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Integrated Signals Between IL-13, IL-4, and IL-5 Regulate Airways Hyperreactivity

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In this investigation, we have examined the integrated relationship between IL-13, IL-4, and IL-5 for the development of airways hyperreactivity (AHR) in a model of asthma in BALB/c mice. Sensitization and aeroallergen challenge of both wild-type (WT) and IL-13 gene-targeted (IL-13−/−) mice induced allergic disease that was characterized by pulmonary eosinophilia and AHR to β-methacholine. Although these responses in IL-13−/− mice were heightened compared with WT, they could be reduced to the level in nonallergic mice by the concomitant neutralization of IL-4. Mice in which both IL-4 and IL-13 were depleted displayed a marked reduction in tissue eosinophils, despite the development of a blood eosinophilia. Similar neutralization of IL-4 in WT mice only partially reduced AHR with no effect on tissue eosinophilia. In addition, neutralization of IL-5 in IL-13−/− mice, but not in WT mice, inhibited AHR, suggesting that tissue eosinophilia is linked to the mechanism underlying AHR only in the absence of IL-13. Additionally, mucus hypersecretion was attenuated in IL-13−/− mice, despite the persistence of AHR. Taken together, our data suggest both a modulatory role for IL-13 during sensitization and a proinflammatory role during aeroallergen challenge. The latter process appears redundant with respect to IL-4.

Asthmatic disease is characterized clinically by the hypersecretion of mucus, obstruction, and inflammation of the airways; enhanced bronchial reactivity (airways hyperreactivity, AHR) to spasmogenic stimuli; and elevated serum IgE (1, 2). Although this inflammatory response is complex, clinical and experimental investigations have highlighted the obligatory role of CD4+ Th2 lymphocytes and the importance of eosinophils in the etiology of asthma (3–6). A strong correlation has been demonstrated between the severity of disease and the level of these leukocytes and their products in the lung (3–5, 7–9). The Th2-type cytokines IL-4 and IL-5 have been directly linked with the immunopathogenesis of asthma (2–4, 10), and murine models have also provided corroborative evidence of the importance of these cytokines as central regulators of allergic disease (11–18). The development of Th2-mediated responses that underlie IgE-dependent inflammation, airway eosinophilia, morphological changes to the respiratory epithelium, and AHR has been linked to IL-4 (11, 13, 18, 19), while IL-5 directly modulates allergic airways disease by regulating eosinophilic inflammation (12, 16). However, although IL-4 and IL-5 are important regulators of eosinophilia and AHR, these cytokines are not obligatory for these processes. In contrast to IL-5−/− C57BL/6 mice, an IL-5-independent mechanism regulates AHR in BALB/c mice deficient in this factor (12, 16). AHR and tissue eosinophilia can also persist in response to allergen provocation of IL-4−/− mice (16, 17, 20). Importantly, the mechanism regulating AHR independently of IL-4 and IL-5 is dependent on Th2 cells (16). Evidence is now emerging that Stat6-mediated signaling by the IL-4Rα subunit is critical for the development of enhanced bronchial reactivity in murine models of asthma (21–25). Thus, there appears to be an IL-4-independent, but IL-4Rα-dependent mechanism for the activation of Stat6 and the induction of AHR.

Recently, IL-13, which also stimulates the activation of Stat6 by signal transduction through the IL-4Rα subunit, has been shown to mediate AHR in the allergic lung (24, 26). However, there appears to be a temporal relationship in the requirement for either IL-4 or IL-13 for the development of AHR. Blockade of IL-13, but not IL-4, before aeroallergen challenge is sufficient to attenuate AHR (11, 24, 26). In addition, while these cytokines induce similar physiological responses through their shared usage of the IL-4Rα subunit, there are distinct mechanisms mediated by either cytokine (reviewed in Ref. 27), and this is corroborated by their different spatial and temporal patterns of expression (28, 29). Therefore, it is likely that IL-4 may be key regulator for the development of a Th2 phenotype during sensitization (11), and that IL-13 may be more important for the induction of AHR during allergen inhalation (24, 26). However, the precise mechanism underlying IL-13-mediated AHR and its integrated relationship with IL-4- and IL-5-mediated processes is unclear. The induction of AHR in naive mice by IL-13 has been shown to correlate with eosinophil accumulation in the airways in a process dependent on signaling through the IL-4Rα subunit (24). In contrast, the role of IL-13 in the development of allergic AHR has been suggested to be dissociated from eosinophilic inflammation, but implicated in other pathophysiological manifestations of the asthma phenotype (26). Notably, while neutralization of IL-13 during challenge has been shown to attenuate mucus hypersecretion concomitantly with AHR (26), the intrinsic association of this secretogogenic process with AHR is yet to be fully delineated.

