Absence of the Common Gamma Chain \((\gamma_c)\), a Critical Component of the Type I IL-4 Receptor, Increases the Severity of Allergic Lung Inflammation

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Abstract

The \(\text{T}_{h2}\) cytokines, IL-4 and IL-13, play critical roles in inducing allergic lung inflammation and drive the alternative activation of macrophages (AAM). Although both cytokines share receptor subunits, IL-4 and IL-13 have differential roles in asthma pathogenesis: IL-4 regulates \(\text{T}_{h2}\) cell differentiation, while IL-13 regulates airway hyperreactivity and mucus production. Aside from controlling \(\text{T}_{h2}\) differentiation, the unique contribution of IL-4 signaling via the Type I receptor in airway inflammation remains unclear. Therefore, we analyzed responses in mice deficient in gamma \(c\) (\(\gamma_c\)) to elucidate the role of the Type I IL-4 receptor. OVA primed CD4\(^+\) OT-II T cells were adoptively transferred into RAG2\(^{-/-}\) and \(\gamma_c^{-/-}\) mice and allergic lung disease was induced. Both \(\gamma_c^{-/-}\) and \(\gamma_c^{\text{XRAG2}^{-/-}}\) mice developed increased pulmonary inflammation and eosinophilia upon OVA challenge, compared to RAG2\(^{-/-}\) mice. Characteristic AAM proteins FIZZ1 and YM1 were expressed in lung epithelial cells in both mouse strains, but greater numbers of FIZZ1\(^+\) or YM1\(^+\) airways were present in \(\gamma_c^{-/-}\) mice. Absence of \(\gamma_c\) in macrophages, however, resulted in reduced YM1 expression. We observed higher \(\text{T}_{h2}\) cytokine levels in the BAL and an altered DC phenotype in the \(\gamma_c^{-/-}\) recipient mice suggesting the potential for dysregulated T cell and dendritic cell (DC) activation in the \(\gamma_c\)-deficient environment. These results demonstrate that in absence of the Type I IL-4R, the Type II R can mediate allergic responses in the presence of \(\text{T}_{h2}\) effectors. However, the Type I R regulates AAM protein expression in macrophages.

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Introduction

IL-4 and IL-13 are central mediators of asthmatic responses. They initiate and propagate hallmark features of asthma such as pulmonary inflammation, eosinophilia, mucus hypersecretion and airway hyperreactivity by engaging shared receptor complexes and signaling proteins [1,2,3,4]. IL-4 alone, binds to the Type I receptor (R), composed of the IL-4R\(\alpha\) chain and IL-2R\(\gamma\) (common gamma \((\gamma_c)\)) chain [reviewed in [5]]. Both IL-4 and IL-13, however, can signal through the Type II R (composed of IL-4R\(\alpha\) and IL-13R\(\alpha\)) [5].

While both IL-4 and IL-13 can elicit asthma pathology when provided exogenously, it is evident that they mediate different responses in vivo [2,3,6,7]. IL-4 is critical for \(\text{T}_{h2}\) cell differentiation and IgE synthesis, while IL-13 is predominantly responsible for inducing airway hyperresponsiveness and mucus secretion. The reason for this separation of duties is not well understood; relative abundance and differential usage of receptor complexes and signaling pathways in different cell types, together with greater quantities of IL-13 (than IL-4) produced during \(\text{T}_{h2}\) responses have been proposed to explain these observations (reviewed in [9]). A recent publication also suggested that there may be distinct cellular expression and localization of IL-4 and IL-13 [9].

The relative contributions of the Type I R and the Type II R to asthma pathophysiology are only now being investigated. The unique contributions of the Type II receptor in allergic lung inflammation were examined using IL-13R\(\alpha\) mice. It was reported that mucus secretion, airway resistance, eosinophilia, mucus hypersecretion and induction of pro-fibrotic mediators such as Th2 pathway (reviewed in [10]). However, Th2 cytokine production, IgE secretion, and recruitment of eosinophils into the lungs could occur independently of IL-13R\(\alpha\).

