Maintenance of Airway Hyperresponsiveness in Chronic Asthma May Be Mediated by Th2-Independent Mechanisms

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Abstract: CD4+, Th2-mediated inflammation is an important component of airway hyperresponsiveness (AHR) in allergic airway disease. IL-4 specifically interacts with the Type 1 IL-4 receptor comprised of IL-4Rα and the common γ chain, whereas IL-4 and IL-13 mediate their effects through a common receptor complex made up of IL-4Rα and IL-13Rα1 (i.e. the Type II IL-4 receptor). In this study, we examined the effects of impaired Th2 signaling on AHR using IL4Rα$^{-/-}$ mice in a murine model of allergic asthma. IL-4Rα$^{-/-}$ mice and control BALB/c (IL-4Rα$^{+/+}$) mice were sensitized to and challenged with *Aspergillus fumigatus*. Airway disease was assessed at days 14, 28, 51, and 57 after intratracheal conidia challenge. AHR was evaluated by plethysmography after intravenous methacholine. Whole lung levels of cytokines and chemokines, and serum immunoglobulins were measured by specific ELISA. Paraffin-embedded lung sections were stained for histology. Bronchoalveolar lavage (BAL) fluid was cytospun for differential cell counts. While AHR was significantly reduced in IL-4Rα$^{-/-}$ mice ($p<0.01$) at days 14 and 28 after conidia challenge, it was increased when compared to controls at later time points (days 51 and 57) even though Th2 cytokines were significantly decreased at day 57, and total IgE and IgG1 levels were markedly decreased throughout the study ($p<0.0001$). Goblet cell metaplasia was also evident at days 51 and 57 in the knockout groups. These results demonstrate that airway hyperresponsiveness and mucus cell metaplasia in a model of allergic asthma can develop in the absence of a predominant Th2 signaling pathway, suggesting that Th2-independent mechanisms may arbitrate chronic stages of airway disease due to *A. fumigatus*.

Keywords: Th1/Th2, asthma, mice, IL-4Rα.

INTRODUCTION

IL-4 and IL-13 are considered to be key players in the pathophysiology of asthma and allergic disorders, although their individual roles are still unclear. Both are cytokines produced by T helper type 2 cells (Th2) cells, mast cells, and basophils, usually in response to antigen challenge [1] Because of their importance in orchestrating the airway inflammation and airway hyperresponsiveness seen in asthma [2-4], there has been considerable attention focused on research involving therapeutic targeting of IL-4, IL-13, or their signaling pathways.

IL-4 and IL-13 share several overlapping biological functions [1,5]. There are two known IL-4 receptors (type I and type II). IL-4 binds with high affinity to the 140-kD IL4Rα chain, and subsequent dimerization with the common gamma chain (γc) forms the type I receptor, found on hematopoietic cells [1,6]. Formation of this IL4Rα/γc heterodimer then initiates signaling through the IL4Rα chain, leading to STAT6 activation and gene transcription [6]. The IL4Rα chain can also dimerize with IL-13Rα1 instead of γc, which forms the type II receptor, typically found on nonhematopoietic cells [1,6]. IL-13 binds to this heterodimer with low affinity. The IL4Rα chain serves as a common signaling component of both the IL-4 and IL-13 receptors that explains much of their overlapping functions.

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Because of its central role in the IL-4 and IL-13 system, we investigated the effect of IL4Rα chain deficiency in mice on airway hyperresponsiveness, goblet cell hyperplasia, and TH2 responses *in vivo*. Although others have utilized IL4Rα$^{-/-}$ mice to demonstrate that Th2 responses can and do develop in the absence of IL4Rα in both parasite models [7, 8] and allergy models [9, 10], these studies have largely employed either more acute models or models using a non-aeroallergen as the sensitizing agent. We have previously described a murine model of chronic asthma induced by aeroallergen whereby there are sustained features of airway hyperresponsiveness, goblet cell metaplasia, and peribronchial fibrosis [11].

MATERIALS AND METHODS

Mice

Specific pathogen-free (SPF) female BALB/c (abbreviated IL-4R$^{+/+}$) mice (8-12 weeks) were used in all experiments and were obtained from The Jackson Laboratory (Bar Harbor, ME). IL4Rα$^{-/-}$ mice were obtained from The Jackson Laboratory and had a BALB/c genetic background. Animals were housed and maintained (5 mice per cage) under SPF conditions at the University Laboratory of Animal Medicine at The University of Michigan Medical School (Ann Arbor, MI) in accordance with the Institutional Animal Care and Use Committee guidelines.