In this investigation, we have employed IL-13−/− mice to examine the role of this cytokine and its integrated relationship with IL-4 and IL-5 in the development of pulmonary eosinophilia,
mucus hypersecretion, and AHR. Our data suggest dual roles for IL-13: one that is modulatory during sensitization and lymphocyte priming and one that is proinflammatory during aeroallergen challenge. This latter process appears redundant with respect to IL-4, suggesting that both cytokines, or their common receptor subunit IL-4Rα, may need to be targeted for successful therapeutic intervention.

Materials and Methods
Induction of allergic airways inflammation
IL-13−/− mice were generated from (129 × C57BL/6) mice (30) that were backcrossed for five generations onto the BALB/c strain. WT mice were obtained from a similar number of backcrosses of the same genetic background. Mice were sensitized at 6 wk of age by i.p. injection with 50 μg of OVA/1 mg Alhydrogel (CSL, Parkville, Australia) in 0.9% sterile saline. Nonsensitized mice received 1 mg of Alhydrogel in 0.9% saline (Sal). WT and IL-13−/− mice were also injected i.p. with either IL-4 Ab (1 mg of GL113) 24 h before the i.p. injection with OVA or Sal and then weekly throughout the experimental period. Abs were administered in sterile saline. On days 12, 14, 16, and 18, all groups of mice were aeroallergen challenged with OVA, as previously described (12, 16). Twenty-four hours after the last challenge, AHR was measured, and then mice were sacrificed by cervical dislocation and the inflammation and morphological changes to the airways were characterized. Mice were treated according to Australian National University Animal Welfare guidelines and were housed in a specific pathogen-free facility.

Characterization of lung morphology and leukocytes in blood, tissue, and bronchoalveolar lavage fluid
Lung tissue representing the central (bronchi-bronchiole) and peripheral (alveoli) airways was fixed in 10% phosphate-buffered Formalin, sectioned, and stained with Alcian blue-periodic-acid Schiff for the enumeration of mucin-secreting cells or Carbol’s Chromotrope-Hematoxylin for the identification of eosinophils. Leukocytes in the blood, bronchoalveolar lavage fluid, and lung were identified by morphological criteria and quantified, as previously described (12, 16).

Measurement of AHR
Responsiveness to β-methacholine was assessed in conscious, unrestrained mice by barometric plethysmography, using apparatus and software supplied by Buxco (Troy, NY). This system yields a dimensionless parameter known as enhanced pause (Penh), reflecting changes in waveform of the pressure signal from the plethysmography chamber combined with a timing comparison of early and late expiration. Measurement was performed essentially as previously described (31). Briefly, mice were placed in the plethysmograph chamber and exposed to an aerosol of water (baseline readings) and then cumulative concentrations of β-methacholine ranging from 3.125 to 25 mg/ml. The aerosol was generated by an ultrasonic nebulizer and drawn through the chamber for 2 min. The inlet was then closed and Penh readings taken for 3 min and averaged. Values were reported as the percentage increase over baseline.

Measurement of cytokine production by peribronchial lymph nodes (PBLN)
Cells from the PBLN were isolated and stimulated with 1 ng/ml OVA in mixed lymphocyte culture medium for 72 h, as described previously (32). The concentration of IL-4, IL-5, and IFN-γ in the cell-free supernatants was measured with ELISA (32). The concentration of IL-13 was also determined with ELISA using the Abs AB-413-NA and AF-413-NA (R&D Systems, Minneapolis, MN) and following the manufacturer’s recommendations. The sensitivity of detection was 0.5 ng/ml for IL-5, IL-13, and IFN-γ, and 0.1 ng/ml for IL-4.

Statistical analysis
The significance of differences between experimental groups was analyzed using Student’s unpaired t test. Values were reported as the mean ± SEM. Differences in means were considered significant if p < 0.05.

