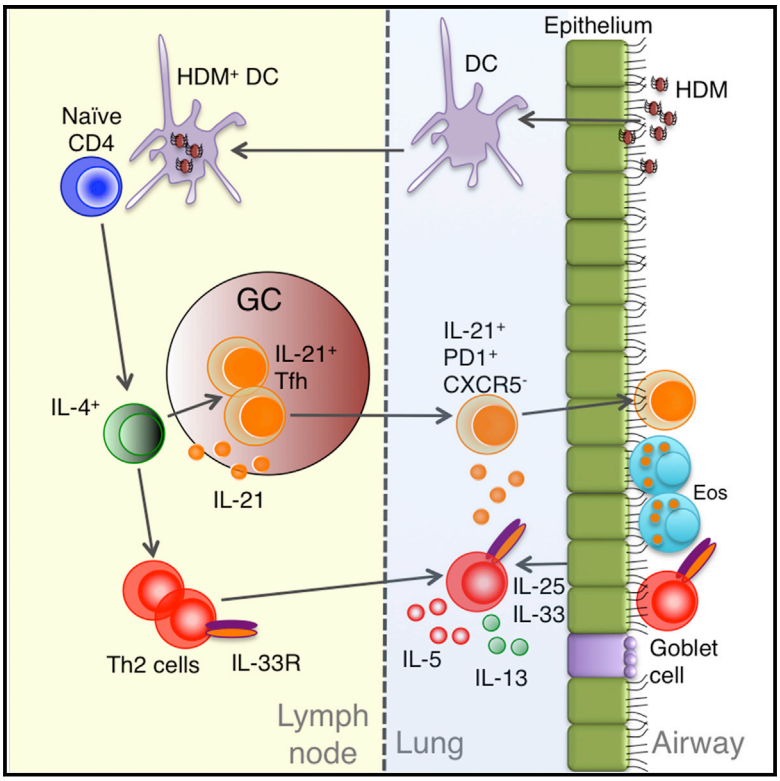
Article

Immunity

Interleukin-21-Producing CD4+ T Cells Promote Type 2 Immunity to House Dust Mites

Graphical Abstract



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In Brief

Asthma is a well-described Th2-cell-mediated disease. Coquet, Lambrecht, and colleagues demonstrate that the allergen house dust mite induces pronounced IL-21 production in lymph nodes draining the lung, and also in lung tissue and in the airways. IL-21 and its receptor promote type 2 responses from adaptive CD4+ T cells.

Highlights

- House dust mites induce potent IL-21 production by Tfh cells in lymphoid organs
- IL-21+ cells are distinct from Th2 cells and migrate to the lung tissue and airway
- IL-21R provides cell-intrinsic cues for Th2 cell functions
- IL-21 synergizes with innate signals to promote adaptive Th2 cell responses

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Interleukin-21-Producing CD4+ T Cells Promote Type 2 Immunity to House Dust Mites

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SUMMARY

Asthma is a T helper 2 (Th2)-cell-mediated disease; however, recent findings implicate Th17 and innate lymphoid cells also in regulating airway inflammation. Herein, we have demonstrated profound interleukin-21 (IL-21) production after house dust mite (HDM)-driven asthma by using T cell receptor (TCR) transgenic mice reactive to *Dermatophagoides pteronyssinus 1* and an IL-21GFP reporter mouse. IL-21-producing cells in the mediastinal lymph node (mLN) bore characteristics of T follicular helper (Tfh) cells, whereas IL-21$^+$ cells in the lung did not express CXCR5 (a chemokine receptor expressed by Tfh cells) and were distinct from effector Th2 or Th17 cells. *Il21r*$^{-/-}$ mice developed reduced type 2 responses and the IL-21 receptor (IL-21R) enhanced Th2 cell function in a cell-intrinsic manner. Finally, administration of recombinant IL-21 and IL-25 synergistically promoted airway eosinophilia primarily via effects on CD4$^+$ lymphocytes. This highlights an important Th2-cell-amplifying function of IL-21-producing CD4$^+$ T cells in allergic airway inflammation.

INTRODUCTION

Asthma is an ever-increasing global health problem characterized by the development of robust T helper 2 (Th2)-cell-mediated immunity (Lambrecht and Hammad, 2015). Th2 cells secrete cytokines including interleukin-4 (IL-4), IL-5, IL-9, and IL-13, which promote airway inflammation, stimulate mucus production by lung epithelial cells, and modulate antibody secretion by B cells (Paul and Zhu, 2010). Critical for the differentiation of Th2 cells from naive CD4$^+$ T cells are the transcription factors signal transducer and activator of transcription (STAT) 6 and GATA-3 (Kaplan et al., 1996; Zheng and Flavell, 1997), which remodel chromatin at critical Th2 cell gene loci and can directly influence Th2-cell-associated cytokine gene expression (Paul and Zhu, 2010). A number of soluble factors such as IL-4 can activate and induce the expression of STAT-6 and GATA-3, whereas IL-25 and IL-33 directly induce secretion of IL-5 and IL-13 from differentiated Th2 cells (Fort et al., 2001; Schmitz et al., 2005). Studies in mice and humans have provided conclusive evidence that Th2 cells are the major cell population driving asthma.

Other T helper cell subsets also regulate the development of asthma (Lambrecht and Hammad, 2015). Th1 cells that secrete interferon-γ (IFN-γ) are thought to prevent airway inflammation and can impede the differentiation of Th2 cells (Gajewski and Fitch, 1988). IL-17 secreted by Th17 cells has a potentially dual function, on the one hand enhancing T cell priming in lung-draining lymph nodes (Schnyder-Candrian et al., 2006) and triggering bronchial smooth muscle cell contraction (Kudo et al., 2012), but on the other hand reducing eosinophilic infiltration of the airways (Schnyder-Candrian et al., 2006), a hallmark of asthma. More recently, the innate lymphoid cell type 2 (ILC2) population, which shares many characteristics of Th2 cells including pronounced secretion of IL-5 and IL-13, has also been demonstrated to promote many features of asthma (Barlow et al., 2012; Neill et al., 2010). Because ILC2s lack a T cell receptor (TCR), they react to allergen challenge in an antigen non-specific, yet cytokine-driven, manner, mainly responding to the cytokines IL-25 and IL-33 made by barrier epithelial cells (Hammad and Lambrecht, 2015). Hence, although Th2 cells remain central to the asthmatic response, a range of cell types might influence their activity.