Chronic Model of *A. fumigatus*-Induced Allergic Asthma

All mice were sensitized using a commercially available preparation of soluble *Aspergillus fumigatus* antigen per-

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formed as previously described in detail [11]. Seven days after the third intranasal challenge, each mouse received 5.0 x 10^6 *A. fumigatus* conidia suspended in 30 µl of 0.1% Tween 80 *via* the intratracheal route [11]. Although our previous studies in this model involved the analysis at various times up to approximately one month after the conidia challenge [11], in the present study we elected to follow wild-type and knockout mice up to day 57 after conidia. The rationale for this extension stemmed from our preliminary observation that greater amounts of fungal material were present in the lungs of the IL-4Rα$^{-/-}$ group at day 28 after conidia compared with the IL-4Rα$^{+/+}$ group at the same time after conidia (data not shown). Thus, we were interested to observe whether this increased fungal burden in the knockout group had an adverse effect on their chronic lung function and morphology.

Measurement of Airway Hyper-Responsiveness (AHR)

Immediately before and at days 14, 28, 51, and 57 after intra-tracheal *A. fumigatus* conidia challenge, AHR was assessed in a Buxco plethysmograph (Buxco Electronics, Troy NY) as previously described [11]. Sodium pentobarbital (Butler Co., Columbus OH; 0.04 mg/g mouse body weight) was used to anesthetize mice before their intubation and ventilation with a Harvard pump ventilator (Harvard Apparatus, Reno NV). Once baseline airway resistance was established, each mouse received a low dose (210µg/kg) and high dose (420 µg/kg) of methacholine *via* tail vein injection, and corresponding changes in airway hyper-responsiveness were monitored for approximately 3 minutes and calculated. The peak increase in airway resistance was recorded. After AHR analysis was completed, approximately 500 µl of blood was removed from each mouse *via* ocular bleed and centrifuged at 15,000 *x* g for 10 minutes to yield serum. A bronchoalveolar lavage (BAL) was then performed using 1 ml of normal saline. Finally, whole lungs were dissected from each mouse and snap frozen in liquid N_2 or fixed in 10% formalin for histological analysis (see below).

Morphometric Analysis of Leukocyte Accumulation in BAL Samples

Macrophages, lymphocytes, eosinophils, and neutrophils were enumerated in BAL samples cytospun (Shandon Scientific, Runcorn, UK) onto coded microscope slides. Each slide was stained with a Wright-Giemsa differential stain, and the average number of each cell type was determined after counting a total of 300 cells in 10 to 20 high-powered fields (x1000) per slide. A total of 1 x 10^6 BAL cells were cytospun onto each slide to compensate for differences in cell retrieval.

Elisa and Serologic Analysis

Murine IL-4, IL-5, IL-13, macrophage-derived chemokine (MDC), thymus and activation-regulated chemokine (TARC), and IFN-γ protein levels were determined in 50 µl samples from whole lung homogenates using a standardized sandwich enzyme-linked immunosorbent assay (ELISA) technique previously described in detail [12]. All ELISAs were screened to ensure the specificity of each antibody used. Recombinant murine cytokines and chemokines (R&D Systems, Rochester MN) were used to generate the standard curves from which the sample concentrations were derived. The limit of ELISA detection for each cytokine was consistently above 50 pg/ml.

Total Ig levels were measured in serum samples using a specific ELISA. Serum levels of IgE, IgG1, and IgG2a at days 14, 28, 51, and 57 after conidia in all mice were analyzed using complementary capture and detection antibody pairs for IgE, IgG1, and IgG2a (BD PharMingen, San Diego CA). Ig ELISAs were performed according to the IgE determination and 1/10 for determination of IgG levels. Ig levels were then calculated from OD readings at 492 nm, and Ig concentrations were calculated from a standard curve generated using rIgE, rIgG1, or rIgG2a (both standard curves ranged from 5 to 2000 pg/ml).

Whole Lung Histological Analysis

Whole lungs from *A. fumigatus*-sensitized IL4Rα$^{+/+}$ and IL4Rα$^{-/-}$ mice before and at various times after *A. fumigatus* conidia challenge were fully inflated with 10% formalin, dissected, and placed in fresh 10% formalin for 24 hours. Routine histological techniques were used to embed the entire lung with paraffin, and 5 µm sections of whole lung were stained with periodic acid-Schiff reagent (PAS). Morphological evaluations of inflammatory infiltrates and structural alterations were determined around blood vessels and airways using light microscopy at a magnification of x 1000.

Statistical Analysis

All results are expressed as means ± SEM (SE). A Student's unpaired t-test (Prism GraphPad software, San Diego, CA) was used to determine statistical significance between IL4Rα$^{+/+}$ and IL4Rα$^{-/-}$ mice at various times after the conidia challenge. Two separate, full time course (i.e. prior to and at days 14, 28, 51, and 57 after conidia) experiments were performed. $P<0.05$ was considered statistically significant.