IL-4 and IL-13 also stimulate alternative activation of macrophages (AAM). AAM express a distinctive set of proteins such as Arginase 1 (Arg1), found in inflammatory zone (FIZZ); 1-4 and...
some members of the chitinase family such as acidic mammalian chitinase (AMCase) and YM1/2. DNA microarray analysis of cells isolated from allergen or IL-4 treated WT or IL-13Rα1−/− mice revealed that several AAM genes were differentially regulated by the Type I and Type II R. Munitz et al. showed that allergen- and IL-4-induced FIZZ1 (Retnla) expression levels were similar in both WT and IL-13Rα1−/− mice, but induction of chitinase (ChiA) was completely dependent on IL-13Rα1 [10]. Our studies indicated that IL-4 induces significantly greater expression of AAM genes (FIZZ1, YM1 and Arg1) when compared to IL-13 [12].

The above findings clearly demonstrated that IL-4 or IL-13 signaling through the Type II R is not required for mediating pulmonary inflammation and eosinophilia, and suggest the hypothesis that the Type I receptor is responsible for controlling the inflammatory response. Therefore, to test the specific role of the Type I R, we assessed the degree of airway inflammation, eosinophilia, and AAM stimulation upon allergen priming and challenge in mice lacking γc (and the Type I R). Here we report that γc deficient mice developed increased pulmonary inflammation and eosinophilia upon OVA challenge when compared to RAG2−/− mice when provided with OVA-specific T-cells. Although significantly higher numbers of FIZZ1+ or YM1+ airways were detected in γc−/− mice, absence of the Type I R in macrophages caused reduced YM1 expression in these cells. These results suggest that the Type I and Type II receptors have redundant functions in vivo and the Type II R can mediate effector allergic responses in absence of the Type I R. However, in macrophages the Type I R regulates YM1 protein expression.

Materials and Methods

Ethics Statement
All experimental procedures on mice were performed in accordance to guidelines issued by the National Institutes of Health Guide for the Care and Use of Laboratory Animals and approved by the Institutional Animal Care and Use Committee at UMB.

Mice
γc−/− mice and OT-II transgenic mice on a C57BL/6 background were acquired from Jackson Labs and bred in the animal care facility at the University of Maryland, Baltimore (UMB). Mice deficient in RAG2 (B6.RAG2−/−) were purchased from Taconic (Germantown, NY). γc−/− mice were obtained from Dr. Paul Antony at UMB and bred in house.

Adoptive Transfer of in vivo Primed CD4 T Cells
OT-II transgenic mice were immunized with 100 μg of chicken egg ovalbumin (OVA; Sigma-Aldrich, St. Louis, MO) adsorbed to aluminum hydroxide (alum; Sigma-Aldrich) intraperitoneally (i.p) and LN and spleens were harvested 10 days later. CD4+ T cells present in these tissues were purified by negative selection (Easy Sep kit, Stem Cell Technologies, Vancouver, Canada). Following this, in vivo primed CD4+ T cells were injected intravenously (i.v) via the tail vein in recipient mice (5×10⁶ cells/mouse).

Antigen Sensitization and Challenge
Mice were sensitized and challenged with OVA using a protocol described earlier [13]. Briefly, mice were immunized with either 100 μg of OVA/alum or alum alone on day 1 and day 6. After the last sensitization step, mice were challenged with aerosolized 1% OVA in PBS for 40 minutes each day on days 12 and 14.

Evaluation of Airway Inflammation
Bronchial lavage was performed 48 hours after the last OVA challenge as described previously [13]. The cellular component of the bronchoalveolar lavage (BAL) was used to determine total and differential cell counts and the supernatant was used for cytokine analysis.

Lung Histology and Immunohistochemistry
Lung histology sections were prepared as described [13]. Briefly, mouse lungs were perfused with 10–15 ml of PBS followed by fixation with 10% formalin. The tissues were then processed, embedded in paraffin and sectioned. After deparaffinization, slides were stained with Hematoxylin and Eosin (H&E) or Periodic acid Schiff (PAS). For immunohistochemistry, deparaffinized sections were incubated with 10% goat serum and stained with a 1:100 dilution of rabbit anti-mouse FIZZ1 (Abcam, Cambridge, MA) or 1:100 dilution of rabbit anti-mouse YM1 (Stem Cell Technologies, Vancouver, Canada). The histology sections were prepared by E.S., who generated unique slide numbers. The sections were then evaluated by P.D. without knowledge of the identity of the experimental groups. Areas of the slide that were representative for the whole group were photographed and digitally processed using CoolSnap (Roper Scientific, Trenton, NJ). For cell counts, photomicrographs of 10 100x fields were taken per mouse and average number of cells per high power field were calculated and graphed.