The role of follicular T helper (Tfh) cells in the development of asthma remains more ambiguous. Tfh cells are characterized by the expression of the chemokine receptor CXCR5 as well as PD-1 (Chtanova et al., 2004; Schaerli et al., 2000). An important feature of Tfh cells is their ability to secrete high amounts of IL-21 (Vogelzang et al., 2008). IL-21, originally identified as a CD4$^+$ T-cell- (Parrish-Novak et al., 2000) and NKT-cell- (Coquet et al., 2007; Harada et al., 2006) derived cytokine, has broad immune-stimulatory properties on a range of cell types including

T and B cells (Spolski and Leonard, 2008). The expression of CXCR5 on the surface of Tfh cells allows for their migration to B cell areas in primary lymphoid organs, where IL-21 promotes antibody affinity maturation in germinal center (GC) B cells (Linterman et al., 2010; Zotos et al., 2010). The important role for IgE production by plasma cells in some forms of asthma (Burrows et al., 1989) has led to the presumption that Tfh cells might play an important role in allergic diseases, even if IgE GC reactions are very short lived (Dullaers et al., 2012).

A number of experimental mouse models have alluded to close co-operation between Th2 and Tfh cells. The experimental antigen chicken ovalbumin (OVA) in combination with alum induces potent Th2 and Tfh cell responses (Baumjohann et al., 2011). Furthermore, in models of nematode infection, robust Th2-cell-mediated immunity is known to coincide with Tfh cell responses (King and Mohrs, 2009; León et al., 2012; Reinhardt et al., 2009). In these models, effector IL-4-producing Th2 cells are known to develop at the T cell-B cell border of the priming lymph node and this has been shown to depend on CXCR5 expression on DCs (León et al., 2012; Liang et al., 2012). These studies imply that Th2 and Tfh cells develop in close co-operation during Th2-cell-mediated immune responses. However, the potential role(s) of Tfh cells in clinically relevant models of asthma has not been systematically examined.

Herein, we investigated the role of IL-21 in a mouse model of asthma induced by house dust mites (HDMs). By using IL-21GFP reporter mice (Lüthje et al., 2012) and T cell receptor (TCR) transgenic mice expressing a TCR reactive to the Der p1 peptide of HDM, we demonstrate that IL-21 was highly expressed in mice sensitized and challenged with HDMs. IL-21$^+$CD4$^+$ T cells were prominent in the airways, lung tissue, and lung-draining lymph nodes and IL-21R promoted Th2 cell function in a cell-intrinsic manner. These findings suggest that IL-21-producing cells are crucial for promoting type-2-cell-mediated immune responses in asthma.

RESULTS

Th2-, Th17-, and Tfh-Cell-Associated Gene Expression Is Increased in Lung CD4$^+$ T Cells during House-Dust-Mite-Induced Asthma

Recent evidence has implicated a variety of cell types in the asthmatic response (Lambrecht and Hammad, 2015). To gain greater insight into the role of other T helper cell subsets during asthma, we conducted gene expression profiling of lung CD4$^+$ T cells in naive and asthmatic mice sensitized to HDMs on day 0 and challenged daily intranasally (i.n.) between days 7 and 11. Lung CD4$^+$ T cells from HDM-exposed asthmatic mice had upregulated the expression of numerous Th2-cell-associated factors such as *Il13*, *Il4*, *Il5*, *Il1rl1* (part of IL-33R), and *Il17rb* (part of IL-25R) (Figures 1A, 1B, and S1). Classical Th17-cell-associated genes were also upregulated in lung CD4$^+$ T cells from asthmatic mice. Flow cytometric analysis of CD4$^+$ T cells infiltrating the airways showed that Th17 cells were largely distinct from IL-4$^+$ or IL-5$^+$ cells and did not express the classical Th2 cell marker, IL-33R (Figure 1C). In addition to Th2 and Th17 cells, *Il21* and *Pdcd1* (PD-1) were increased in lung CD4$^+$ T cells (Figure 1B), suggesting that Tfh cell responses might be involved in the development of asthma. Gene expression changes in lung CD4$^+$ T cells were confirmed with limited quantitative PCR in separate experiments (Figure S1). Th1-cell-associated gene expression was generally unchanged or decreased in mice receiving HDMs. These data imply that CD4$^+$ T cells from asthmatic mice acquire features not only of Th2 cells, but also of the Th17 and Tfh cell lineages.

IL-21R Is Necessary for the Development of Eosinophilic Airway Inflammation

To investigate the role of Tfh cells and IL-21 in HDM-induced asthma, we analyzed the development of asthmatic features in IL-21R-deficient mice. WT or *Il21r*$^{-/-}$ mice were sensitized either via the airways or skin, and subsequently challenged with HDMs i.n. on days 7–11. At day 15, inflammation of the airways was analyzed by flow cytometry. Actively sensitized mice mounted robust airway responses characterized by an influx of eosinophils and lymphocytes to the airways, accessible by bronchoalveolar lavage (BAL). The degree of eosinophilia and lymphocytosis was identical irrespective of the route of sensitization. *Il21r*$^{-/-}$ mice sensitized via the skin or airways showed a significant reduction in airway eosinophilia (Figure 2A), and skin-sensitized *Il21r*$^{-/-}$ mice also had a significant reduction in T cell infiltration of the airways. Of note, *Il21r*$^{-/-}$ mice even showed a slight reduction in airway eosinophilia in the non-sensitized setting, suggesting that IL-21 can promote airway eosinophilia via effects on innate immunity. The induction of BAL fluid eosinophilia is a reflection of adaptive Th2 cell immunity to HDMs (Lambrecht and Hammad, 2015). To address this further, we measured cytokine production by lymphocytes in the mediastinal lymph nodes (mLNs) at day 15. IL-5 and IL-13 production by mLN cells restimulated in the presence of HDMs was greatly reduced in the absence of IL-21R (Figure 2B). Compared with WT mice, *Il21r*$^{-/-}$ mice also had higher amounts of total serum IgG2c and lower IgG1 (Figure 2C). As previously reported, serum IgE concentrations were higher in *Il21r*$^{-/-}$ compared with WT mice (Ozaki et al., 2002). Allergen-specific IgG1 was increased in WT but not *Il21r*$^{-/-}$ mice administered HDMs, but HDM-specific IgG2c, IgA, and IgE were not significantly elevated at day 15. Lung function was measured by assessing bronchial hyperreactivity to methacholine. HDM-sensitized and -challenged WT mice developed considerably more hyperreactivity than *Il21r*$^{-/-}$ mice (Figure 2D). Together, these results indicate that Th2 cell responses are impaired in the absence of IL-21R.