RESULTS

IL4Rα$^{-/-}$ Mice Develop Increased Airway Hyperresponsiveness at the Later Stages of Chronic Fungal Asthma

Methacholine challenge induces airway hyper-responsiveness in mice with fungus-induced asthma [11]. As shown in Fig. (**1**), IL4Rα$^{+/+}$ wild type mice at days 14 and 28 had significantly greater airway resistance ($p<0.05$) in response to methacholine when compared to the IL4Rα$^{-/-}$ mice (Day 14: 24.3 ± 1.8 *vs* 8.3 ± 4.1; Day 28: 29.5 ± 1.4 *vs* 8.2 ± 3.8). However, at the later stages of disease – day 57 after conidia challenge – IL4Rα$^{-/-}$ mice exhibited significantly more AHR ($p<0.05$) than their wild type counterparts after methacholine administration (22.9 ± 3.8 *vs* 10.1 ± 3.2).

IL4Rα$^{-/-}$ Mice had Significantly Reduced T Cell Recruitment into the Airways at Late Stages of Disease

Previous studies have shown that T lymphocytes are the major effector cells that mediate AHR in allergic airway disease [13]. Both IL-4[2] and IL-13 [14,15] play important, possibly independent, roles in this process. We examined T cell recruitment into the airways of IL4Rα$^{+/+}$ wild type mice and IL4Rα$^{-/-}$ mice at both acute (days 14 and 28) and chronic (days 51 and 57) stages of *Aspergillus*-induced asthma. On days 28 and 57, there are significantly reduced numbers of T cells found in the BAL samples of IL4Rα$^{-/-}$ mice compared