Assessment of Airway Remodeling
Lung sections were stained with Masson’s Trichrome to detect collagen deposition. The collagen content around the airways was quantified using NIH Image J software (National Institutes of Health, Bethesda, MD) [13]. Airway smooth muscle thickness was measured using H&E stained lung sections as described previously [13].

NK Cell Depletion
Anti-asialo GM1 antibody was obtained from Wako Chemicals USA. Mice were injected intraperitoneally with 30 μg anti-asialo GM1 antibody in a volume of 300 μl, starting on day -2 and every 5 days thereafter over the course of the study.

NK Cell Isolation and Transfer
Splenocytes from WT C57BL/6 mice or B6.STAT6−/− mice were enriched for NK cells using the Stem Cell Technologies NK cell enrichment kit. WT or STAT6−/− NK cells (1×10⁶) were transferred into γc−/− mice through the tail vein at the time CD4+ T cell adoptive transfer.

Preparation of Lung Digests
Lung tissue samples were harvested from mice 48 hours after the last challenge. The tissue was minced into small pieces and incubated with serum-free RPMI medium containing 150 U/ml Collagenase Type IV (Worthington Biochemicals) and 10 U/ml DNase (Roche) for 1 hour at 37°C. Cells were spun down and RBC lysis performed. After washing, cells were resuspended in complete medium and counted before use.

Cytokine and Chemokine Analysis
Cytokines in the BAL fluid or cell culture supernatants were analyzed by using individual ELISA kits for IL-4 (Pierce Thermo Scientific, Rockford, IL; BioLegend, San Diego, CA), IL-5, IL-13 and IFNγ (all from R&D Systems, Minneapolis, MN).
FACS Analysis

Single cell suspensions of splenocytes or BAL cells were incubated with Fc Block (2.4G2, BD Biosciences) followed by staining with fluorochrome-conjugated antibodies to surface markers (from BD Biosciences: CD4-PE, CD4-Alexa Fluor 647, CD11b-PE, OX10-PE, CD11c-ITC, F4/80-Alexa Fluor 647 and CD69-PerCP-Cy5.5. From eBioscience: CD44-PerCP-Cy5.5 and CD62L-PerCP-Cy5.5). Cells were washed twice with FACS buffer and analyzed directly or after fixing with 4% paraformaldehyde by using a FACSCalibur machine (Becton Dickinson, Franklin Lakes, NJ). For measurement of intracellular cytokine staining, cells were fixed and permeabilized using a BD Cytofix/Cytoperm kit (BD Biosciences), followed by staining with antibodies for intracellular proteins. After incubation with antibodies, cells were washed twice with Perm Wash Buffer and resuspended in FACS buffer before acquisition. Data was analyzed by using FlowJo software (Treestar, CostaMesa, CA).

Statistical Analysis

Anova single factor data analysis tool was used to compare the differences between two groups and to calculate significance values. p values of ≤0.05 were considered statistically significant.

Results

To determine the contribution of the Type I R in inducing features of allergic lung disease, we utilized \( \gamma_c \)−/− mice. Since T cells play a critical role in initiating and propagating asthma and airway disease, we induced allergic inflammation in \( \gamma_c \)−/− mice by using FlowJo software (Treestar, CostaMesa, CA). The differences in eosinophil counts in the BAL (Figure 1C) in the two mouse strains were recapitulated in the lung tissue. The number of eosinophils recruited to the airways and blood vessels in both alum- and OVA-primed and OVA-challenged \( \gamma_c \)−/− mice were significantly increased in comparison to their RAG2−/− counterparts (Figure 2).

To rule out the possibility that small differences in the genetic background of these mice were causing the differences in allergic lung inflammation seen in these mice, we repeated the above experiment with RAG2−/− mice and \( \gamma_c \times RAG2−/− \) mice. \( \gamma_c \)−/− mice on a RAG2−/− background still developed significantly higher pulmonary inflammation and eosinophilia (Figure 3A and 3B). Thus, the responses seen in \( \gamma_c \)−/− and \( \gamma_c \times RAG2−/− \) mice were essentially the same in all aspects and these mice were used interchangeably. These results show that the Type I R is not required for inflammatory response. They further suggest that in the absence of \( \gamma_c \) and the Type I R, the Type II R can mediate the pulmonary inflammatory response as well as mucus production. Moreover, deficiency of \( \gamma_c \) in cells other than T cells led to exaggerated asthma pathology.