Results
AHR persists in the absence of IL-13, but not in the absence of both IL-4 and IL-13
To determine the requirement for IL-13 in the development of allergic disease of the lung, IL-13−/− and WT mice were sensitized and aeroallergen challenged with OVA. Twenty-four hours after the last challenge, airway responsiveness to β-methacholine was determined (Fig. 1). Both groups of sensitized mice developed AHR when compared with nonsensitized mice, suggesting that IL-13 is not obligatory for the development of AHR. In fact, IL-13-deficient mice demonstrated a heightened response relative to WT mice. We next determined the influence of both IL-4 and IL-5 in the processes underlying AHR in WT and IL-13−/− mice. In

FIGURE 1. AHR to cholinergic stimuli in WT (A) and IL-13−/− (B) mice that were treated with isotype control, IL-4, or IL-5 Abs. Groups of mice were sensitized with saline (Sal) or OVA and aeroallergen challenged with OVA. Mice were treated with Ab before the first i.p. sensitization and then weekly throughout the experimental period. Reactivity to β-methacholine was measured by barometric plethysmography, and the data (mean of 6–7 mice ± SEM) represent the percentage increase in enhanced pause (Penh) over baseline reactivity in the absence of cholinergic stimuli. Heightened reactivity was seen at some concentrations of β-methacholine by OVA-sensitized IL-13−/− mice when compared with the corresponding WT mice (*, p < 0.05).
contrast to WT mice in which neutralization of IL-4 before sensitization only moderately inhibited AHR (Fig. 1A), similar neutralization of IL-4 in IL-13-deficient mice reduced bronchial responsiveness to the level seen in nonsensitized IL-13−/− mice (Fig. 1B). Similarly, neutralization of IL-5 in IL-13−/− mice, but not in WT mice, significantly reduced AHR compared with the isotype control-treated mice. Thus, these data demonstrate that the AHR that develops in response to sensitization in IL-13−/− mice is dependent on IL-4 and to a lesser extent IL-5-mediated processes.

Cytokine production in PBLN of WT and IL-13−/− mice

To elucidate the cytokine network underlying AHR, the production of IL-4, IL-13, IL-5, and IFN-γ by in vitro Ag-stimulated PBLN cells from OVA-sensitized and challenged WT and IL-13−/− mice treated with anti-IL-4, IL-5, or isotype control Abs was determined. IL-13−/− mice produced similar amounts of IL-4 and IL-5 when compared with WT mice (Fig. 2A and B). Interestingly, neutralization of IL-5 significantly reduced the production of IL-4 in both WT and IL-13−/− mice (Fig. 2A), suggesting that eosinophils may influence the production of IL-4. In contrast to WT mice in which neutralization of IL-4 reduced IL-5 production to near baseline levels, similar treatment of IL-13−/− mice was less effective at inhibiting the production of this cytokine (Fig. 2B), indicating that IL-4-independent cells may be an important source of IL-5 in IL-13−/− mice. In addition, while neutralization of IL-4 in WT mice inhibited the production of IL-13, significant levels persisted (Fig. 2C), suggesting that IL-4-independent mechanisms may also contribute to the production of this cytokine. A deficiency of IL-5 in WT mice had little impact on IL-13 levels, indicating that eosinophils are not associated with the production of this cytokine, at least in the PBLN. No Ag-specific production of IFN-γ could be detected in any samples.

Pulmonary tissue eosinophilia is inhibited in the absence of both IL-4 and IL-13

The numbers of eosinophils in the vasculature, the peribronchial/perivascular region, and the airway lumen were determined to assess the relationship between AHR and eosinophilia, and the role of IL-4 and IL-13 in the recruitment of these cells. Sensitized and challenged IL-13−/− mice responded with a strong eosinophilia in the blood, tissue, and airway compartments that was somewhat heightened, but paralleled the responses seen in WT mice (Fig. 3). However, the most apparent discrepancy between WT and IL-13−/− mice was the effect of neutralizing IL-4 on tissue eosinophilia (Fig. 3B). Although the deficiency of IL-4 in WT mice significantly impaired the permeation of eosinophils into the airway lumen (Fig. 3C), the development of tissue eosinophilia was unchanged relative to IL-4-replete WT mice. This suggests that IL-4 may provide an important signal in the permeation of eosinophils into the airway lumen, and that the absence of eosinophils in this compartment is not necessarily reflective of their tissue occupancy. In sharp contrast, similar neutralization of IL-4 in IL-13−/− mice inhibited the accumulation of tissue eosinophils despite the development of a pronounced blood eosinophilia (Fig. 3A). This indicates that IL-4 and IL-13 elicit redundant responses associated with the transendothelial migration of eosinophils. Notably, this low level of tissue eosinophils correlated with a reduction in AHR in IL-13−/− mice. Neutralization of IL-5 in both WT and IL-13−/− mice reduced the levels of eosinophils in all compartments. However, in contrast to WT mice, in which the inhibition of an IL-5-induced eosinophilia had no effect on AHR, the low levels of this cell in the tissues of IL-13−/− mice coincided with a loss in bronchial hyperresponsiveness.