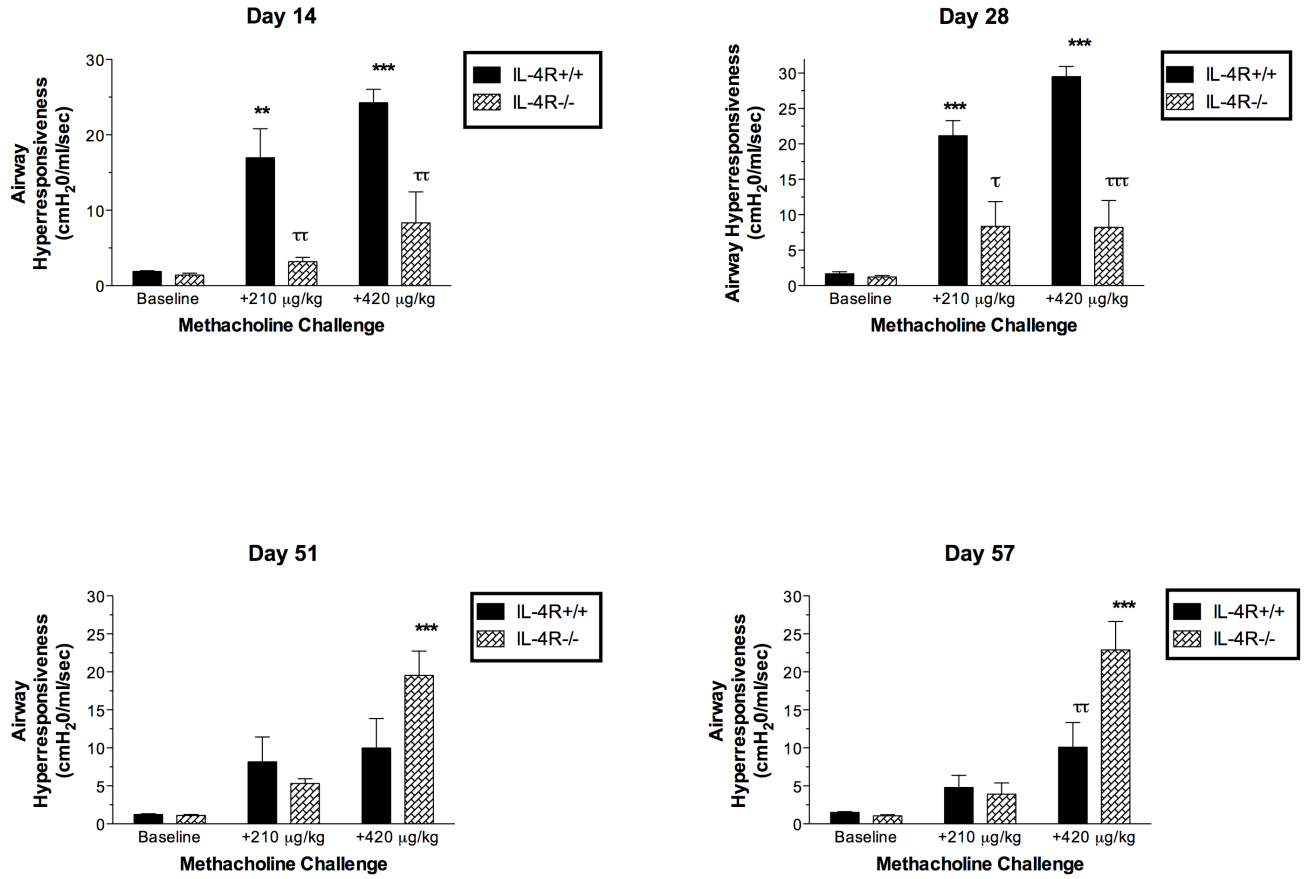

Fig. (1). Airway hyperresponsiveness in *A. fumigatus* sensitized IL-4Rα wild-type ($^{+/+}$) and IL-4Rα knockout ($^{-/-}$) mice prior to and at various times after challenge with *A. fumigatus* conidia. Airway resistance was calculated at each time point before (i.e. baseline) and after intravenous methacholine challenge. Values are expressed as mean ± SEM; n=5/group/time point. Significant differences are as follows: ** $P \leq 0.01$, *** $P \leq 0.001$ compared with baseline resistance in the appropriate group. τ $P \leq 0.05$, ττ ≤ 0.01, ττττ ≤ 0.001 compared with airway resistance in IL-4Rα$^{+/+}$ mice after methacholine challenge.

with wild type mice (Fig. **2**). BAL eosinophils were also reduced in IL4Rα$^{-/-}$ mice at all time points, but this difference did not reach statistical significance (Fig. **2**). Interestingly, although neutrophils were low to undetectable on days 14, 28, and 51 after conidia, there was marked infiltration ($p<0.0001$) of neutrophils at day 57 after conidia in the IL4Rα$^{-/-}$ mice compared to wild type mice (Fig. **2**).

Increased Airway Hyperresponsive-Ness in IL4Rα$^{-/-}$ Mice is Not Mediated by Th2 Cytokines and Chemokines

Both IL-4 and IL-13 share the IL4Rα chain as part of their receptor complex. Signaling through the IL4Rα chain leads to downstream STAT6 activation and production of Th2 cytokines [16]. We examined the Th1 and Th2 cytokine levels in whole lungs of both IL4Rα$^{+/+}$ wild type mice and IL4Rα$^{-/-}$ mice had lower IL-4 levels at days 14, 28, and 57 after conidia and significantly reduced IL-4 levels ($p<0.05$) in the lung at day 57 post conidia (Fig. **3**). IL-5 and IL-13 were also decreased at this and earlier time points, but this decrease did not reach statistical significance (Fig. **3**). Similar to the Th2 cytokines, Th2 chemokines were also significantly decreased in IL4Rα$^{-/-}$ mice. MDC was significantly decreased ($p<0.05$) at days 51 and 57 after conidia challenge, corresponding with the same time points at which increased AHR was seen in the IL4Rα$^{-/-}$ mice (Fig. **3**). Interestingly, TARC was significantly decreased ($p<0.05$) in the IL4Rα$^{-/-}$ mice at all time points throughout the study (Fig. **3**). IFN-γ levels were not significantly changed throughout the study, with the exception of day 14 after conidia challenge (Fig. **3**).

IL4Rα$^{-/-}$ Mice Develop Goblet Cell Metaplasia at Days 51 and 57 After Conidia

Consistent with previous studies [17], IL4Rα$^{-/-}$ mice showed a markedly significant decrease in serum IgE and IgG1 production at all times in the chronic fungal model (Fig. **4A**,**B**). IgG2a was not significantly different in the two groups of mice (Fig. **4C**).

As previously shown in the chronic fungal model, goblet cell metaplasia is a characteristic finding in *A. fumigatus*-sensitized mice challenged with live conidia [11]. PAS staining of whole lung sections from *A. fumigatus*-sensitized IL4Rα$^{+/+}$ mice showed prominent goblet cell metaplasia in large airways at days 14 (Fig. **5A**) and 51 (Fig. **5C**) after conidia. In contrast, mice deficient in the IL4Rα chain did not show goblet cell staining at day 14 (Fig. **5B**) but interestingly, goblet cells were apparent at days 51 (Fig. **5D**) and 57 (not shown) in the large airways of these mice.