Effect of \( \gamma_c \) Deficiency on FIZZ1 and YM1 Protein Expression

Gene expression profiling of lungs from allergen challenged IL-13Rα1 deficient mice had identified FIZZ1 upregulation as being completely independent of the Type II R while YM1 was only partially dependent [10]. This suggested that the Type I R may contribute to AAM gene expression. We have previously found that bone marrow-derived macrophages (BMM) isolated from WT mice induced robust induction of FIZZ1 and YM1 transcripts when stimulated with IL-4 in vitro [12]. In contrast, IL-4 treated BMM from \( \gamma_c \)−/− mice demonstrated significantly reduced AAM gene expression. Thus, we examined if expression of FIZZ1 and YM1 protein was reduced in vivo during allergic lung inflammation when \( \gamma_c \) and the Type I R were absent.

To determine if macrophages or epithelial cells or both were producing FIZZ1 and YM1, immunohistochemical staining was performed on serial lung sections from alum- or OVA-primed and OVA-challenged RAG2−/− and \( \gamma_c \)−/− mice. No YM1 or FIZZ1 was detected in lung epithelial cells in RAG2−/− mice in the absence of OVA priming (Figure 4A, panels a & c), but expression of these proteins was increased upon OVA priming (panels b & f). However, both alum- and OVA-primed \( \gamma_c \)−/− epithelial cells stained strongly for YM1 and FIZZ1 after OVA challenge (panels c-d & g-h). Quantification of YM1 or FIZZ1 in both mice showed a significant increase in the numbers of airways.
expressing these proteins in $\gamma_c^{−/−}$ mice over RAG2$^{−/−}$ mice (Figure 4B).

Unlike epithelial cells, which expressed both YM1 and FIZZ1 protein, macrophages expressed only YM1 (Figure 5A). In contrast to the epithelial cells, however, YM1 expression in $\gamma_c^{−/−}$ macrophages was found to be less intense than macrophages present in RAG2$^{−/−}$ mice (panels b-d). This observation was confirmed by monitoring YM1 expression in BAL macrophages by flow cytometry. Although the percentages of CD11b$^+$YM1$^+$ cells were similar in both groups of mice, the mean fluorescence intensity (MFI) of YM1 staining was reduced by half when these cells lacked $\gamma_c$ (Figure 5B). The differences in YM1 staining intensity in the two mouse strains were significant (Figure 5C).

Taken together, these results indicate that the Type I R regulates YM1 protein expression in macrophages in vivo. This is consistent with in vitro studies that have demonstrated that IL-4 induced greater YM1 gene and protein expression than IL-13 in BMM cells [12]. Epithelial cells, which express only the Type II R,
can still express both YM1 and FIZZ1. Moreover, deficiency of γc led to enhanced YM1 and FIZZ1 production in these cells after OVA challenge whether or not the recipients were primed with OVA.

Effect of γc Deficiency on Airway Remodeling

Excessive IL-4/IL-13 signaling in many different cell types during pulmonary inflammation can cause airway remodeling (reviewed in [14]). Overexpression of IL-4 or IL-13 in the lungs of mice led to induction of profibrotic mediators and myofibroblast activation [6,15]. However, the contribution of the individual receptors (Type I vs Type II R) is still unclear. Studies have shown that the Type II R is required for TGFβ production but not for fibroblast activation in vivo [10,11]. To determine the role of the Type I R in airway remodeling in vivo, we analyzed the amount of collagen deposition and airway smooth muscle thickness in RAG2−/− and γc−/− mice. Masson’s Trichrome staining of lung sections revealed that a small amount of collagen (shown in blue) was present around the airways in RAG2−/− mice primed with alum (Figure 6A, panel a), which increased significantly upon priming with OVA (panel b). However, both alum- and OVA-primed γc−/− mice showed extensive collagen deposition, when compared with RAG2−/− mice (panels c&d). Quantification of collagen staining using image analysis software showed that the differences were significant (Figure 6B). Furthermore, there was a marked increase in Airway Smooth Muscle thickness in mice lacking γc in comparison to their RAG2−/− counterparts (panels e-h and 6C). Interestingly, there is a correlation between the degree of airway remodeling and the extent of inflammation (Figure 1) observed in the above mice. These results demonstrate that airway remodeling can occur independently of the Type I R in vivo. In fact, absence of γc chain enhanced collagen deposition and increased the diameter of the airway smooth muscle layer.