HDMs Induce IL-21 Expression from CD4$^+$ T Cells that Are Distinct from Effector Th2 Cells in the Lung

To more clearly characterize the response to HDMs, IL-21GFP reporter mice were analyzed (Lüthje et al., 2012). Very little IL-21 was detected in naive *Il21*$^{gfp/+}$ mice (Figure 3A). After exposure of mice to HDMs, IL-21GFP was exclusively expressed by CD4$^+$ cells in the mLN, spleen, lung, and BAL (Figure 3A). Invariant NKT cells (identified with PBS57-loaded CD1d tetramers) showed no signs of IL-21 expression (data not shown), suggesting that HDMs induced IL-21 production in conventional CD4$^+$ T cells. The expression of IL-21GFP in mLNs correlated highly with the expression of the Tfh-cell-associated markers CXCR5 and PD-1 (Figures 3A and 3B). This was also true of IL-21GFP$^+$ cells from the spleen, although to a lesser extent. IL-21GFP$^+$ cells in the lung and BAL expressed high amounts of PD-1 in accord with the findings of gene expression from lung

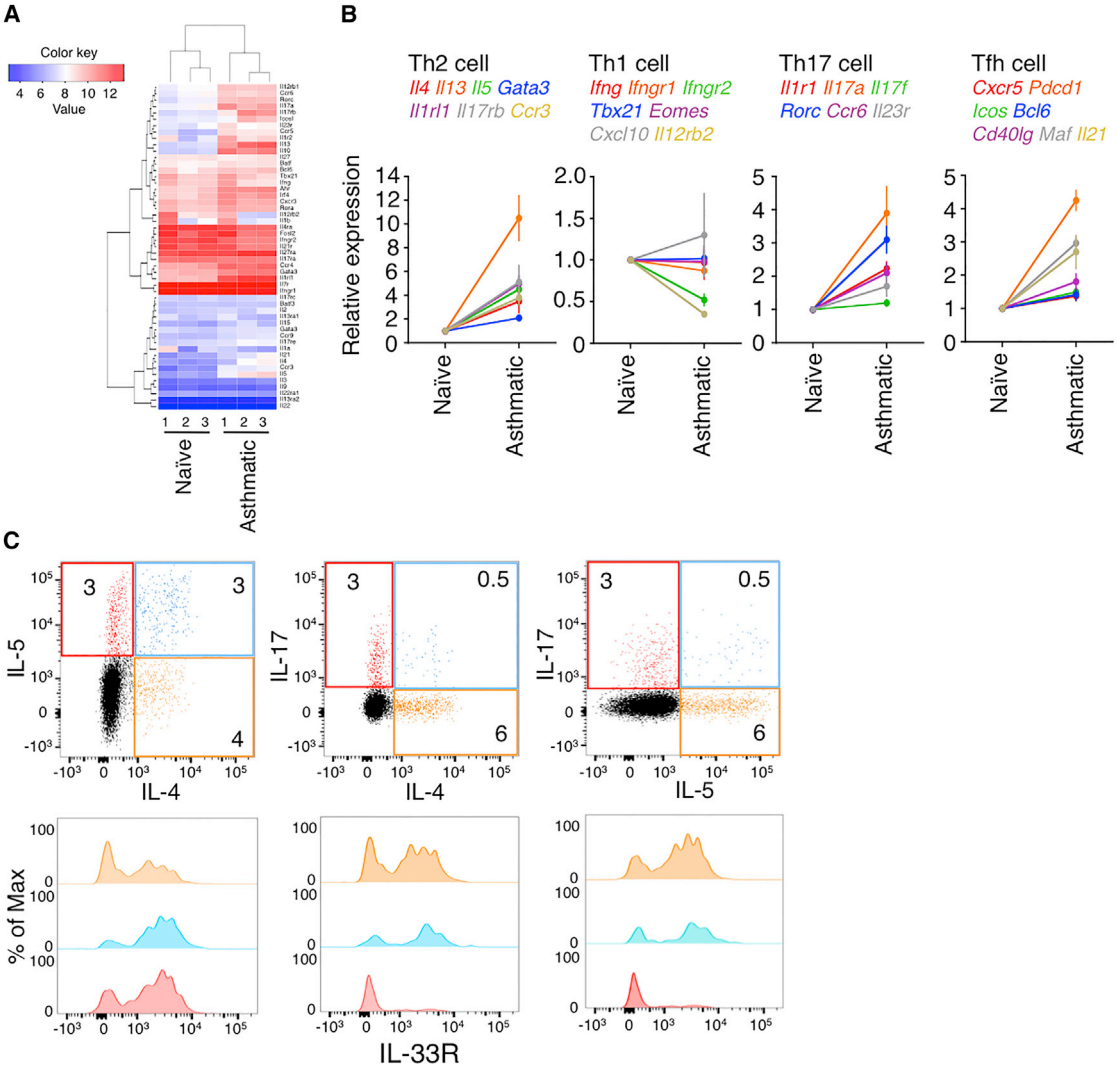

Figure 1. Upregulation of Genes Associated with Th2, Th17, and Tfh Cells in Asthmatic Mice

WT mice were sensitized i.n. with PBS or HDM extract (1 μg) on day 0 and challenged i.n. with PBS or HDM extract (10 μg) daily between days 7 and 11 to induce signs of asthma. On day 15, lungs were isolated and $CD4^+CD45^+CD11b^-$ cells were purified by flow cytometry. RNA was extracted and analyzed on the Affymetrix platform.

