretion of Th2 cytokines, whereas IL-13 enhanced the capacity of DC to suppress T cell secretion of IFN- γ . Because IL-4R α is critical for IL-4 and IL-13 signaling, we also determined how variants of IL-4R α influenced immune cell function. T cells derived from allergic mice expressing a high-affinity IL-4R α variant produced higher levels of IL-5 and IL-13 compared with T cells derived from allergic mice expressing a low-affinity IL-4R α variant. Although DC expressing different IL-4R α variants did not differ in their capacity to influence Th2 cytokine production, they varied in their capacity to inhibit IFN- γ production by T cells. Thus, IL-4 and IL-13 differentially regulate DC function and the way these cells regulate T cells. The affinity of IL-4R α also appears to be a determinant in the balance between Th2 and IFN- γ responses and thus the severity of allergic disease. *The Journal of Immunology*, 2007, 178: 219–227.

Asthma is a chronic inflammatory disease in which CD4⁺ Th2 lymphocytes form an important component of the cellular infiltrate to the pulmonary compartment (1, 2). The Th2 cytokines produced by these cells are thought to induce asthma-associated pathology, of which eosinophilia, allergen-specific IgE, mucus hypersecretion, airway remodeling, and changes in the contractility of the smooth muscle layer surrounding the airways are key phenotypic features.

The intense research effort to elucidate the mechanisms by which Th2 cytokines regulate these processes has focused in recent times on IL-4 (3–8) and IL-13 (8–10), cytokines that are functionally linked by their requirement for the IL-4 receptor α subunit (IL-4R α) for signaling. The type I receptor comprising IL-4R α and the γ common chain is expressed by hemopoietic cells and is exclusively responsive to IL-4. IL-4 is pivotal in down-regulating IFN- γ gene transcription in early activated lymphocytes to induce differentiation to a Th2 bias (11) and in switching B cells to IgE production (12, 13). In contrast, the type II receptor comprising IL-4R α and IL-13R α 1 is responsive to both IL-4 and IL-13 (14) and is expressed by a diverse range of cells including those of nonhemopoietic origin (15, 16). However, despite IL-4 and IL-13 potentially targeting common cells expressing the type II receptor, the transient early peak in the level of IL-4 compared with

the delayed, but more sustained, expression of IL-13 following T cell stimulation (17, 18) suggests that the latter cytokine plays a more predominant role in downstream effector responses. In fact, experimental models have shown that the IL-13 produced by IL-4-biased Th2 cells is a pluripotent asthma-inducing molecule that stimulates eotaxin-regulated pulmonary eosinophilia, mucus hypersecretion, airways hyperreactivity (AHR),³ remodeling, and a cascade of proinflammatory mediators (9, 10, 19–23).

Although the potency of IL-13 in regulating asthma pathology is widely recognized, the role that this cytokine plays in regulating T cell function is less clear. T cells do not bind IL-13 or express IL-13R α 1 (17), although IL-13 stimulation of anti-CD3-activated splenocytes can drive Th2 differentiation in a STAT6-dependent manner (24). The explanation of this apparent paradox may be that IL-13 acts on a cell population in these cultures that promotes Th2 development. Notably, very recent studies suggest that IL-13 regulates the maturation of dendritic cells (DC) (25), which in turn may modulate T cell function.

DC play a critical role in the development of pulmonary inflammation in experimentally induced allergic airways disease. The dampening of AHR, inflammation, cytokine production, and fibrosis that occurs following sustained Ag exposure and aeroallergen tolerance is paralleled by a reduction in the numbers of myeloid CD80- and CD86-expressing DC in the lungs (26). However, instillation of bone marrow-derived DC into mice in which this dampening has occurred restores AHR and eosinophilic inflammation (26). Furthermore, depletion of CD11c⁺ DC during aeroallergen challenge abolishes AHR, inflammation, and the hypersecretion of mucus (27).

Characterization of the role of IL-4 and IL-13 in regulating the phenotype of DC that drive allergic responses and defining mechanisms that switch their maturation from Ag-uptake to Ag-presenting

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³ Abbreviations used in this paper: AHR, airways hyperreactivity; DC, dendritic cell; MHCII, MHC class II; WT, wild type; BALF, bronchoalveolar lavage fluid; MLN, mediastinal lymph node.

modes is central to understanding the development of allergic disease. However, mixed conclusions have been drawn from studies concerning the comparative power of either IL-4 or IL-13 in regulating the maturation of DC. Ahn and Agrawal (28) demonstrated that IL-4 was more effective than IL-13 in inducing expression of the surface molecules DEC-205, CD86, and MHC class II (MHCII) on DC matured *in vitro*, and this correlated with higher endocytic activity and greater efficacy at inducing allogeneic T cell proliferation. In contrast, other studies have shown that IL-4 and IL-13 were similarly effective in generating mature functional DC *in vitro* in an IL-4R α -dependent manner (29, 30). Notably, few studies have examined the roles of these cytokines and the influence of IL-4R α on the maturation of endogenous DC in the allergic lung and their role in generating Th2 responses. Padilla et al. (25) showed that administering an IL-13 inhibitor (sIL-13R α 2) to mice before or during aeroallergen exposure inhibited maturation of DC found within the conducting airways. These authors suggested that neutralization of IL-13 function was sufficient to inhibit DC maturation and that the contribution of IL-4 was limited. However, this study did not address the influence of these cytokines on DC maturation in the tissue compartment of the lung and did not specifically assess the impact that IL-4 deficiency has on the phenotypic and functional maturation of pulmonary DC during the development of allergic airways disease. Furthermore, how cytokine deficiency at initiation of sensitization rather than just during the allergen challenge phase impacts on DC maturation was not investigated. This temporal aspect of cytokine use is an important factor, considering that activated immune cells generated during sensitization drive secondary immune responses in the lung and produce factors that are likely to enhance localized activation of DC during allergen inhalation. We propose that another important determinant of DC maturation and effector function (i.e., the ability to engage, activate, and differentiate CD4⁺ T cell subtypes) is the molecular structure of IL-4R α . We have recently shown that polymorphisms in IL-4R α can lead to discrete changes in the ability of the immune system to generate defined aspects of allergic disease. For example, the capacity of IL-4 to substitute for IL-13 is dependent on the variant of IL-4R α that is expressed in IL-13^{-/-} mice and its avidity for IL-4 (31). Thus, differential use of ligands in combination with the molecular structure of IL-4R α may critically determine DC and subsequent CD4⁺ T cell function and the development of allergic disease.

The aims of the current study were to 1) directly compare the phenotypic maturation of DC in the lung (nonlymphoid) compartment during allergic airway inflammation in mice deficient in either IL-4 or IL-13 and 2) establish the capacity of DC from these cytokine-deficient mice to stimulate cytokine production by memory CD4⁺ T cells. Furthermore, by generating congenic mice expressing different polymorphic forms of IL-4R α , we also address the important issue of whether subtle molecular differences in this receptor component, and thus signaling potential, impact on DC activation and CD4⁺ Th2 cell function.