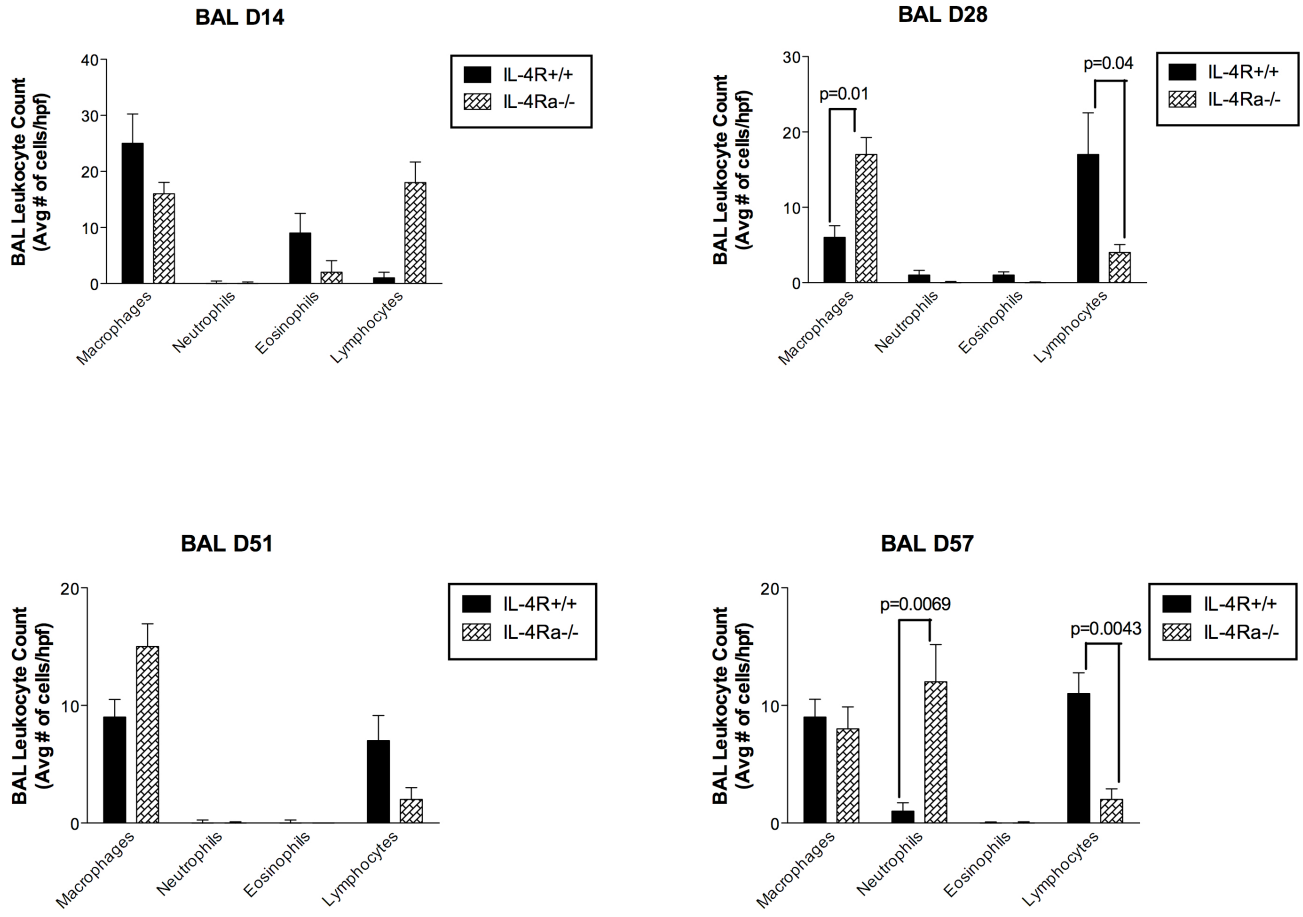

Fig. (2). Leukocyte counts in BAL samples in *A. fumigatus* sensitized IL-4Rα wild-type ($^{+/+}$) and IL-4Rα knockout ($^{-/-}$) mice prior to and at days 14, 28, 51, and 57 after challenge with *A. fumigatus* conidia. BAL samples were dispersed onto microscope slides *via* cytospin, and cells were differentially stained with Wright-Giemsa stain. A total of 1 x 10^6 cells were cytospun onto each slide and a minimum of 15 high-powered fields or 300 cells were examined in each cytospin. Values are expressed as mean ± SEM. Significant differences between wild-type and knockout groups are indicated.

DISCUSSION

In the present study, we showed that in an established model of chronic asthma, deficiency of the IL4Rα chain in *A. fumigatus*-sensitized mice did not result in the long-term elimination of AHR and goblet cell metaplasia as might be expected given the previously established importance of IL-4 and IL-13 signaling in the pathogenesis of asthma [14, 15, 18-20]. We observed that IL4Rα$^{-/-}$ mice had increased AHR and mucus cell metaplasia in the more chronic stages of disease (i.e. days 51 and 57 after conidia challenge) but not in the earlier stages (i.e. days 14 and 28 after conidia). This is in contrast to previous studies that showed that the maintenance of AHR in IL4Rα$^{-/-}$ mice in levels comparable to IL4Rα$^{+/+}$ wild type mice after sensitization and challenge [10]. Their study, however, had important differences in methodology, including use of ovalbumin as the sensitizing antigen, and determination of AHR at much earlier time points.