(A) Clustering analysis on a restricted set of Th-cell-associated genes.

(B) Normalized gene expression of samples isolated from three independent experiments. Graphs depict the expression of genes typically associated with Th1, Th2, Th17, or Tfh cells.

(C) Intracellular staining for IL-4, IL-5, and IL-17 in combination with IL-33R on $CD4^+$ cells in the BAL of mice. Expression of IL-33R on different color-coded populations is shown in histograms.

Data are representative of four individual mice. Also refer to Figure S1.

$CD4^+$ T cells (Figures 1 and S1); however, they expressed little CXCR5, indicating that these were extrafollicular cells. The intensity of IL-21GFP expression from the lung and BAL was reduced compared with the intensity of those from the mLN (Figure 3C), suggesting that IL-21 protein expression might be reduced in peripheral organs compared to in mLN follicles.

Previous studies have suggested that Th2 and Tfh cells develop along closely related lines (Baumjohann et al., 2011) and that IL-21 might promote Th2 cell responses (Fröhlich et al., 2007; Pesce et al., 2006). Furthermore, IL-21 can be co-expressed by Th2 and Th17 cells in vitro (Suto et al., 2008), although whether this is the case in a physiological disease setting is unknown. In mice sensitized and challenged with HDMs, IL-21 mRNA was enriched in IL-4GFP$^+$CD4$^+$ T cells (Figure S2A), suggesting that IL-21 was Th2 cell product. However, IL-4 expression alone is not an accurate measure of effector Th2 cells because IL-5$^+$ and IL-13$^+$ effector cells in the airways might not always express IL-4 (Figure 1C; Liang et al., 2012). To more accurately elucidate the relationship between IL-21$^+$ cells and effector Th2 cells, we analyzed IL-33R expression on IL-21GFP$^+$ and IL-21GFP$^-$ CD4$^+$ T cells because IL-33R is a reliable marker of differentiated effector Th2 cells (Löhning et al., 1998).

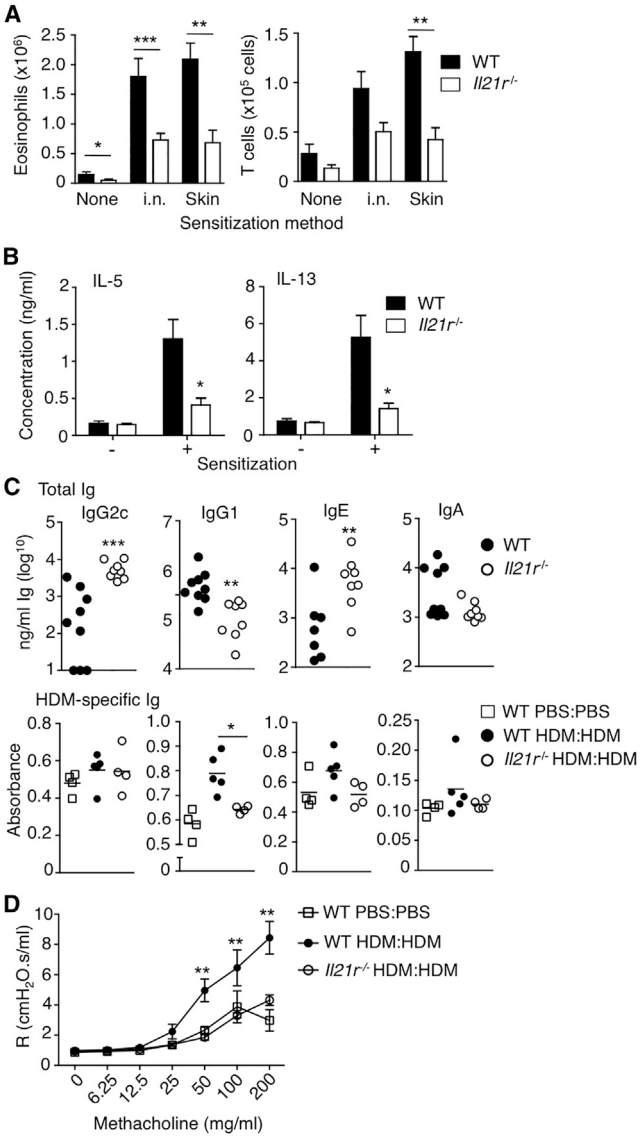

Figure 2. Mice Deficient in IL-21R Develop Reduced Airway Eosinophilia and Poor Th2 Cell Responses

WT and *Il21r*$^{-/-}$ mice were sensitized via the skin (100 μg) or i.n. (1 μg) with HDM extract or PBS on day 0. Mice were then challenged i.n. with 10 μg HDM extract daily between days 7 and 11, and on day 15 bronchoalveolar lavage (BAL) was performed.

(A) Shown are the numbers of eosinophils (SiglecF$^+$CD11c$^-$) and T cells (SSC^{lo}FSC^{lo}CD3$^+$MHCII$^-$) as assessed by flow cytometry. Pooled results from 2–4 experiments with 9–20 mice per group.

(B) 1 × 10^6 lung-draining mLN cells from skin-sensitized or non-sensitized mice at day 15 were cultured for 48 hr in the presence of 20 μg/ml HDM extract and cytokines in the supernatant were measured by ELISA. Graphs depict the amount of IL-5 and IL-13 in supernatants. One representative of three experiments is shown.