Materials and Methods

Mouse strains

Wild-type (WT) BALB/c mice were obtained from the Animal Resource Centre (Canning Vale, Western Australia). N5 BALB/c IL-13^{-/-} mice were supplied by Dr. A. McKenzie (Medical Research Council Laboratories, Cambridge, U.K.) (32). These mice were crossed for an additional five generations onto the BALB/c background. BALB/c IL-4^{-/-} mice were supplied by Dr. N. Noben-Trauth (National Institutes of Health, Bethesda, MD) (33). Mice expressing variants of IL-4R α were derived from N5 IL-13^{-/-} mice (32), which express the C57BL/6 form of IL-4R α (31). To generate congenic WT mice expressing either the BALB/c or the C57BL/6 form of IL-4R α , N5 IL-13^{-/-} mice were crossed with WT BALB/c mice.

Heterozygous F₁ offspring were then interbred and F₂ pups were positively selected by PCR screening for IL-13^{+/-}, and then further screened by *Eco47III* digestion of PCR-amplified exon 4 of IL-4R α , which distinguishes the two isoforms of IL-4R α (31).

Induction of allergic airways inflammation

Allergic airways inflammation was induced in equal numbers of 6- to 8-wk-old male and female mice by i.p. sensitization (day 0) with 50 μ g of OVA (fraction V; Sigma-Aldrich) mixed with 20 μ l of Rehydralgel (2% Al₂O₃, binding capacity 2.5 mg of BSA/mg; Reheis) made up to a total volume of 200 μ l with 0.9% sterile saline. Control mice received the same inoculum without OVA. On days 12, 14, 16, and 19, all mice were challenged with an aerosol of 10 mg/ml OVA in 0.9% saline for three times for 30 min/day with 30-min breaks between aerosols as previously described (34). Twenty-four hours after the last challenge, mice were sacrificed by cervical dislocation and the lungs were either removed for histology or the bronchoalveolar lavage fluid (BALF) was obtained by cannulating the trachea and gently flushing the airways with two 1-ml volumes of PBS. All mice were treated according to Australian National University Animal Welfare guidelines (Protocol JMB18/04) and were housed in a specific pathogen-free facility.

Enumeration of eosinophils and mucin-producing cells

Lungs were fixed in 10% phosphate-buffered formalin, sectioned, and then stained with Carbol's chromotrope-hematoxylin, and the number of eosinophils in the peribronchial region was counted. Sections were also stained with Alcian blue periodic-acid Schiff for the enumeration of mucin-containing cells. Leukocytes in blood smears and BALF cytopins were identified by morphological criteria and quantitated as previously described (8).

Measurement of Ag-specific cytokine production by draining lymph nodes

Cells from the mediastinal lymph nodes (MLN) were isolated, washed in MLC medium (DMEM; Invitrogen Life Technologies) with 10% heat-inactivated FBS, 4 mg/ml D-glucose, 13.6 μ M folic acid, 2.7 μ M L-asparagine, 0.67 mM L-arginine, 2 mM glutamine, 2 mg/ml sodium (HCO₃)₂, 1 mM sodium pyruvate, 10 mM HEPES (pH 7.4), 27 μ M 2-ME, 1/1000 PSN antibiotics (Invitrogen Life Technologies), and 1/1000 Fungizone (Invitrogen Life Technologies) and stimulated with 1 mg/ml OVA in MLC medium for 68 h at 37°C and 5% CO₂ in 96-well plates with 1 $\times$ 10⁶ cells/well. The concentration of IL-5, IL-13, and IFN- γ in the cell-free supernatants was measured by ELISA as described elsewhere (8). The sensitivity of detection was 24 pg/ml. The paired capture and detection IL-5 and IFN- γ Abs were obtained from BD Pharmingen, and the IL-13 Abs were obtained from R&D Systems.

Proliferation and cytokine production by Th2 CD4⁺ cells cultured *in vitro*

Equal numbers of male and female mice were sensitized at 6–8 wk of age by i.p. injection with 50 μ g of OVA mixed with 20 μ l of Rehydralgel in 0.9% sterile saline. Splenocytes were recovered after 7 days, erythrocytes were lysed, and washed splenocytes were stimulated with 200 μ g/ml OVA and polarized toward a Th2 phenotype with 40 ng/ml rIL-4 (a gift from S. Ford and I. G. Young, John Curtin School of Medical Research, Canberra, Australian Capital Territory, Australia), and 70 μ g/ml anti-IFN- γ Ab (clone R46A2) in MLC medium for 7 days at 37°C and 5% CO₂. CD4⁺ T cells were then purified using the Minimaacs magnetic bead system according to the manufacturer's recommendations (Miltenyi Biotec). CD4⁺ T cells (5 $\times$ 10⁶/ml) were then incubated in MLC medium containing 200 μ g/ml OVA with 1 $\times$ 10⁶/ml mitomycin C-treated (25 μ g/ml for 60 min at 37°C, followed by extensive washing in PBS) naive splenocytes to serve as APCs. Cells were cultured for 2 days, when proliferation was determined with the CellTiter 96 reagent (Promega) according to the manufacturer's recommendations. Supernatants were also collected and assayed for IL-5, IL-13, and IFN- γ by ELISA.

Purification of pulmonary tissue and MLN DC

Twenty-four hours after the last OVA challenge, lungs were lavaged to remove alveolar macrophages and then dissected into ~2-mm pieces. Tissue fragments from each lung were incubated at 37°C for 30 min in 2.5 ml of collagenase buffer (10 mM HEPES (pH 7.4), 150 mM NaCl, 5 mM KCl, 1 mM MgCl₂, and 1.8 mM CaCl₂) containing 300 U/ml Collagenase Type IV (Worthington Biochemical) and 200 U/ml DNase I (Roche Diagnostic Systems). A cell suspension was prepared from the collagenase-digested

lung tissue or from MLN by gently pushing the tissue through a cell strainer. After erythrocyte lysis, cells were washed and incubated with Fc block (Fc γ II/III receptor; BD Biosciences) and then enriched for expression of CD11c using the Minimacs magnetic bead system according to the manufacturer's recommendations (Miltenyi Biotec). Care was taken to keep the tissue samples cold before and after collagenase incubation and during purification to reduce any possible DC activation. The DC-enriched cells were then analyzed by FACSCalibur (BD Biosciences) for expression of I-A^d MHCII (clone AMS-32.1; BD Biosciences), CD8 α (clone 53-6.7; BD Biosciences), CD11c (clone HL3; BD Biosciences), CD19 (clone 6D5; Chemicon), CD86 (clone GL1; eBioscience), and F4/80 (clone CI: A3-1; Caltag Laboratories). For detection of CD205 (clone NLDC0145; Serotec), cells were incubated sequentially with unconjugated anti-CD205, biotinylated anti-rat IgG2a, then a combination of streptavidin-PerCP and anti-CD11c FITC. If DC were to be used for mixed cell cultures, the eluate from the Minimax column was passed through a second column to enhance purity of CD11c⁺ cells, which was consistently >90% of live cells as determined by FACS.