Certain cytokines and many chemokines are well known for their overlapping functions and receptor binding patterns [21]. This is perhaps especially true in the setting of allergic airway disease [2, 9, 14]. To investigate whether a compensatory Th2 milieu was created in the absence of IL4Rα signaling, we measured whole lungs levels of IL-4, IL-5, IL-13, as well as the Th2 Chemokines MDC and TARC. Interestingly, the increased AHR and goblet cell hyperplasia observed in the IL4Rα$^{-/-}$ mice developed in the absence of a predominant Th2 cytokine and chemokine environment, as manifested by significantly decreased whole lung levels of IL-4, MDC and TARC. Serum levels of IgE and IgG1 were also markedly depressed. This is consistent with previous studies demonstrating that IL-4 [2], and IL-5 [4,22], and IgE [23] are not essential for development of antigen-induced AHR. Whole lung levels of IL-5 and IL-13 in our study approached, but did not reach statistically significant decreased levels in the later time points of the study. Such low level Th2 responses have been demonstrated in other studies using IL4Rα$^{-/-}$ mice [17], suggesting that there are alternative sources of Th2 cytokines, such as NKT cells [17], that do not require signaling through the IL4Rα chain.

Airway hyper-responsiveness in IL4Rα$^{-/-}$ mice was significantly increased compared to IL4Rα$^{+/+}$ wild-type mice at