(C) Serum total (pooled data from two experiments) and HDM-specific (representative data from one of two experiments) Ig concentrations in WT and *Il21r*$^{-/-}$ mice sensitized and challenged with HDM extract or PBS.

(D) Lung function test of WT and *Il21r*$^{-/-}$ mice that were sensitized via the skin and challenged via the airways. 24 hr prior to lung function testing, mice were administered 10 μg HDM extract i.n.

WT and *Il21r*$^{-/-}$ mice were compared by Mann-Whitney U test. *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$.

The expression of IL-33R was not enhanced on IL-21GFP$^+$ CD4$^+$ T cells in the lung, BAL, or spleen, but was moderately enhanced on IL-21GFP$^+$ CD4$^+$ T cells in the mLN, compared with IL-21GFP$^-$ CD4$^+$ T cells (Figure 3A). IL-21 protein expression was also verified by intracellular staining with chimeric IL-21R.Fc. IL-21$^+$ cells in the BAL were distinct from effector IL-4- and IL-5-producing cells and appeared distinct from Th17 cells (Figure S2B). Moreover, IL-21$^+$ cells did not express IL-33R (Figure S2B). Thus, HDMs induce the production of IL-21 by Tfh cells in the lung-draining mLN and lead to the development of an IL-21-producing CD4$^+$ T cell population in the lung that is distinct from Th2 or Th17 cells.

Tfh Cells Induce Eosinophilia but Do Not Productively Develop into Effector Th2 Cells

Previous studies have demonstrated that Tfh cells transferred into mice inoculated with influenza can efficiently produce IFN-γ and contribute to the effector Th1 cell pool (Lüthje et al., 2012). To analyze whether Tfh cells can contribute to the effector Th2 cell pool, IL-21$^+$ and IL-21$^-$ Tfh cells were purified from *Il21*$^{gfp/+}$ mice that had been exposed to HDMs. 5 × 10^4 cells were transferred into naive CD45.1$^+$ mice, which were subsequently challenged daily with HDMs between days 0 and 4. Cellular infiltration into the airways was assessed on day 8. Mice that received no cell transfer developed very little eosinophilic infiltration of the airways, whereas mice that received IL-21$^+$ Tfh cells developed noticeable eosinophilia (Figure 3D). IL-21$^-$ Tfh cell transfer did not significantly enhance airway eosinophilia. Phenotypic analysis of the transferred Tfh cells revealed that only approximately 25% of transferred IL-21$^+$ Tfh cells remained IL-21GFP$^+$ and around 20% of transferred IL-21$^-$ Tfh cells had acquired IL-21GFP expression (Figure 3E) in the mLN. This is in line with the notion that IL-21$^-$ Tfh cells are precursors for IL-21$^+$ Tfh cells (Lüthje et al., 2012). Thus, although Tfh cells effectively develop into Th1 cells after influenza infection (Lüthje et al., 2012), transferred Tfh cells did not productively upregulate IL-33R (Figures 3E and S2C), nor express IL-5 or IL-13 (data not shown) following challenge with HDMs. These data indicate that IL-21$^+$ Tfh cells amplify type 2 responses to HDMs, but do not themselves readily differentiate into IL-33R$^+$ effector cells.

Der p1-Reactive CD4$^+$ T Cells in the mLN Strongly Acquire Tfh Cell Characteristics

To explore the kinetics and antigen specificity of the CD4$^+$ T cell response to HDMs, we crossed IL-21GFP reporter mice to the recently constructed "1-DER" mouse, which expresses a TCR specific to the Der p1 peptide of HDM (Plantinga et al., 2013). In their initial description, 1-DERβ mice expressed only the TCR Vβ chain of a selected Der p1-reactive hybridoma, leading to a precursor frequency of 5%–7% of Ag-reactive CD4$^+$ T cells. However, after new rounds of microinjection, we obtained mice TCR Tg for both the Vβ4 and Vα8 chain of the hybridoma leading to a precursor frequency of 60%–70% of Der p1-reactive CD4$^+$ T cells (unpublished data). *Il21*$^{gfp/+}$ 1-DER CD4$^+$ T cells were transferred into naive CD45.1 congenic mice. Purified CD4$^+$ T cells did not express GFP before transfer (Figure S3A). In the first instance, the expression of IL-21GFP by transferred 1-DER T cells was analyzed at day 15, subsequent

to skin sensitization and intranasal challenge with HDMs (Figure 4A). Around 20% of transferred 1-DER cells in the mLN, lung, and BAL expressed GFP (Figures 4B and 4C). This frequency was lower in the spleen of recipient mice (Figures 4B and 4C). In addition, the proportion of transferred 1-DER T cells expressing PD-1 and CXCR5 (bona fide Tfh cells) was close to 50% in the mLN, compared to fewer than 10% in the spleen, lung, and BAL (Figure 4C). The intensity of IL-21GFP was greater in 1-DER T cells from the mLN compared with other organs, similar to our findings in IL-21GFP reporter mice expressing heterogeneous TCRs (Figure 3). Hence, Der p1-reactive $CD4^+$ T cells exhibit a high capacity to upregulate the expression of IL-21 after HDM administration and this population is prevalent in the lung tissue and airspace.

To gain further insight into the kinetics of IL-21 expression, *Il21*$^{gfp/+}$ 1-DER $CD4^+$ T cells were transferred into naive mice and recipients were challenged daily with HDMs for 5 days. The expression of IL-21GFP among transferred cells was analyzed at days 5, 8, and 11 (Figures 4D and 4E). The frequency of 1-DER T cells peaked around 5 days after transfer and declined thereafter in the mLN and spleen whereas the frequency of transferred cells in the lung and BAL was highest at day 8 (Figures 4D and 4E). Around 8% of 1-DER T cells in the mLN expressed IL-21GFP at day 5 after transfer and this proportion increased to more than 30% by day 11 (Figure 4E). Virtually all IL-21GFP$^+$ cells in the mLN expressed PD-1 and CXCR5 at these time points (data not shown). Furthermore, more than 30% of transferred 1-DER T cells in the mLN already expressed PD-1 and CXCR5 at day 5. This proportion increased over time such that more than 60% of 1-DER T cells in the mLN at day 11 were PD-1$^+$CXCR5$^+$. Very few 1-DER T cells recovered from lung tissue or BAL expressed IL-21GFP or markers consistent with Tfh cells using this HDM administration protocol (Figure 4E). This suggests that the migration of IL-21-producing $CD4^+$ T cells to the lung and BAL requires additional time or (more likely) multiple rounds of antigen stimulation.