Purification of CD4⁺ cells and mixed cell cultures

MLN were removed from allergic mice, pooled, and then incubated for 10 min at 37°C in 2.5 ml of collagenase buffer containing 300 U/ml Collagenase Type IV and 200 U/ml DNase I. A cell suspension was prepared by gently pushing the digested nodes through a cell strainer. Cells were incubated with Fc block (Fc γ II/III receptor; BD Biosciences) and then enriched for expression of CD4 using the Minimacs magnetic bead system and following the manufacturer's recommendations (Miltenyi Biotec). Cells were assessed for expression of CD4 (clone GK1.5; BD Biosciences) by FACS. If cells were to be used for mixed cell cultures, the eluate from the Minimax column was passed through a second column to enhance purity of the CD4⁺ cells, which was consistently >95% of live cells as determined by FACS. For mixed cell cultures, 2×10^5 CD4⁺ cells and 4×10^4 CD11c⁺ cells in 200 μ l of MLC containing 200 μ g/ml OVA were plated per well of round-bottom 96-well plates and then incubated for 5 days at 37°C and 5% CO₂. Negative controls consisted of the same numbers of either CD4⁺ cells or CD11c⁺ cells.

Statistical analysis

The significance of differences between experimental groups was analyzed using Student's unpaired *t* test. Values are reported as the mean $\pm$ SEM. Differences in means were considered significant if *p* < 0.05.

Results

Comparison of allergic responses in WT, IL-4^{-/-}, and IL-13^{-/-} mice

We directly compared the degree of inflammation and Th2 cytokine secretion in allergic IL-4^{-/-} and allergic IL-13^{-/-} mice to obtain initial insight into the potential roles of these cytokines in modulating DC function and Th2 biasing. Compared with WT mice, deficiency in IL-4 impaired the development of blood eosinophilia (Fig. 1A), whereas deficiency in either IL-4 or IL-13 impaired the development of tissue and airway eosinophilia, the numbers of mucus-containing cells and Th2 cytokine production (IL-5 and IL-13) by the MLN (Fig. 1, B–E). However, an important observation was that T cells from IL-13^{-/-} mice secreted the highest levels of IFN- γ (Fig. 1E), suggesting that endogenous IL-13 controls IFN- γ levels. Notably, except for airway eosinophilia in allergic IL-4^{-/-} and IL-13^{-/-} mice and mucus in allergic IL-13^{-/-} mice, all response parameters measured in all groups of allergic mice were significantly higher relative to controls (data not shown).

Maturation of DC in the pulmonary compartment of allergic WT, IL-4^{-/-}, and IL-13^{-/-} mice

Since T cells do not bind IL-13, we next determined whether the effects of IL-4 and IL-13 on production of cytokines by MLN was attributable to the influence of these cytokines on DC activation. We specifically focused on the tissue compartment because interdigitating DC are known to form a contiguous immune surveillance network that is anchored within the bronchial epithelium

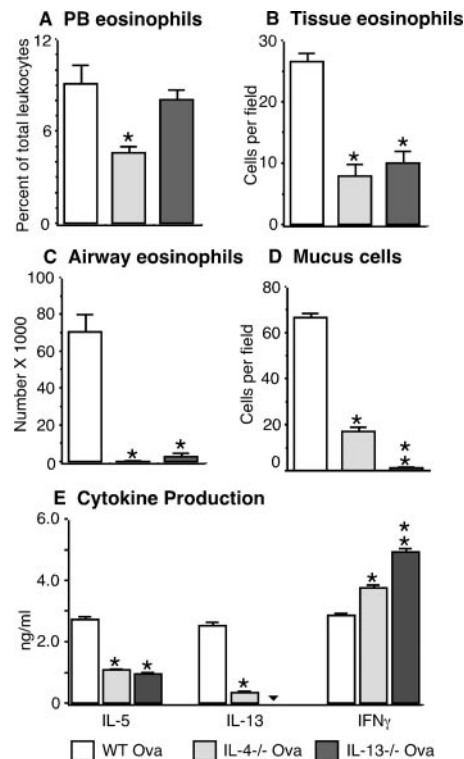


FIGURE 1. IL-4 and IL-13 regulate eosinophilia, mucus hypersecretion, and Th2 cytokine production in allergic mice. A–C, Eosinophilic inflammation in allergic WT, IL-4^{-/-}, and IL-13^{-/-} mice. The percentages of blood eosinophils were determined by morphological criteria in May-Grünwald Giemsa-stained smears. The numbers of airway eosinophils were determined by correlating differential counts of May-Grünwald Giemsa-stained cytopins with total cell counts in the BALF. Lung sections were stained with Carbol's chromotrope and the numbers of eosinophils were counted in the peribronchial region of 10 similar fields of $\times 1000$ magnification per mouse. D, Mucus-containing cells. Sections were stained with Alcian blue and periodic-acid Schiff and the numbers of mucin-containing cells in the peribronchial region were determined in 10 similar fields of $\times 1000$ magnification per mouse. E, Th2 cytokine production by MLN cells cultured ex vivo. Cell suspensions of MLN were cultured with OVA for 68 h. Supernatants were harvested and assayed for IL-5, IL-13, and IFN- γ by ELISA. A–D, Values represent the mean $\pm$ SEM of five to six mice per group and are representative of two independent experiments. E, Values represent the mean $\pm$ SEM of three assays. Because insufficient MLN cells per mouse could be obtained to generate sufficient sample for replicate ELISA, MLN were pooled from six mice per group before culture. Assays from two independent experiments generated similar findings. *, *p* < 0.05 compared with WT group; **, *p* < 0.05 compared with WT and IL-4^{-/-} groups; ∇ , Not detected. PB, Peripheral blood.

(35). CD11c⁺ cells were purified from allergic lungs, which had been lavaged to reduce copurification of the alveolar macrophage population. Importantly, CD19 or F4/80 was undetectable on 94–98% of CD11c⁺ cells isolated using this method (Fig. 2A). However, in contrast to WT DC where the majority of cells stained positive for the endocytic marker CD205, expression of this marker on CD11c⁺ cells isolated from the lungs of allergic IL-4^{-/-} and IL-13^{-/-} mice was considerably lower (Fig. 2A). Analysis of the morphology of lung CD11c⁺ cells showed that these cells were 10–12 μ m in diameter and exhibited an eccentric nucleus with some cells displaying delicate filamentous projections (Fig. 2B). This morphology, which is typical of that described for DC (36, 37), combined with the pattern of surface marker expression, suggested that these populations of cells were highly enriched for DC.