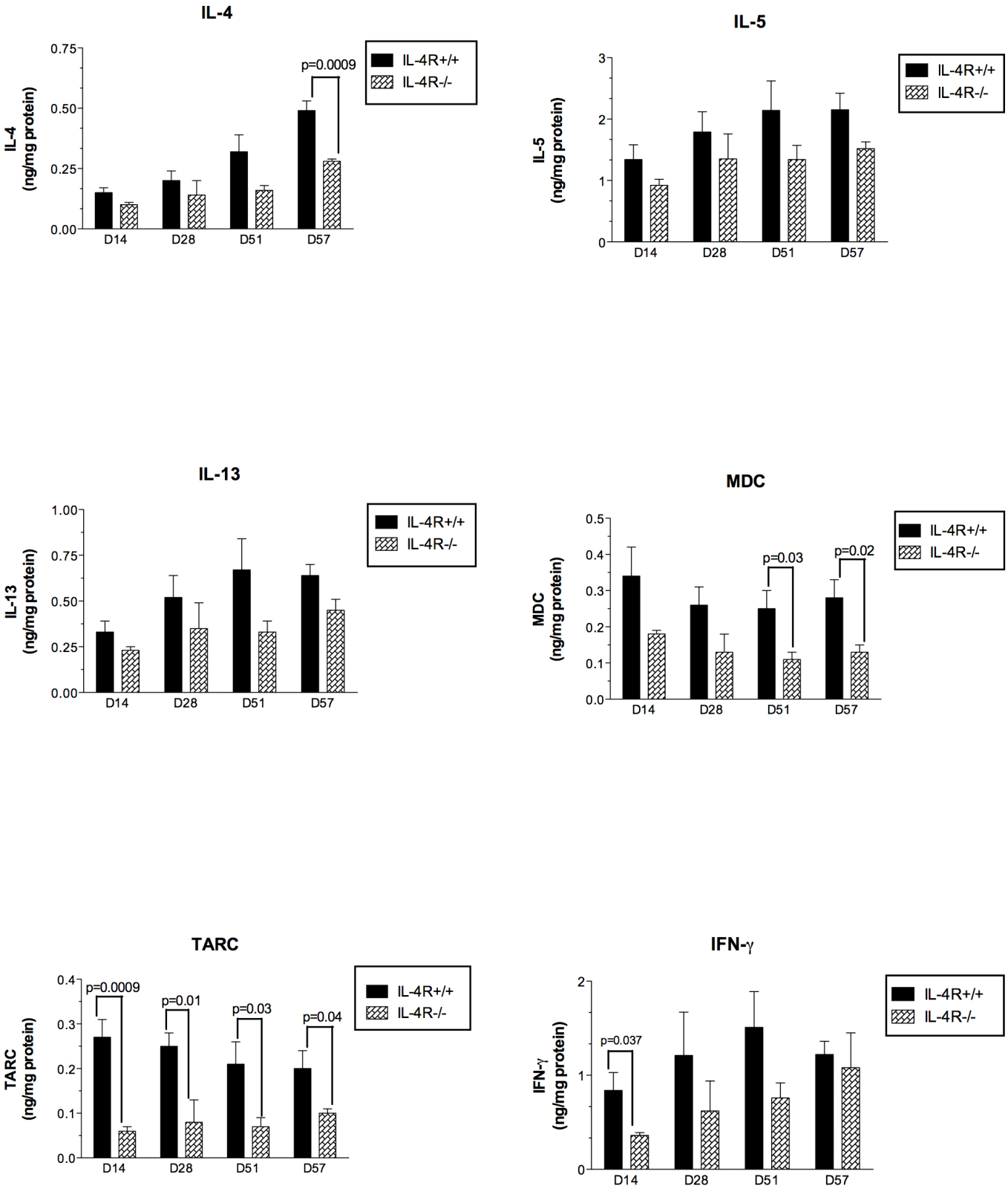

Fig. (3). Whole lung levels of IL-4, IL-5, IL-13, MDC, TARC, and IFN-γ in *A. fumigatus*-sensitized IL-4Rα wild-type ($^{+/+}$) and IL-4Rα knockout ($^{-/-}$) mice prior to and at days 14, 28, 51, and 57 after challenge with *A. fumigatus* conidia. Cytokine and chemokine levels were measured by a specific ELISA as described in the Materials and Methods section. Values are expressed as mean ± SEM; n=5/group/timepoint. Significant differences between wild-type and knockout groups are indicated.

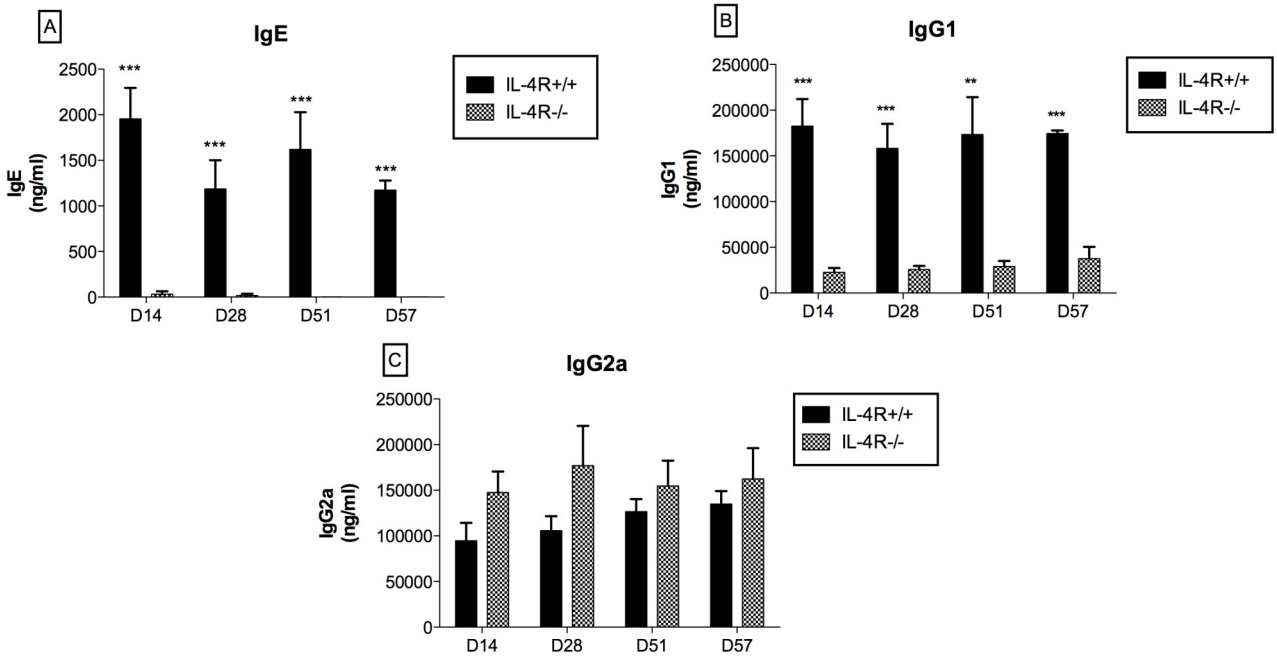

Fig. (4). Serum levels of IgE (**A**), IgG1 (**B**), and IgG2a (**C**) in *A. fumigatus*-sensitized IL-4Rα wild-type ($^{+/+}$) and IL-4Rα knockout ($^{-/-}$) mice prior to and at days 14, 28, 51, and 57 after challenge with *A. fumigatus* conidia. Ig levels were measured by a specific ELISA as described in the Materials and Methods section. Values are expressed as mean ± SEM; n=5/group/time point. Significant differences between wild-type and knockout groups are indicated; ** $P \leq 0.01$, *** $P \leq 0.001$.