Cytokine Production by Control and γc Deficient Mice

Secretion of Th2 cytokines generally positively correlates with the degree of inflammation. Since γc deficient mice developed severe lung pathology, we assessed the amount of IL-4, IL-5 and IL-13 present in the BAL. In the BAL, IL-13 levels were significantly greater in both alum- or OVA-primed γc−/− mice in comparison to RAG2−/− mice; IL-4 and IL-13 levels showed a similar trend although the values did not meet the threshold for significance (Figure 7A). Along with the increase in Th2 cytokine levels, we observed a decrease in IFNγ secretion in the absence of γc. Thus, in the absence of γc, in recipient mice there was an increase in Th2 cytokines, even in the absence of OVA priming.

Role of NK Cells in the γc Deficient Asthma Model

NK cell numbers are reduced in mice deficient in γc [16]. Previous studies have reported that IL-4 signaling through the Type I R induces IFNγ production by NK cells in a STAT6 dependent manner [17,18]. Since we observed reduced levels of IFNγ in the BAL fluid of γc−/− mice, we tested whether reduced numbers of NK cells in these mice was responsible for enhanced Th2 cell activation and allergic lung inflammation. We first depleted NK cells in RAG2−/− mice using antibodies against asialo GM1 using the regimen depicted in Figure S2A. If lack of IFNγ production by NK cells was causing the enhanced asthma phenotype observed in γc deficient mice, then we would expect to see the same phenotype in NK cell depleted RAG2−/− mice. Treatment of RAG2−/− mice with asialo GM1 antibodies against NK cells transferred WT or STAT6-deficient CD49b+ (also known as DX5) NK cells into γc−/− mice. The purity of NK cells before
adoptive transfer was evaluated (Figure S3A) and the mice were then subjected to our asthma protocol (Figure S3B). We confirmed that the NK cells had repopulated into the recipient mice (Figure S3C). As observed previously, greater numbers of eosinophils were present in the BAL in \( \gamma_c^{+/-} \) mice upon OVA priming and challenge when compared to \( \gamma_c^{-/-} \) mice (Figure S3D). Transfer of WT or STAT6 \( ^{-/-} \) NK cells into \( \gamma_c^{-/-} \) mice, however, did not change the numbers or percentages of eosinophils significantly. Thus, an alteration in NK cell numbers was likely not the reason for the enhanced allergic lung disease phenotype seen in mice deficient in \( \gamma_c \).

**Figure 3.** \( \gamma_c^{+/-} \) mice also develop enhanced allergic lung inflammation. (A) H&E (10X and 100X) and PAS (10X) stained lung sections of \( \gamma_c^{-/-} \) and \( \gamma_c^{+/-} \) mice are shown here. (B) The number of macrophages, eosinophils, lymphocytes and neutrophils present in the BAL in these mice are represented in the form of bar graphs. *p<0.05. n = 5 for OVA-primed mice, n = 3 for alum-primed. Representative data from one of two experiments is shown.

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**Analysis of Treg and DC Populations in RAG2\(^{-/-}\) and \( \gamma_c^{+/-} \) Mice**

We transferred a population of *in vivo* primed CD4\(^+\) T cells that were not depleted of CD4\(^+\) CD25\(^+\) regulatory T (Treg) cells. Since regulatory T cells can suppress Th2 and Th1 cell function, we evaluated if there was a decrease in the numbers of Tregs after transfer into mice lacking \( \gamma_c \). We found, however, that the percentage of CD4\(^+\) CD25\(^+\) cells and Foxp3\(^+\) cells was generally low in RAG2\(^{-/-}\) mice and was a three fold increase in their numbers in the \( \gamma_c^{+/-} \) recipient mice. Thus, there was no evidence of a reduction in Treg numbers after adoptive transfer to
The transferred T cells expressed γc, but were placed into a γc deficient environment that could impact their subsequent responses to OVA. It is well known that dendritic cells play an important role in T cell priming. Thus, we investigated whether there was any change in the phenotype of DCs in γcxRAG22/2 mice. DCs in the lung were identified as CD11c+F4/802 (Figure 8B) and CD11b and OX40L expression in these cells were monitored. While we observed an overall increase in CD11c+F4/802 DC in γxRAG22/2 mice, there was a reduction in the percentages of CD11b+ DCs in γc deficient mice from 23% to 15%. In addition, the percentage of DCs expressing OX40L was modestly increased from 11% to 17% (Figure 8B). Taken together, these results suggest the possibility that enhanced allergic lung inflammation occurring in mice deficient in γc may be caused by dysregulated activation of the adaptively transferred γc+ T-cells.