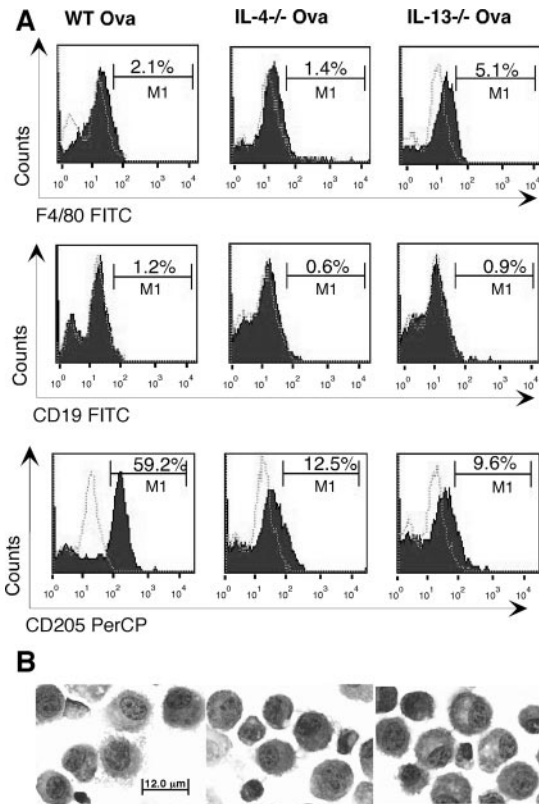


FIGURE 2. Expression of F4/80, CD19, and CD205 and morphology of CD11c⁺ cells isolated from the lung tissue compartment of allergic WT, IL-4^{-/-}, and IL-13^{-/-} mice. **A**, Cells from collagenase-digested lungs were enriched for expression of CD11c using Minimax beads and then stained with F4/80 FITC, CD19 FITC, or CD205 PerCP for FACS analysis of gated live CD11c⁺ cells. The indicated percentages of positive cells in the M1 gate was determined after subtracting the percentages of cells staining with similarly labeled isotype control Abs. Samples were derived from a pool of CD11c⁺ cells from three lungs per group. **B**, Cytospins were prepared from CD11c⁺ cells isolated from the lung tissue compartment of allergic WT, IL-4^{-/-}, and IL-13^{-/-} mice and stained with May-Grünwald Giemsa. A typical field is shown for each group of mice.

We then examined whether IL-4 or IL-13 regulated the activation of DC in the allergic lung. Although similar numbers of CD11c⁺ cells were isolated from allergic WT, IL-4^{-/-}, and IL-13^{-/-} mice (data not shown), deficiency in either IL-4 or IL-13 significantly impacted on the expression of MHCII by these cells and coexpression of the T cell stimulatory receptor CD86 (Fig. 3, *A* and *C*). Although the numbers of MHCII/CD86⁺ cells seen in allergic IL-13^{-/-} mice were significantly reduced compared with allergic WT mice, significantly lower levels were seen in allergic IL-4^{-/-} mice, demonstrating that IL-4 is more potent than IL-13 in regulating the activation of DC in the allergic lung. However, it is probable that because IL-4 also regulates IL-13 expression, the effects of the deficiency in IL-4 on DC maturation is compounded by the impairment in IL-13 production in IL-4^{-/-} mice.

Because expression of CD8α can help discriminate myeloid from lymphoid DC, we next determined whether deficiency in IL-4 or IL-13 influenced the myeloid phenotype of DC in the allergic lung (Fig. 3, *B* and *C*). Low numbers of DC expressing high MHCII and CD8α were observed in the lungs of allergic WT, IL-4^{-/-}, and IL-13^{-/-} mice; however, there were no differences between groups in the numbers of these cells (Fig. 3, *B* and *C*). Collectively, these findings suggest that IL-4 or IL-13 enhance the maturation of myeloid DC and their expression of CD205 in the

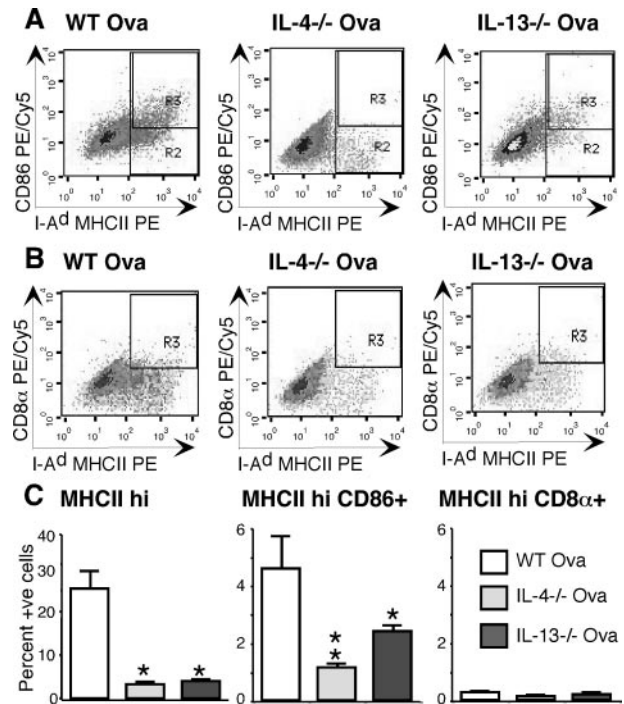


FIGURE 3. Activated DC in the tissue compartment of lungs from allergic WT, IL-4^{-/-}, and IL-13^{-/-} mice. **A–C**, CD11c⁺-enriched cells were isolated from collagenase-digested lungs, stained for CD11c FITC, MHCII PE, and CD86 PE/Cy5 or CD8α PE/Cy5, then analyzed by FACS. Cells were gated on live cells and CD11c FITC⁺ staining. The percentage of CD11c cells expressing high levels of MHCII was determined by the R2 gate (*A*), and the percentage of CD11c cells expressing high MHCII and CD86 (*A*) or CD8α (*B*) was determined by the R3 gate. Histograms (*C*) represent the mean $\pm$ SEM of the percentages of cells in the corresponding R2 or R3 gates (*A* and *B*) for four to five mice per group. *, $p < 0.05$ compared with WT group; **, $p < 0.05$ compared with WT and IL-13^{-/-} groups.

allergic lung. Notably, the mechanism whereby DC in the allergic lung are of a predominately myeloid phenotype is independent of the influence of IL-4 or IL-13.