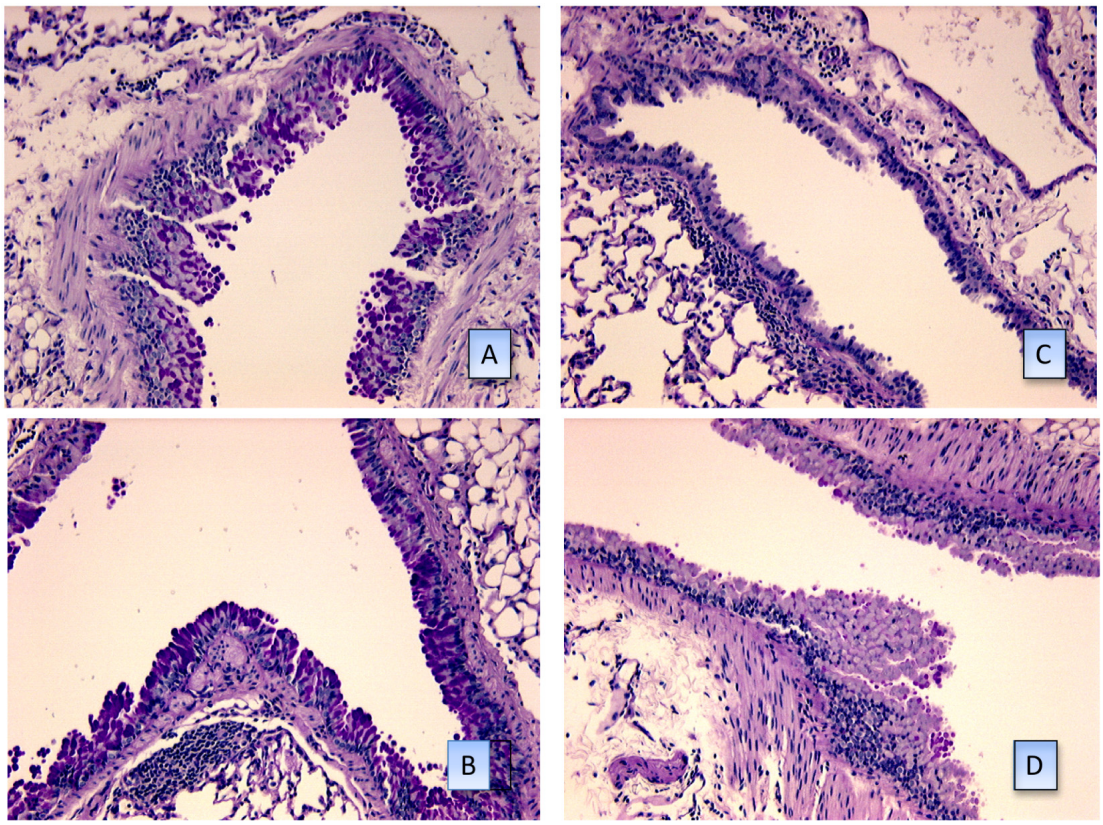

Fig. (5). Representative photomicrographs of PAS-stained whole lung sections from *A. fumigatus*-sensitized IL-4Rα wild-type ($^{+/+}$) (**A** and **C**) and IL-4Rα knockout ($^{-/-}$) (**B** and **D**) mice at days 14 (**A** and **B**) and 51 (**C** and **D**) after challenge with *A. fumigatus* conidia. Goblet cells (stained magenta) were prominent in the airways of the IL-4Rα$^{+/+}$ mice at both times after the conidia challenge. In contrast, PAS-stained cells were observed in the IL-4Rα$^{-/-}$ group at day 51 (**D**) but not at day 14 after conidia challenge. Original magnification was 200x for each photomicrograph.

days 51 and 57 after conidia challenge. Explanatory mechanisms were not apparent from our cellular and proteomic analysis in the present study. Although BAL neutrophil numbers were not different between the wild-type and knockout groups at day 51 after conidia, we were surprised by the marked neutrophilia in the bronchoalveolar lavage fluid in the IL4R$\alpha^{-/-}$ mice at day 57 after conidia (Fig. **2**). A similar pattern of sputum neutrophilia has been described in patients with severe, fatal asthma [24]. It is unclear at this time whether this phenotype represents a subtype of general asthma, or if there are other inflammatory mechanisms involved. The pattern of BAL neutrophilia, combined with mucus cell metaplasia and airway inflammation in a non-Th2 environment is also reminiscent of chronic obstructive pulmonary disease (COPD). Additional supportive findings of COPD would require quantitation of other inflammatory mediators including IL-8, TGF-B, matrix metalloproteinases, among others [25]. Nevertheless, it is unlikely that the increase in airway hyper-responsiveness observed in this study was due to increased neutrophil recruitment and/or activation in the knockout group. Further studies are required to elucidate the mechanism leading to increased airway reactivity to methacholine in the absence of IL-4Rα, and these future studies will be directed toward elucidating the significance of increased fungal material in the lungs of the knockout mice.

In summary, we have shown that IL4R$\alpha^{-/-}$ mice develop AHR and mucus cell metaplasia in the absence of a Th2 cytokine environment. These findings suggest that certain features of chronic asthma including AHR and mucus cell metaplasia might be mediated by Th2 independent mechanisms.

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