Discussion

There is overwhelming evidence linking IL-4 and IL-13 signaling to allergic asthma responses. Since IL-4 and IL-13 share receptor complexes, however, the exact contribution of the individual receptor complexes in inducing asthma pathophysiology is unclear. In this study, we used mice deficient in γc to elucidate the role of the Type I IL-4 receptor.

In addition to lacking the Type I IL-4 receptor, the γc−/− mice are also deficient in IL-2R, IL-7R, IL-9R, IL-15R and IL-21R. IL-2 and IL-7 signaling play an important role in lymphocyte development and survival (reviewed in [19,20]): γc−/− mice lack T and B cells. Therefore, we used RAG22/2 mice as controls for all our experiments. Since T, B cells, and the cytokines they produce, are essential for initiation and propagation of allergic responses, we adoptively transferred in vivo-primed CD4+ T cells from OT-II (OVA specific) transgenic mice. These transferred T cells express γc and thus, receptors for IL-2, IL-4, IL-7 and IL-15. Therefore,
they can respond to these cytokines even in a γc deficient environment. We have demonstrated previously that TCR transgenic mice can be immunized with OVA/alum and that transfer of in vivo primed CD4+ T cells into mice followed by OVA/alum priming and OVA challenge was sufficient to induce features of allergic lung inflammation [13].

Mice lacking the Type II IL-4/IL-13 receptor (IL-13Rα1−/− mice) still developed pulmonary inflammation and eosinophilia upon allergen challenge [10,11]. Therefore, we hypothesized that IL-4 signals through the Type I R may be uniquely responsible for inducing these effects. However, our results show that the absence of the γc chain caused no defect in these processes, suggesting that the Type I R is not absolutely required for mediating inflammatory responses and eosinophil recruitment into the lung. Since these responses are dependent on the IL-4Rα [1], the Type I and

Figure 5. FIZZ1 and YM1 expression in macrophages. Allergic lung disease was induced in RAG2−/− and γc−/− mice as shown in Figure 1. Serial sections of mouse lungs were stained for FIZZ1 and YM1 by immunohistochemistry. Photomicrographs (100X magnification) of YM1 (panels a-d) and FIZZ1 (panels e-h) expression in macrophages in representative lung sections are shown. YM1+ macrophages are indicated by arrows. (B) BAL cells from RAG2−/− and γcXRAG2−/− mice immunized and challenged with OVA were collected and analyzed by FACS. Cells were labeled with a fluorochrome-conjugated antibody to CD11b, stained with an antibody to YM1, followed by a secondary antibody conjugated to Alexa Fluor 647 (solid histogram). Secondary antibody staining alone was used as control (dashed histogram). Macrophages were gated based on forward by side scatter and then on CD11b expression. MFI = Mean Fluorescence Intensity of YM1 expression. (C) The average MFI of YM1 staining in macrophages from RAG2−/− and γcXRAG2−/− mice from different experiments is shown. *p<0.05, n=5 for OVA-primed mice, n=3 for alum primed. Representative data from one of two experiments is shown.

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Type II receptors must mediate redundant functions for the inflammatory response. Interestingly, we found that c c deficiency significantly enhanced lung pathology mediated by the transferred OT-II helper T-cells. While the exact mechanism involved is unknown, the increase in TH2 cytokines in the BAL fluid of c c and c cxRAG2 mice, together with the reduction in IFN \( \gamma \), may contribute to this exaggerated asthma response. The increased amounts of IL-4 and IL-13 present in c c deficient mice could amplify signaling through the Type II R and enhance asthma responses.

We also observed that c c deficiency in mice led to enhanced airway remodeling, leading to excessive collagen deposition and increase in smooth muscle thickness. IL-13 signaling through the Type II R is considered to be the dominant inducer of fibrosis. IL-13 induces macrophages to produce TGF\( \beta \) and can act directly or indirectly on fibroblasts inducing collagen and extracellular matrix deposition (reviewed in [14]). It has also been reported that both eosinophils and FIZZ1 and YM1 can cause lung fibrosis and smooth muscle thickening [21,22,23]. In our model, the extent of inflammation in mice correlates well with the degree of airway remodeling.