CD4⁺ memory T cell stimulation by DC from allergic WT, IL-4^{-/-}, and IL-13^{-/-} mice

We next determined the importance of IL-4 and IL-13 for facilitating the capacity of DC to stimulate memory CD4⁺ T cells. CD11c⁺ cells were enriched from the MLN of allergic WT, IL-4^{-/-}, and IL-13^{-/-} mice and characterized for surface markers and morphology. Despite poorer maturation of DC in the lungs of allergic IL-4^{-/-} and IL-13^{-/-} mice, the majority of CD11c⁺ cells in the MLN expressed high levels of MHCII (74.1–81.3%; Fig. 4*A*, upper panel) with undetectable expression of F4/80 or CD19 on 91–95% of live cells (Fig. 4*A*, second and third panels). Additionally, as identified in lung DC, expression of CD205 on DC was dependent on IL-13 and, to a lesser extent, IL-4 (Fig. 4*A*, fourth panel). These cells were also morphologically similar to lung DC as described above, but with enhanced nuclear lobulation and longer filamentous structures than observed in the lung (Fig. 4*B*). When cocultured with memory WT CD4⁺ T cells, both WT and IL-13^{-/-} DC were more adept at stimulating Th2 cytokine production than DC derived from IL-4^{-/-} mice (Fig. 4*C*). Interestingly, in contrast to WT and IL-4^{-/-} DC, costimulation with IL-13^{-/-} DC significantly promoted expression of IFN-γ, suggesting that IL-13 or an IL-13-dependent factor produced by these cells is important in suppressing IFN-γ production by memory CD4⁺ T cells. Importantly, there was no clear trend in the level of residual B cell or

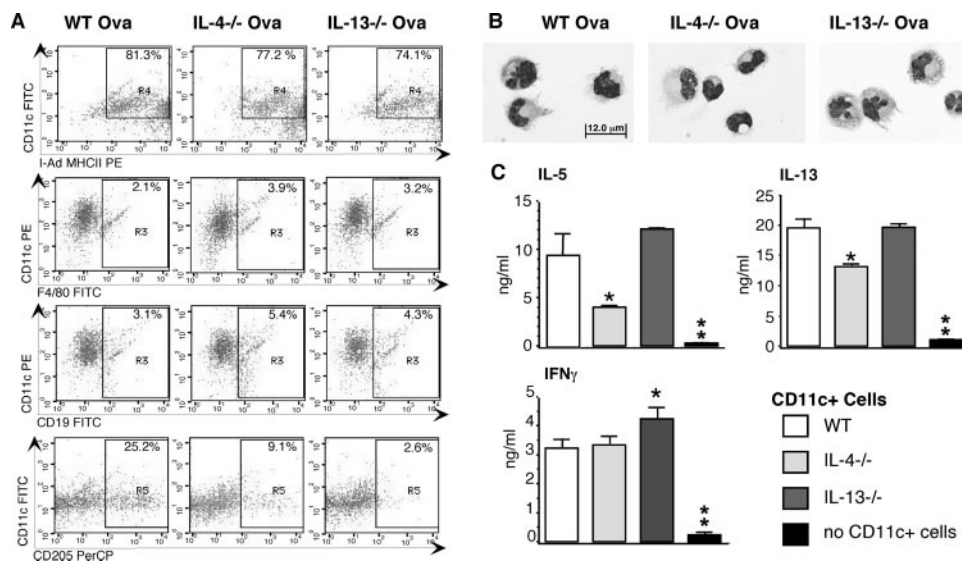


FIGURE 4. Surface marker profile, morphology, and WT CD4⁺ T cell stimulatory capacity of CD11c⁺ cells enriched from MLN of allergic WT, IL-4^{-/-}, and IL-13^{-/-} mice. *A*, CD11c⁺-enriched cells were isolated from collagenase-digested MLN, stained for CD11c FITC or CD11c PE and MHCII PE, F4/80 FITC, CD19 FITC, or CD205 PerCP, and then were analyzed by FACS. Cells were gated on live cells and the percentages of cells in the respective gates were determined after subtracting the percentages of cells staining positive with the appropriate isotype control Ab. *B*, Cytopins were prepared from CD11c⁺ cells isolated from the MLN of allergic WT, IL-4^{-/-}, and IL-13^{-/-} mice and stained with May-Grünwald Giemsa. A typical field is shown for each group of mice. Analyses of MLN CD11c⁺ cells were performed on a pool from 9 to 10 mice/group. *C*, CD11c⁺ cells from allergic WT and cytokine-deficient mice and CD4⁺ cells from allergic WT mice were purified from the MLN and then cocultured for 5 days. Supernatants were harvested and the levels of IL-5, IL-13, and IFN- γ were determined by ELISA. No IL-5, IL-13, or IFN- γ was detected in cultures containing CD11c⁺ cells in the absence of CD4⁺ cells (data not shown). Values represent mean $\pm$ SEM of triplicate assays. Assays from two independent experiments generated similar findings.

macrophage populations in WT, IL-4^{-/-}, or IL-13^{-/-} CD11c⁺ cells that could explain the differences in the functional interaction of these cells with memory CD4⁺ T cells. Collectively, these observations suggest differential roles for IL-4 and IL-13 in modulating the influence of DC on cytokine production by memory CD4⁺ T cells. IL-4 appears critical in maintaining IL-5 and IL-13 production. In contrast, IL-13 is more important than IL-4 in modulating the ability of DC to suppress IFN- γ production by cocultured T cells.

Comparison of allergic responses in mice expressing variants of IL-4R α

Since IL-4R α is a critical conduit of IL-4 and IL-13 signaling, we next investigated what impact subtle changes in the molecular structure of IL-4R α had on DC maturation and Th2 cytokine production. We and others have shown that C57BL/6 and BALB/c mice express distinct variants of IL-4R α that differ in glycosylation of the extracellular domain, the avidity of the receptor for IL-4, and the efficacy by which IL-4 can substitute for IL-13 (31, 38). Notably, the C57BL/6-type IL-4R α (BL6-4R) has a higher affinity for IL-4 than the BALB/c-type IL-4R α (Blb-4R) (38). Therefore, to enable direct comparison of the effects of the IL-4R α variants on immune cell function, we generated congenic mice expressing either BL6-4R or Blb-4R. Whereas no differences were seen in the development of blood or tissue eosinophilia or in the numbers of mucus-containing cells when allergic responses were induced in these mice (Fig. 5, *A*, *B*, and *D*), allergic BL6-4R mice developed significantly higher airway eosinophilia (numbers in BALF) and Ag-specific Th2 cytokine production in comparison to allergic Blb-4R mice (Fig. 5, *C* and *E*). In contrast, cultures of MLN from Blb-4R mice produced significantly higher levels of IFN- γ (Fig. 5*E*). Although these differential responses are subtle, our observations support earlier data from allergic N5 and N10 IL-13^{-/-} mice, which demonstrated that the capacity of IL-4R α to

drive Th2 responses and airway inflammation in the absence of IL-13 relates to the affinity of this receptor for IL-4 (31). To confirm that Th2 cell function was differentially regulated directly by