Cell-Intrinsic IL-21R Signaling Supports the Acquisition of Th2 Cell Functions

Given that HDM challenge induced potent Tfh cell responses and because *Il21r*$^{-/-}$ mice developed weak Th2 cell responses, we analyzed whether this might be due to a defect in Tfh cell differentiation. WT and *Il21r*$^{-/-}$ mice that had been sensitized and challenged with HDMs had similar amounts of total $CD4^+$ T, germinal center (GC) B, and Tfh cells in the mLN (Figure 5A), suggesting that the impaired Th2 cell response in *Il21r*$^{-/-}$ mice was not due to a deficiency in Tfh cells. Despite the normal frequency of $CD4^+$ T cells in the mLN, the proportion of IL-33R$^+$ cells among CD44$^+$ memory $CD4^+$ T cells was significantly greater in WT compared with *Il21r*$^{-/-}$ mice (Figures 5B and 5C), again indicative of a defect in effector Th2 cell differentiation.

To investigate whether IL-21R provided cell-intrinsic signals to $CD4^+$ T cells responding to HDMs, CD45.1$^+$CD45.2$^+$ double congenic mice were lethally irradiated and reconstituted 1:1 with WT (CD45.1) and *Il21r*$^{-/-}$ (CD45.2) bone marrow cells. Eight weeks after reconstitution, mice were sensitized and challenged with HDMs and the $CD4^+$ T cell response was analyzed. WT and *Il21r*$^{-/-}$ cells accounted for a roughly equal proportion of $CD4^+$ and $CD8^+$ T cells and the percentage of memory CD44$^+$ cells among $CD4^+$ T cells from each fraction was similar (Figure 5D). However, a greater proportion of WT cells expressed IL-33R compared with *Il21r*$^{-/-}$ cells within the same mouse (Figure 5E). In addition, purified WT $CD4^+$ T cells from the mLN expressed significantly more IL-13 and IL-21 and moderately more IL-4 mRNA than *Il21r*$^{-/-}$ cells from the same mouse (Figure 5F). IL-17 expression was similar in WT and *Il21r*$^{-/-}$ cells suggesting that IL-21R preferentially supports the acquisition of Th2, rather than of Th17, cell effector functions. These results indicate that IL-21R provides cell-intrinsic signals that induce the acquisition of Th2 cell functions.

IL21R Signaling Supports ILC2 Function and Number in a Non-Cell-Intrinsic Manner

ILC2s are an innate population of cells, which share many features of conventional Th2 cells and have been proposed to play an important role in controlling eosinophilic lung inflammation (Halim et al., 2014; Neill et al., 2010; Van Dyken et al., 2014). As such, the frequency and function of ILC2s was analyzed in WT and *Il21r*$^{-/-}$ mice. In the mLN and lung of HDM-sensitized and challenged mice, the frequency of ILC2s among gated lineage$^-$CD45$^+$CD90.2$^+$ cells was reduced in *Il21r*$^{-/-}$ compared with WT mice (Figure 6A). In addition, the ability of ILC2s in the mLN to produce IL-5 and IL-13 was significantly reduced in *Il21r*$^{-/-}$ mice (Figure 6B), although the cytokine-producing capacity of the lung ILC2 population was similar between WT and *Il21r*$^{-/-}$ mice. This suggested that IL-21R might numerically and functionally support the ILC2 population. To test whether IL-21R also provided cell-intrinsic inputs to developing ILC2s, we analyzed the frequency of ILC2s in 1:1 chimeras as described in Figure 5. WT and *Il21r*$^{-/-}$ cells evenly contributed to the ILC2 pool in the mLN and lung of 1:1 chimeras (Figure 6C), suggesting that although IL-21R can augment ILC2 responses, it does not provide cell-intrinsic inputs as it does for Th2 cells.

Recombinant IL-21 and rIL-25 Synergize to Promote Lung Eosinophilia

The presence of IL-21$^+$ cells that were distinct from Th2 or Th17 cells and were not precursors of Th2 cells in the lung was indicative of an extra-follicular function for IL-21. Because IL-21R promoted the acquisition of Th2 cell function in a cell-intrinsic manner and because transferred $CD4^+$IL-21-GFP$^+$ cells induced lung eosinophilia in a short-term challenge model (Figure 3), we hypothesized that IL-21 might directly promote type-2-cell-mediated immunity in the lungs. Recombinant IL-21 was administered to mice in isolation or in combination with the innate cytokine IL-25, because the receptor for IL-25 (*Il17rb*) was upregulated on Th2 cells in mice immunized with HDMs (Figures 1A and S1). IL-25 is made by epithelial cells in response to HDMs or protease allergens and can potentiate ILC2 precursors in the lung (Hammad et al., 2009; Huang et al., 2015; Willart et al., 2012). Cytokines were administered i.n. for 3 consecutive days and eosinophilia of the airways was assessed on day 4. rIL-21 and rIL-25 both induced little eosinophilia in the airways of WT mice, but the combination of rIL-21 and rIL-25 synergistically promoted airway eosinophilia (Figure 7A). Expression of *Muc5ac* mRNA in lung tissue, which is indicative of IL-13-induced mucus production by lung epithelial cells, was also induced in response to rIL-21 and rIL-25 co-administration (Figure S4A). In *Rag2*$^{-/-}$