Previous studies had indicated that gene expression of AAM products in the lung were differentially regulated by the Type I and Type II receptors: YM1 mRNA expression was partially dependent on IL-13R\( \alpha \)1, while FIZZ1 mRNA induction was completely independent of this chain [10]. Here we show that epithelial cells in both RAG2\( ^{-/-} \) and c c mice were able to produce FIZZ1 and YM1, suggesting that induction of these proteins can occur independently of IL-4 signaling through the Type I R. We also observed that greater numbers of airways were YM1+ or FIZZ1+ in c c mice. This was surprising, as epithelial cells usually lack c c expression. It is possible however, that the increased levels of IL-4 and IL-13 present in these mice results in greater engagement of these cytokines with the Type II R on epithelial cells, thus enhancing YM1 and FIZZ1 protein expression. Conversely, the decreased levels of IFN\( \gamma \) observed in the BAL fluid may be causing the enhanced T\( H2 \) responses seen in

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**Figure 6. Enhanced airway remodeling in c c deficient mice.** RAG2\( ^{-/-} \) and c c mice were subjected to the asthma protocol as described in Figure 1. (A) Lung sections of mice were stained with Masson’s Trichrome. Photomicrographs of collagen deposition around the airways (panels a-d) in RAG2\( ^{-/-} \) and c c mice primed with Alum or OVA/alum are shown. Panels e-h: photomicrographs (40X) of the airway smooth muscle (ASM) layer in H&E stained lung sections from each mouse group. Arrows depict the thickness of the ASM layer (transverse section). (B) Collagen deposition in the lung was quantified using NIH Image J software. Data is represented as area of collagen (blue stain) ± SEM. (C) The distance between the innermost and outermost aspect of the smooth muscle was measured at 3 different positions around each airway, using NIH Image J software. Data is represented as airway smooth muscle thickness in \( \mu m \) ± SEM. n=3 mice/group. An average of 10 airways was analyzed per mouse. *\( p<0.05 \), **\( p<0.01 \), # (\( p<0.05 \)) represents statistically significant differences between the OVA and Alum primed mice.

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epithelial cells. IFNγ signaling in airway epithelial cells has been reported to suppress STAT6 activation [24].

Our group had shown earlier that IL-4 induced robust AAM gene expression in BMM cells in vitro while IL-13 was less potent in inducing the same responses [12]. When macrophages lacked the γc chain and the Type I R, however, their response to IL-4 was reduced, yet IL-13 responses were intact in these cells [12]. Consistent with these in vitro studies, we found that YM1 protein expression in airway macrophages was reduced by half in the absence of the γc chain, suggesting that the Type I R regulates YM1 protein expression in macrophages in vivo.

The enhanced TH2 responses and allergic lung inflammation occurring as a result of γc deficiency was puzzling. In addition to the Type I IL-4R, the IL-9R, IL-15R and IL-21R are also absent in γc−/− mice. Studies in IL-9 deficient mice have demonstrated that TH2 differentiation, eosinophilic inflammation, AHR, mucus production in NK cells [17,18] and IFNγc deficiency was puzzling. In addition to the Type I IL-4R, the IL-9R, IL-15R and IL-21R are also absent in γc−/− mice. Studies in IL-9 deficient mice have demonstrated that TH2 differentiation, eosinophilic inflammation, AHR, mucus production in NK cells [17,18] and IFNγc−/− mice have demonstrated that TH2 differentiation, eosinophilic inflammation, AHR, mucus production in NK cells [17,18] and IFNγc−/− mice have demonstrated that TH2 differentiation, eosinophilic inflammation, AHR, mucus production in NK cells [17,18] and IFNγc−/− mice have demonstrated that TH2 differentiation, eosinophilic inflammation, AHR, mucus production in NK cells [17,18] and IFNγc−/− mice have demonstrated that TH2 differentiation, eosinophilic inflammation, AHR, mucus production in NK cells [17,18] and IFNγ.

Recent studies in Type II T cells that have been primed only once in vivo, we performed additional rounds of OVA/alum priming in the host after adoptive transfer. Dendritic cells play an integral role in T cell priming, and therefore, we postulated that the absence of γc in DCs may cause dysregulated T cell priming. Indeed, we observed an increase in the TH2 cytokine production and an increase in the percentage of Foxp3γ cells when T cells were primed and challenged with OVA in the γc−/− deficient environment. It will be interesting to determine whether the Foxp3γ cells maintain suppressor function or acquire the ability to make effector cytokines. In addition to the T-cell changes, we found that the number of CD11b+CD11c+ DCs was reduced in γc×RAG2−/− mice while OX40Lγ DC11c+ cells were modestly increased in these mice. DCs express both the Type I and Type II receptor and it has been established that these two receptors have differential roles in DC function. Lutz et. al. demonstrated that both IL-4 and IL-13 promote DC maturation by signaling mainly through the Type II R [31]. In contrast, the Type I R induces IL-12 production in DCs. It is conceivable that in γc deficient mice, absence of this negative signaling loop causes enhanced TH2 priming. OX40L expression in DCs is associated with increased TH2 differentiation in absence of IL-12 [32]. It has