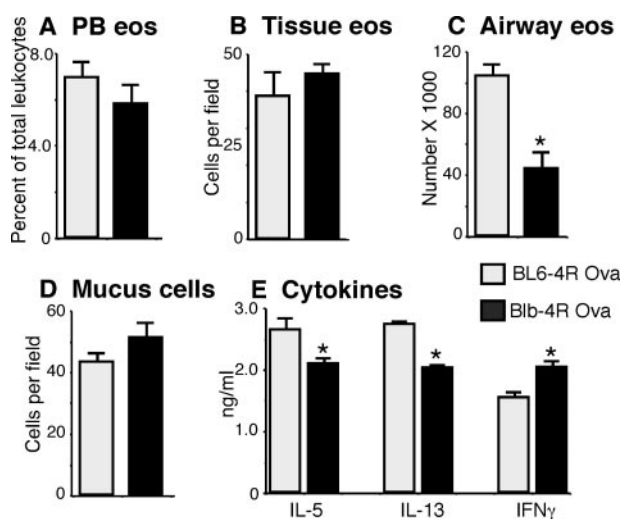


FIGURE 5. Impact of IL-4R α variants on eosinophilia, mucus hypersecretion, and Th2 cytokine production. *A–E*, Eosinophilic inflammation, mucus cells, and Th2 cytokine production in allergic BL6-4R and Blb-4R mice. Blood, lung tissue and airway eosinophilia, mucus-containing cells, and Th2 cytokine production in allergic BL6-4R and Blb-4R mice were determined as described in the legend to Fig. 1. *A–D*, Values represent the mean $\pm$ SEM of six mice per group and are representative of two independent experiments. *E*, Values represent the mean $\pm$ SEM of three assays. Assays from two independent experiments generated similar findings. *, $p < 0.05$ for allergic Blb-4R mice compared with allergic BL6-4R mice. PB, Peripheral blood.

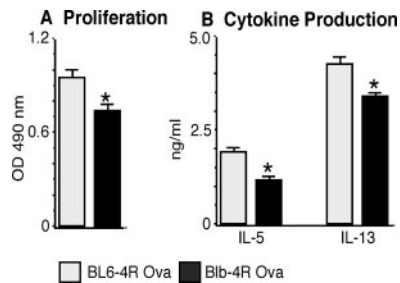


FIGURE 6. Proliferation and Th2 cytokine production by BL6-4R and Blb-4R CD4⁺ Th2 cells biased in vitro. Splenocytes were recovered from OVA-sensitized BL6-4R and Blb-4R mice and then biased in vitro with IL-4 and anti-IFN- γ Ab. CD4⁺ T cells were purified and then cocultured with Ag-loaded mitomycin C-treated naive splenocytes for 2 days when cell proliferation and cytokine levels in the supernatants were determined. **A**, Proliferation of CD4⁺ Th2 cells from BL6-4R and Blb-4R mice. **B**, Th2 cytokine levels in the supernatants of restimulated Th2-biased CD4 cells from BL6-4R and Blb-4R mice. **A** and **B**, Values represent the mean $\pm$ SEM of three cultures. No IFN- γ was detected. *, $p < 0.05$ for allergic Blb-4R mice compared with allergic BL6-4R mice.

IL-4 α , rather than some effect associated with cells trafficking from the lung to the draining nodes or in expression of IL-4, we used IL-4 to bias splenocytes expressing the different IL-4 α variants to a Th2 phenotype. Supporting the data from MLN cultures, proliferation (Fig. 6, **A**) and Th2 cytokine production (Fig. 6**B**) by biased splenocytes was significantly lower with splenocytes from Blb-4R mice in comparison to splenocytes from BL6-4R mice. These observations confirm that variants in the mouse IL-4 α influence the efficacy with which IL-4 can bias Th2 lymphocytes.

Role of IL-4 α polymorphisms in regulating IL-13-induced responses

Previous studies have shown that intratracheal instillation of IL-13 induces tissue eosinophilia, and mucus hypersecretion in naive mice (9, 10, 39). To investigate whether polymorphisms in IL-4 α influence IL-13-dependent processes, we delivered exogenous IL-13 to naive Blb-4R and BL6-4R mice. However, no differences were seen in tissue eosinophilia (Fig. 7**A**) or in the numbers of mucus-containing cells (Fig. 7**B**; $p = 0.09$) between these strains. These data show that the naturally occurring polymorphisms in IL-4 α do not significantly impact on IL-13 signaling pathways when IL-13 is delivered to the lungs of naive mice.

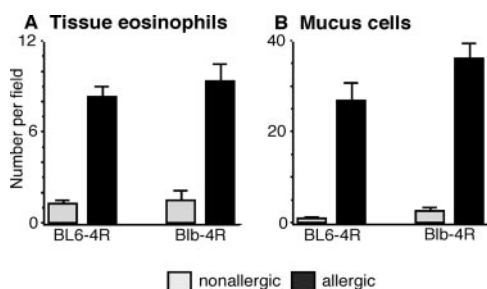


FIGURE 7. Peribronchial eosinophilia and the numbers of mucus cells induced by IL-13 are not influenced by IL-4 α polymorphisms. Exogenous IL-13 was delivered to the lungs of naive BL6-4R or Blb-4R mice and the lungs were removed after 48 h. **A**, The numbers of peribronchial eosinophils in lung sections stained with chromotrope. **B**, The numbers of mucin-containing cells in the peribronchiole region of lung sections stained with Alcian blue and periodic acid-Schiff. Ten comparable sections of $\times 1000$ magnification fields were counted per mouse. Histograms represent the mean of five to six mice per group $\pm$ SEM.