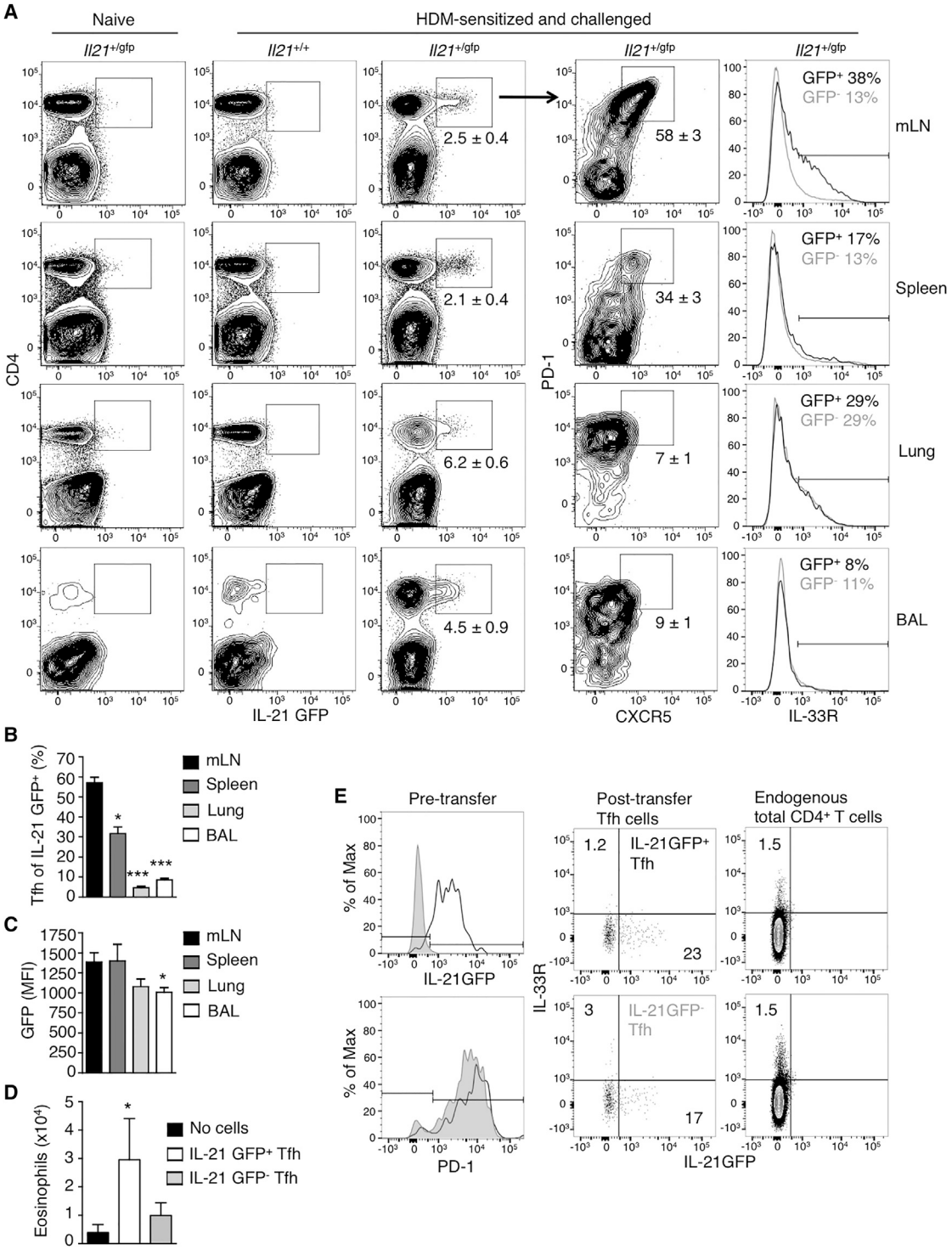

Figure 3. HDMs Induce IL-21 Expression by Tfh Cells in the mLN and Adoptive Transfer of IL-21$^+$ Cells Mediates Airway Eosinophilia

The mLN, spleen, lung, and BAL of naive or HDM-sensitized and challenged *Il21*$^{+/+}$ and *Il21*$^{+/gfp}$ mice was analyzed by flow cytometry.

(A) Contour plots depict IL-21GFP versus CD4 expression in naive or HDM-exposed mice and CXCR5 versus PD-1 expression on gated IL-21GFP$^+$CD4$^+$ cells. Histograms (right-hand) depict IL-33R expression on gated IL-21GFP$^+$CD4$^+$ and IL-21GFP$^-$CD4$^+$ cells. Numbers show the mean percentage of cells in the gated region ± SEM.

(B) The graph represents the percentage of IL-21GFP$^+$CD4$^+$ cells that express CXCR5 and PD-1. Results are representative of 6–15 mice pooled over 2–4 experiments.

(C) The graph represents the mean fluorescence intensity (MFI) of IL-21GFP expression in IL-21GFP$^+$CD4$^+$ cells where n = 6 mice from 2 experiments.

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mice lacking T and B lymphocytes, the number of eosinophils in the BAL of cytokine-administered mice was reduced by an order of magnitude compared with WT mice, yet there was still a significant trend for IL-21 and IL-25 to cooperate in driving eosinophilia (Figure 7A). No eosinophilia was observed in NOD.SCID.*Il2rg*$^{-/-}$ mice. These results suggested that combined rIL-21 and rIL-25 induced airway eosinophilia primarily via RAG-dependent CD4$^+$ T cells. Although IL-25 can promote ILC2 responses (Huang et al., 2015), administration of recombinant cytokines at these doses and for only 3 days had no numerical effect on the lung ILC2 population (Figure S4B). In support of a role for IL-21 in directly promoting Th2 cell functions in lung CD4$^+$ T cells, a significant increase in the frequency of IL-33R$^+$CD4$^+$ T cells was observed in mice administered either rIL-21 alone or both rIL-21 and rIL-25 (Figure 7B). To test whether rIL-21 and rIL-25 induced airway eosinophilia via a Th2-cell- and IL-33R-dependent mechanism, mice were administered anti-IL-5 or soluble ST2 (to block IL-33:L-33R) (Willart et al., 2012). The ability of combined rIL-21 and rIL-25 administration to induce airway eosinophilia was indeed dependent on both IL-5 and the IL-33:IL-33R pathway (Figure 7C). The source of IL-33 in this system is likely to be epithelial, because IL-25 promotes IL-33 production by epithelial cells (Kang et al., 2012).