**Figure 7. Cytokine production on OVA priming and challenge in mice.** Mice were subjected to the asthma protocol mentioned in Figure 1 and Material and Methods. Cytokine levels in BAL fluid from alum- or OVA-primed and OVA-challenged RAG2−/− and γc−/− mice were analyzed. *p<0.05, n = 5 for OVA-primed groups, n = 3 for alum-primed. Representative data from one of two experiments is shown.

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also been reported that the balance between myeloid DCs and plasmacytoid DCs is altered in asthma, with a significant increase in the numbers of pDCs in asthma patients [33]. The reduction in CD11b+ DCs in γcRAG2−/− mice points to a reduction in the numbers of mDCs in these mice, since CD11b is a marker for this subset of cells.

In summary, these results demonstrate that expression of γc is not required for eliciting effector asthma responses such as pulmonary inflammation, recruitment of eosinophils and mucus production. In the absence of the Type I R, the Type II R is sufficient to mediate these responses. In contrast, AAM protein expression in macrophages was dependent on the Type I R. Mice deficient in γc, however, developed a severe asthma phenotype when compared to control mice. Elevated Th2 cytokine production may be responsible for the exacerbated asthma responses seen in γc deficient mice.

Supporting Information

Figure S1 Activation status of in vivo primed CD4+ T cells from OT-II mice. (A) Splenocytes were isolated from unimmunized or OVA/alum-immunized OT-II transgenic mice and cultured in vitro in media alone or with anti-CD3 and anti-CD28 for 48 hours. Cells were stained with fluorochrome-conjugated antibodies and flow cytometry was performed. The OVA-specific T cells (CD4+Vα2+Vβ5+) were gated and expression of CD44, CD62L and CD69 was monitored. (B) Splenocytes isolated from unimmunized or immunized OT-II mice were cultured in presence or absence of PMA/Ionomycin for 18 hours. ELISA was performed on cell culture supernatants.

Figure S2 NK cell depletion in RAG2−/− mice does not enhance allergic lung inflammation. (A) Schematic representation of asthma protocol used in this study. Mice were primed with OVA/alum or alum alone and challenged with aerosolized OVA as mentioned in Materials and Methods. In addition, OVA/alum or alum treated RAG2−/− mice were injected with anti-asialo GM1 antibodies i.p. every 5 days, starting on day -2. (B) Depletion of NK cells in RAG2−/− mice were confirmed by flow cytometry. (C) Differential cell counts of BAL cells isolated from RAG2−/− and γc−/− mice after OVA priming and challenge is depicted. *p<0.05, n = 5 for OVA-primed mice, n = 3 for alum primed.

Figure S3 Transfer of NK cells does not reduce asthma responses. (A) CD49b+ NK cells were enriched from the spleens of STAT6+t+ and STAT6−/− mice as described in Materials and
Methods. After enrichment, the cells were stained with antibodies to CD49b and CD3. The percentage of CD49b+ NK cells before and after enrichment is shown. (B) Schematic representation of the protocol used in this study. Briefly, 5 × 10^6 CD4+ T cells were transferred into recipient mice in the presence or absence of 1 × 10^9 PHA or 1 × 10^9 cytokine-activated NK cells. Mice were primed and challenged twice with OVA on the days indicated. After the last challenge, mice were euthanized and BAL fluid and tissue samples were collected. (C) Splenocytes from recipient mice treated as described above in (B) were harvested and analyzed for expression of CD49b and CD3 by flow cytometry. (D) BAL fluid from recipient mice treated as described above in (B) were harvested. The numbers and percentages of macrophages, eosinophils, lymphocytes (Lym) and neutrophils (PMN) present in the BAL after priming and challenge with OVA in the different groups of mice were enumerated by differential counting after cytopsin. (n = 4 for each group).

(TIF)

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Author Contributions

Conceived and designed the experiments: PD ADK. Performed the experiments: PD XQ EPS. Analyzed the data: PD. Contributed reagents/materials/analysis tools: ADK. Wrote the paper: PD ADK.

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