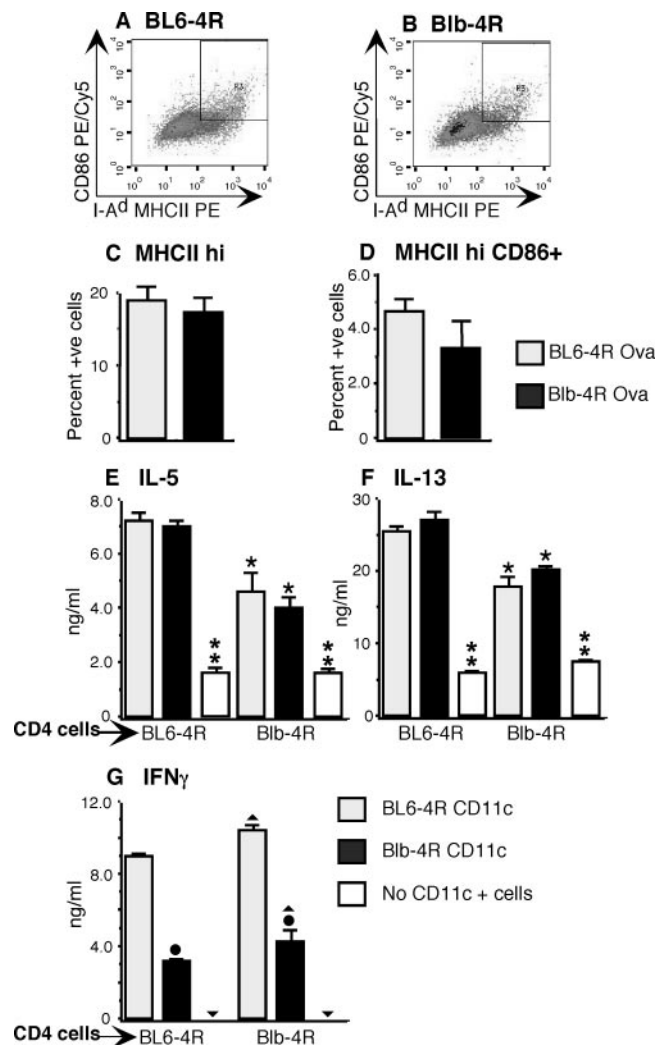


FIGURE 8. Activated pulmonary DC in allergic BL6-4R and Blb-4R mice and their capacity to stimulate memory CD4 T cells. **A** and **B**, Expression of CD86 and MHCII by CD11c⁺ cells isolated from the pulmonary tissues of allergic BL6-4R and Blb-4R mice. CD11c⁺ cells were isolated from the lungs, stained with anti-MHCII PE and anti-CD86 PE/Cy5, and analyzed by FACS as described in the legend to Fig. 3. To assess the activation of these cells, the percentage of live cells expressing high levels of MHCII and CD86 was determined (R3 gate). **C**, Percentage of CD11c⁺ cells expressing MHCII^{high} and **D**, the percentage of CD11c⁺ cells expressing both MHCII^{high} and CD86. **E–G**, CD11c⁺ cells enriched from the MLN of BL6-4R and Blb-4R mice were cocultured with either homologous or heterologous purified BL6-4R or Blb-4R CD4⁺ T cells. Culture supernatants were harvested and IL-5, IL-13, and IFN- γ levels were determined by ELISA. *, $p < 0.05$ for BL6-4R CD4 cells cultured with either BL6-4R or Blb-4R CD11c⁺ cells compared with BL6-4R CD4 cells cultured with either BL6-4R or Blb-4R CD11c⁺ cells. **, $p < 0.05$ for BL6-4R or Blb-4R CD4⁺ cells cultured in the absence of CD11c⁺ cells compared with BL6-4R or Blb-4R CD4⁺ cells cultured in the presence of CD11c⁺ cells. $\blacktriangle$, $p < 0.05$ for Blb-4R CD4⁺ cells cultured with either BL6-4R or Blb-4R CD11c⁺ cells compared with similarly treated BL6-4R CD4⁺ T cells. $\bullet$, $p < 0.05$ for BL6-4R or Blb-4R CD4⁺ cells cultured with Blb-4R CD11c⁺ cells compared with BL6-4R or Blb-4R CD4⁺ T cells cultured with BL6-4R CD11c⁺ cells. $\blacktriangledown$, Not detected. **C** and **D**, Values represent mean $\pm$ SEM of four to five mice per group. **E–G**, Values represent mean $\pm$ SEM of three assays per group and are representative of two independent experiments in which similar differences were observed.

Variants of IL-4 α influence CD4 T cell activation

Because our data show that variants of IL-4 α influence IL-4-induced Th2 biasing and also that IL-4 is an important regulator of

DC maturation and function, we next investigated whether IL-4R α variants impacted on DC activation and the regulation of Th2 memory function. Although similar ratios of MHCII expression on DC were observed (Fig. 8, A–C), a trend toward lower levels of CD86 expression was seen in DC from Blb-4R mice compared with DC from BL6-4R mice (Fig. 8, A, B, and D), but this failed to reach significance. To determine what impact IL-4R α polymorphisms had on the interaction of DC with memory CD4 $^{+}$ T cells, we cross-cultured DC and CD4 $^{+}$ T cells derived from the MLN of allergic BL6-4R or allergic Blb-4R mice. Similar to observations with cultures of whole MLN from allergic BL6-4R and Blb-4R mice, IL-5 and IL-13 produced by BL6-4R CD4 $^{+}$ T cells was significantly higher than IL-5 and IL-13 produced by Blb-4R CD4 $^{+}$ T cells, and this occurred independently of whether the CD4 $^{+}$ T cells were cocultured with DC derived from BL6-4R or Blb-4R mice (Fig. 8, E and F). Notably, this was not the case with IFN- γ where DC appear to play a more important role than the T cell in regulating IFN- γ production (Fig. 8G). More than twice the level of IFN- γ was produced by cocultures with BL6-4R DC than cultures with Blb-4R DC and this was independent of the IL-4R α variant expressed by the CD4 $^{+}$ T cell.

Discussion

Previous studies investigating the efficacy of IL-4 or IL-13 in regulating the maturation of DC *in vitro* have generated conflicting data (28–30). Additionally, little is known of the roles of these cytokines in the maturation of DC *in vivo*, especially in the tissue compartment of the allergic lung. Therefore, our experiments were designed to assess the influence of IL-4 and IL-13 on both DC maturation in the tissue compartment of the allergic lung and the capacity of DC to stimulate memory T cells. Because both IL-4 and IL-13 require IL-4R α for signaling, we also determined how subtle molecular differences in this receptor component impact on DC activation and CD4 $^{+}$ Th2 cell function.

Our data confirmed that IL-4 and IL-13 are important factors in the development of tissue and airway eosinophilia and mucus hypersecretion. Additionally, our studies showed that both cytokines can promote Th2 cytokine production; however, IL-13 is significantly more important than IL-4 for suppressing IFN- γ production by T cells. Previous studies have shown that in contrast to mature DC (30), T cells do not bind IL-13 or express IL-13R α 1 (17, 40). Therefore, our observations showing that IL-13 promoted T cell production of Th2 cytokines and suppressed IFN- γ suggest that IL-13 may indirectly regulate T cells by modulating DC function.

When we examined the phenotype of DC from the lungs of allergic IL-4 $^{-/-}$ or IL-13 $^{-/-}$ mice, it was apparent that either IL-4 or IL-13 enhanced DC expression of MHCII. However, IL-4 was significantly more effective than IL-13 in promoting expression of CD86, an important Th2 costimulatory molecule (41–43). IL-13 and, to a lesser extent, IL-4 also regulated expression of the endocytic marker CD205 (44, 45). Whereas most DC isolated from allergic WT lung expressed CD205, staining for this marker was considerably less in DC from allergic IL-4 $^{-/-}$ and allergic IL-13 $^{-/-}$ mice. In addition, few DC from the lungs of either allergic WT or cytokine-deficient mice expressed CD8 α , suggesting that although IL-4 or IL-13 enhances the maturation and activation of myeloid DC, these cytokines do not affect the balance of CD8 α -negative and CD8 α -positive DC in the lung. Interestingly, the majority of CD11c $^{+}$ cells in the MLN from WT and cytokine-deficient groups expressed MHCII, suggesting that despite poorer expression of MHCII on lung DC in cytokine-deficient mice, only mature DC expressing high levels of MHCII migrate to the draining lymph nodes. In contrast, expression of the endocytic marker CD205 in either the lung or the MLN is partially

dependent on IL-4 or IL-13. However, at this stage it is unclear how this marker influences the function of DC in the allergic lung.

When we investigated what influence IL-4 and IL-13 have on the ability of DC to stimulate memory CD4 $^{+}$ Th2 cells, a clear delineation was seen in the way in which IL-4 or IL-13 modulated DC and their functional interaction with CD4 $^{+}$ T cells. Although IL-4 was required to enable DC to maintain T cell production of Th2 cytokines, IL-13 was more important in directing DC to suppress T cell production of IFN- γ . Interestingly, data from the CD11c $^{+}$ /CD4 $^{+}$ T cell cocultures paralleled data from MLN cultures in which IFN- γ production is enhanced in nodes deficient in IL-13. Therefore, our data strongly indicate that in WT mice, IL-4 and IL-13 act in concert to modulate DC to respectively amplify IL-5 and IL-13 production and suppress IFN- γ expression by memory CD4 $^{+}$ Th2 cells.