The role of IL-4 and IL-13 in regulating mucus hypersecretion

Histological staining was used to determine the effect of Ag stimulation on the hypersecretion of mucus in both WT and IL-13−/− mice that were treated with anti-IL-4 or isotype control Abs. In contrast to both sensitized and challenged WT and IL-4-deficient mice, the numbers of airway epithelial cells that stained positive for mucus were markedly reduced in sensitized and challenged IL-13−/− mice (Fig. 4). The concomitant depletion of IL-4 in IL-13−/− mice did not further reduce airway mucus production. Significantly, AHR persisted in IL-13−/− mice, despite substantially reduced mucus hypersecretion.

Discussion

Analysis of the cytokine network that critically regulates allergic disease is complicated by both the redundant and the multifunctional nature of these molecules. As IL-4, IL-5, and IL-13 have
been circumstantially associated with the etiology of asthma (3, 28, 33), we have examined the interrelationship between these cytokines for the development of allergic pulmonary responses in a mouse model of asthma.

Although neutralization of IL-13 during the airway challenge phase of Ag exposure has been shown to limit AHR (24, 26), we have demonstrated in the present study that AHR develops independently of this cytokine when it is removed at the outset by genetic manipulation. While it is possible that compensatory mechanisms develop in IL-13−/− mice, the levels of IL-4 in Ag-stimulated mice are unchanged from WT, at least in the amount produced in the PBLN. Thus, although the genes encoding IL-4 and IL-13 are proximal (34), it seems that the genomic manipulation that created the IL-13−/− mouse has not induced covert perturbation in the expression of IL-4. However, the significantly increased blood eosinophilia and AHR that develops in IL-13−/− mice suggests that some regulatory function may be compromised. Recently, IL-13 was identified as a modulator of acute inflammatory responses by suppressing the activation of NF-kB (35), a transcription factor that is known to play a role in the expression of IL-5, eotaxin, and eosinophilic inflammation in the lung in an IL-4-independent manner (36). Therefore, it is possible that the enhanced responses seen in IL-13−/− mice may be related to this IL-4-independent mechanism of eosinophilic inflammation overlaid onto IL-4-dependent responses. Notionally, this is supported by our observation that the levels of IL-5 and blood eosinophilia produced in the absence of IL-4 are significantly greater in IL-13−/− mice than in WT mice. In addition, the controversy in the literature regarding the requirement for IL-4 in the development of AHR may relate to the levels of IL-13 induced in response to nuances in the sensitization regimes employed in various models. It could be envisaged that low levels of IL-13 may result in the activation of NF-kB and an IL-4-independent mechanism of IL-5 production and eosinophil mobilization. Conversely, high levels of IL-13 may suppress this transcription factor and make these processes more dependent on IL-4. This mechanism of IL-4-independent inflammation may also be operative in nonallergic asthmatics who have elevated levels of both IL-5 and eosinophils in the absence of similarly elevated IL-4 (37).

Although it seems that an IL-4-independent process for the production of IL-5 occurs in IL-13−/− mice, this does not explain the observation that AHR in these mice is ablated by the neutralization of IL-4. In addition, our observation that eosinophilia and AHR develop in IL-13−/− mice contrasts with data from a previous study in which neutralization of IL-13 before aeroallergen challenge with a soluble form of the IL-13R ameliorated airway eosinophilia and AHR (24). This suggests that alternative mechanisms develop for the recruitment of eosinophils to the airways either when IL-13 is deficient at the outset as in IL-13−/− mice or when the level of IL-13 is modified by methodological variation in...
mouse models employed. Our data indicate that in fact a redundant IL-4 and IL-13 function underpins the migration of eosinophils into the pulmonary compartment in IL-13−/− mice. The absence of both cytokines severely impairs eosinophil migration into the tissues, and this probably relates to the important role these molecules play in stimulating the expression of adhesion molecules and chemokines, particularly eotaxin, within the lung (38–41). These cytokines may also play a role in the activation of eosinophils, either directly or indirectly via the transmigratory process. Notably, while there is a certain redundancy in the function of these cytokines at the endothelial barrier, this is not the case at the airway epithelium in which IL-4 appears a more important regulator of the permeation of eosinophils into the airway lumen. Nonetheless, although a deficiency in IL-4 alone partially inhibited AHR, it was not sufficient to completely abrogate AHR in our model.

While the production of OVA-specific IgE was suppressed in IL-4-deficient mice, this did not occur in IL-13−/− mice, which produced levels of IgE that were similar to those produced in WT mice (data not shown). However, at this stage it is unknown what role IgE plays in the inflammatory responses that develop in IL-13−/− mice.

Although redundant signals elicited by either IL-4 or IL-13 appear to be required for the development of AHR, the role of eosinophils in this process is complex. Clearly, reduced numbers of tissue eosinophils in IL-13−/− mice, whether mediated by the reduced availability of blood eosinophils by the neutralization of IL-5 or by inhibiting the migration of eosinophils into the tissues by the neutralization of IL-4, correlate with a significant reduction in AHR. However, this is not the situation in WT mice in which a deficiency in IL-5 and a massively reduced tissue eosinophilia had little impact on AHR. These results are supported by our previous observations that identified a T cell-regulated mechanism in BALB/c mice that is independent of IL-4 and IL-5 for the induction of AHR (16). Probably two scenarios for the role of eosinophils in the development of AHR could be envisaged: either IL-13 is required to activate low levels of residual tissue eosinophils, or eosinophils provide an important AHR-associated factor that compensates for the deficiency in IL-13. The recent identification of eosinophils as a major source of IL-4 and IL-5 in schistosome granulomas (42, 43) as well as in asthmatics (36) may be particularly relevant to this issue. In the present study, neutralization of IL-5 in both WT and in IL-13−/− mice not only inhibited eosinophilia, but significantly inhibited the production of IL-4 by the PBLN. In contrast, neutralization of IL-5 had little effect on the PBLN secretion of IL-13. This suggests that eosinophils, either by direct secretion, or through a cooperativity with Ag-specific T cells may be an important source of IL-4, but not IL-13. Thus, neutralization of IL-5 in IL-13−/− mice may effectively dampen similar responses as occurs when IL-4 is deficient in these mice. Although the inhibition of IL-4 in WT mice reduced the levels of IL-13 produced by PBLN, significant levels of IL-13 persisted. Therefore, the redundancy in signals elicited by IL-4 and IL-13 may explain why the inhibition of IL-4 in WT mice in which IL-13 is plentiful fails to attenuate AHR. In addition, a deficiency of IL-5 in IL-13−/− mice, but not in WT mice, inhibited the production of IL-5 by PBLNs. This also suggests that eosinophils may be an important source of this cytokine in the absence of IL-13.

As the regional lymph nodes sample the microenvironment of the mucosa, they would be expected to reflect the cytokine production in the lung tissue. Recently, we have observed eosinophils in PBLN (44) and these leukocytes are known to act as APC (45, 46). Furthermore, the function of eosinophils as an important depot of cytokines would be expected to be enhanced in the bronchial mucosa in which these cells have a more predominant profile.

Recently, interest has focused on the role played by IL-4 or IL-13 in the regulation of mucus production. Although IL-4 appears to modulate the accumulation of mucus glycoprotein in the lung, signal transduction via the IL-4Rα subunit is fundamental to this process (14, 47–49). In addition, neutralization of IL-13 with a soluble rIL-13 receptor markedly inhibited both mucus hypersecretion and AHR in response to aeroallergen challenge (26). In the present study, specific histological staining showed that mucus hypersecretion was dramatically reduced in sensitized IL-13−/− mice compared with both WT and IL-4-depleted mice. Significantly, the observation that AHR persisted in these IL-13−/− mice suggests that the mechanism underlying the production of mucus and the presence of secreted mucus in the airways are dissociated from the development of AHR.

In conclusion, the functions of IL-13 in the development of AHR appear to be temporal: a modulatory role during the sensitization process and a proinflammatory role during challenge. The modulatory capacity of IL-13 may relate to suppression of the activation of NF-κB, and concomitant IL-5 induced eosinophilic inflammation in an IL-4-independent manner. In contrast, the proinflammatory role of IL-13 appears redundant with respect to IL-4. Either IL-4 or IL-13 can instigate the transendothelial migration and probably activation of eosinophils. These cells may then provide an important source of IL-4, especially in situations in which IL-13 is deficient. Although the cellular targets of these cytokines and the mechanisms associated with AHR are yet to be clearly defined, the apparent redundancy in the functions of IL-4 and IL-13 for the development of AHR suggests that both cytokines, or their common receptor subunit IL-4Rα, may need to be targeted for successful therapeutic intervention.

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