In contrast to our studies in cytokine-deficient mice, the generation of congenic mice expressing either the BL6 or Blb forms of IL-4R α (which have been shown to bind IL-4 with different avidities (38)) enabled us to analyze subtleties in the ability of IL-4 to signal through IL-4R α to influence the maturation of DC and the biasing of lymphocytes toward a Th2 phenotype. Notably, Schulte et al. (38) showed a marked increase in the dissociation rate of IL-4 from BALB/c IL-4R α (20×10^{-3} /min) compared with C57BL/6 IL-4R α (3×10^{-3} /min), leading to the suggestion that the faster dissociation rate limits signal intensity. Our development of mouse strains expressing variants of IL-4R α more closely parallel the human condition where discrete polymorphisms in immune factors, rather than blanket deletion of genes, are a much more likely occurrence for predisposition to the development of asthma. Notably, several studies have implicated gain of function mutations in the human IL-4R α in asthma pathogenesis (46). In particular, a human IL-4R α with variation in an amino acid adjacent to the corresponding residue that enhances avidity of IL-4 to the mouse IL-4R α has been linked to enhanced STAT6 activation and IgE synthesis (46). We previously reported that differences in the ability of IL-4 to partially compensate for IL-13 correlated with the expression of polymorphic variants of IL-4R α in IL-13 $^{-/-}$ mice and the avidity with which IL-4 is bound (31). Comparison of allergic inflammation in these variants showed that airway, but not tissue or blood eosinophilia, was significantly lower in allergic Blb-4R mice compared with allergic BL6-4R mice and this directly correlated with the avidity of IL-4 for IL-4R α . Since IL-4 is known to modulate airway inflammation by regulating the adhesion molecule VCAM-1, which binds to the VLA-4 integrin expressed on eosinophils (3, 47), it is likely that reduced airway inflammation in Blb-4R mice is associated with IL-4-mediated adhesion processes. In fact, IL-4 is known to play a key role in migration of eosinophils from the lung tissue to the airway lumen (5, 8, 48). Although subtle, the differential modulation of Th2 biasing in mice expressing variants of IL-4R α also correlated with its affinity for IL-4. Th2 cytokine production by cultured MLN was higher and IFN- γ was lower in allergic BL6-4R mice compared with allergic Blb-4R mice. Treatment of BL6-4R splenocytes with IL-4 also stimulated higher Th2 cytokine production than IL-4 treatment of Blb-4R splenocytes. Thus, the avidity of the interaction between IL-4 and IL-4R α directly correlated with the intensity of the Th2 response and airway inflammation.

Although the influence of IL-4R α polymorphisms on the avidity for IL-4 is recognized (31, 38), nothing is known about how these variants modulate the function of IL-13. Therefore, to compare differences in BL6-4R and Blb-4R in response to IL-13, we delivered IL-13 to the lungs of naive mice expressing these different forms of IL-4R α . Although we observed a trend toward greater numbers of mucus-containing cells in allergic Blb-4R mice ($p =$

0.09), no significant difference was seen in the development of tissue eosinophilia, suggesting that at least in naive mice, variants of IL-4R α respond similarly to IL-13.

We also compared the phenotype and effector function of DC isolated from the lungs of allergic BL6-4R mice and allergic Blb-4R mice. Whereas a trend toward reduced numbers of MHCII/CD86-expressing CD11c⁺ cells in the lungs of allergic Blb-4R mice was observed, this failed to reach significance. However, cross-culture of DC and CD4⁺ T cells derived from these mice generated some interesting findings. CD4⁺ T cells from BL6-4R mice produced higher levels of Th2 cytokines and lower IFN- γ than Blb-4R T cells. However, the magnitude of Th2 cytokine production occurred independently of whether these T cells were cocultured with BL6-4R or Blb-4R DC. In contrast, the genetic background of the DC markedly influenced IFN- γ production. IFN- γ was significantly higher in cultures of BL6-4R or Blb-4R CD4⁺ Th2 memory cells cocultured with BL6-4R CD11c⁺ cells than when these memory T cells were incubated with Blb-4R CD11c⁺ cells. Collectively, these findings show that the avidity of IL-4 for the IL-4R α expressed by CD4⁺ Th2 cells directly correlates with the magnitude of Th2 cytokine production, whereas avidity of IL-4 for the IL-4R α expressed by DC directly correlates with the magnitude of IFN- γ expression.

So can our observations showing a relationship between the avidity of IL-4 for the IL-4R α expressed on CD11c⁺ cells and their influence on the level of IFN- γ expressed by CD4⁺ T cells be reconciled with data from cultures of MLN from cytokine-deficient mice showing that IL-13 suppresses IFN- γ ? We observed insignificant differences when exogenous IL-13 was delivered to the airways of naive BL6-4R or Blb-4R mice, suggesting that both IL-4R α variants had similar avidities to IL-13. However, the situation in naive mice, in which little IL-4 expression would be expected, may not necessarily be the same in allergic mice, where IL-4 production is enhanced and thus likely to compete with IL-13 for access to IL-4R α , especially on cells such as DC, which express the type II (IL-4R α /IL-13R α 1) receptor. Therefore, it is conceivable that the weaker IL-4/IL-4R α interaction on Blb-4R DC would allow greater access of IL-13 to IL-4R α to suppress IFN- γ production by the cocultured CD4⁺ T cells than access of IL-13 to BL6-4R DC, in which the avidity of IL-4 for IL-4R α is greater.

In summary, our experiments show that both IL-4 and IL-13 are important factors in maintaining the function of memory CD4⁺ Th2 cells in the allergic lung. However, the major impact of these cytokines occurs by influencing different mechanistic pathways. IL-4 directly modulates Th2 cytokine production by binding to the IL-4R α complex on memory CD4⁺ T cells. Additionally, IL-4 stimulates the maturation of DC and enhances their ability to induce Th2 cytokine production by cocultured T cells. In contrast, the role of IL-13 appears to be more important in influencing DC function to suppress IFN- γ production by cocultured CD4⁺ T cells. Although not proven, the efficacy of IL-13 in suppressing IFN- γ may relate inversely to the avidity of IL-4 to IL-4R α expressed on DC and thus the availability of the receptor complex to signaling by IL-13. Because T cells do not express IL-13R α 1, the role of DC in influencing T cell production of IFN- γ is not likely to be due to IL-13 produced by DC acting on T cells, but more likely to be due to soluble factors or receptors expressed by DC in an IL-13-dependent manner. We will now use these observations as a platform to further characterize IL-13-dependent pathways in DC and how these pathways are associated with DC function.

Disclosures

The authors have no financial conflict of interest.

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