Collectively, these data illustrate that the allergen HDM potently induces IL-21 production that promotes the acquisition of Th2 cell effector functions. IL-21-producing cells in the mLN are primarily Tfh cells, but IL-21-producing CD4$^+$ T cells are present in large numbers in the lungs of asthmatic mice and appear capable of potentiating type 2 immunity in this organ directly.

DISCUSSION

Asthma is a complex disease comprising of diverse clinical and biological symptoms, which are triggered by the activity of cytokines produced by Th2 cells (Lambrecht and Hammad, 2015). Although the majority of research has centered on the role of Th2 cells in asthma, roles for Th17 cells and ILCs have also recently been described, both in the pathogenesis and potentially the alleviation of typical signs of asthma (Halim et al., 2014; Neill et al., 2010; Schnyder-Candrian et al., 2006; Van Dyken et al., 2014). Herein, we have persuasively demonstrated that HDMs potently induced IL-21$^+$ Tfh cells in the lung-draining mLN and promoted the emergence of a distinct CXCR5$^-$PD1$^+$IL-21$^+$ cell population in the lung tissue and airway.

Because Tfh cells are generally regarded to provide help to B cells, it has been logical to assume that Tfh cells play an amplificatory role in allergic asthma. However, there is little evidence that HDMs induce robust IgE production in mice in short-term models (Birrell et al., 2010). Furthermore, mice deficient in IL-21R developed normal GC B cell responses and displayed higher concentrations of circulating total IgE as previously reported (Ozaki et al., 2002), arguing that IgE production from B cells plays a limited role in this model. Reduced HDM-specific IgG1 in *Il21r*$^{-/-}$ mice and the ability of transferred IL-21$^+$ Tfh cells or soluble rIL-21 to rapidly induce IL-5-dependent airway eosinophilia, also strongly supports a role for IL-21-producing T cells in promoting type 2 immunity, rather than in promoting B cell function to HDMs.

A number of studies have described Tfh cell responses under various Th2-cell-inducing conditions (Baumjohann et al., 2011; King and Mohrs, 2009; León et al., 2012; Reinhardt et al., 2009), suggesting that Th2 and Tfh cells share common developmental pathways. Furthermore, IL-21-producing Tfh cells can acquire effector functions associated with other T helper cell lineages (Johansson-Lindbom et al., 2003; Lüthje et al., 2012; Zotos et al., 2010) and vice versa, Th2 cells have been shown to take on Tfh cell characteristics (Glatman Zaretsky et al., 2009). Herein, we observed that even a single dose of HDMs administered through the skin or i.n. could induce the expression of PD-1 and CXCR5 in 1-DER T cells within 5 days (Figure S3B). The relatively rapid induction of IL-21 in CD4$^+$ T cells after HDM administration and the requirement for IL-21R in the acquisition of Th2 effector cell functions suggests that IL-21 expression precedes, or is at least temporally co-regulated, with Th2 cell differentiation.

Although considerable in vitro and in vivo evidence suggested that IL-21-expressing cells would co-express effector Th2 cytokines, we found very little evidence of this in response to HDMs. Recently activated IL-4$^+$ T cells migrate to the T cell-B cell border where they have been shown to receive further Th2 cell priming inputs or enter the follicle to differentiate into IL-21$^+$ Tfh cells (León et al., 2012; Xu et al., 2013). Our results suggest that the entry of cells into the follicle and the expression of IL-21 therein stifles the later acquisition of Th2 effector cell functions in response to HDMs. In line with this, Tfh cells transferred into naive recipients and subsequently challenged with influenza acquired Th1 cell effector functions but lost any ability to produce IL-4 (Lüthje et al., 2012). One possibility is that the nature of CD4$^+$ T cell priming makes it more difficult for cells that have already developed into Tfh cells, to acquire Th2-cell- rather than Th1-cell-associated functions. Studies proposing that Tfh and Th1 cells share overlapping T cell antigen receptors corroborate this idea (Fazilleau et al., 2009; Tubo et al., 2013). High-affinity TCR signals are believed to predispose cells toward the Tfh or Th1 cell fate and away from the Th2 cell fate, which are favored in the presence of lower affinity signals (Ruedl et al., 2000; van Panhuys et al., 2014). Hence, it is possible that after contact of Tfh cells with germinal center B cells expressing high amounts of cognate antigen (Shulman et al., 2014), Tfh cells can no longer acquire functions associated with Th2 cells.

The cell-intrinsic function of IL-21R in promoting IL-13, IL-21, and IL-33R expression but not that of IL-17 suggests that IL-21

(D and E) mLN and spleen was harvested on day 15 from *Il21*$^{+/gfp}$ mice previously sensitized and challenged with HDMs. IL-21GFP$^+$ and IL-21GFP$^-$ PD-1$^+$ cells were purified. Naive CD45.1 mice received 5×10^4 IL-21GFP$^+$ Tfh cells, 5×10^4 IL-21GFP$^-$ Tfh cells, or no cells and were challenged with 10 μg HDM extract i.n. daily from the day of transfer until day 4. On day 8, eosinophilia in the BAL was assessed.

(D) The number of eosinophils in the BAL (n = 4–7 mice). Kruskall-Wallis with post-Dunn's test was used to compare groups. *$p < 0.05$.

(E) Histograms show the expression of IL-21 and PD-1 in IL-21GFP$^+$ Tfh (black unfilled) and IL-21GFP$^-$ Tfh (gray shaded) cells before transfer. Dot and contour plots show the expression of IL-21GFP and IL-33R on transferred cells (CD45.2$^+$CD4$^+$) and endogenous (CD45.2$^-$CD4$^+$) cells from mLN at day 8.

Also refer to Figure S2.

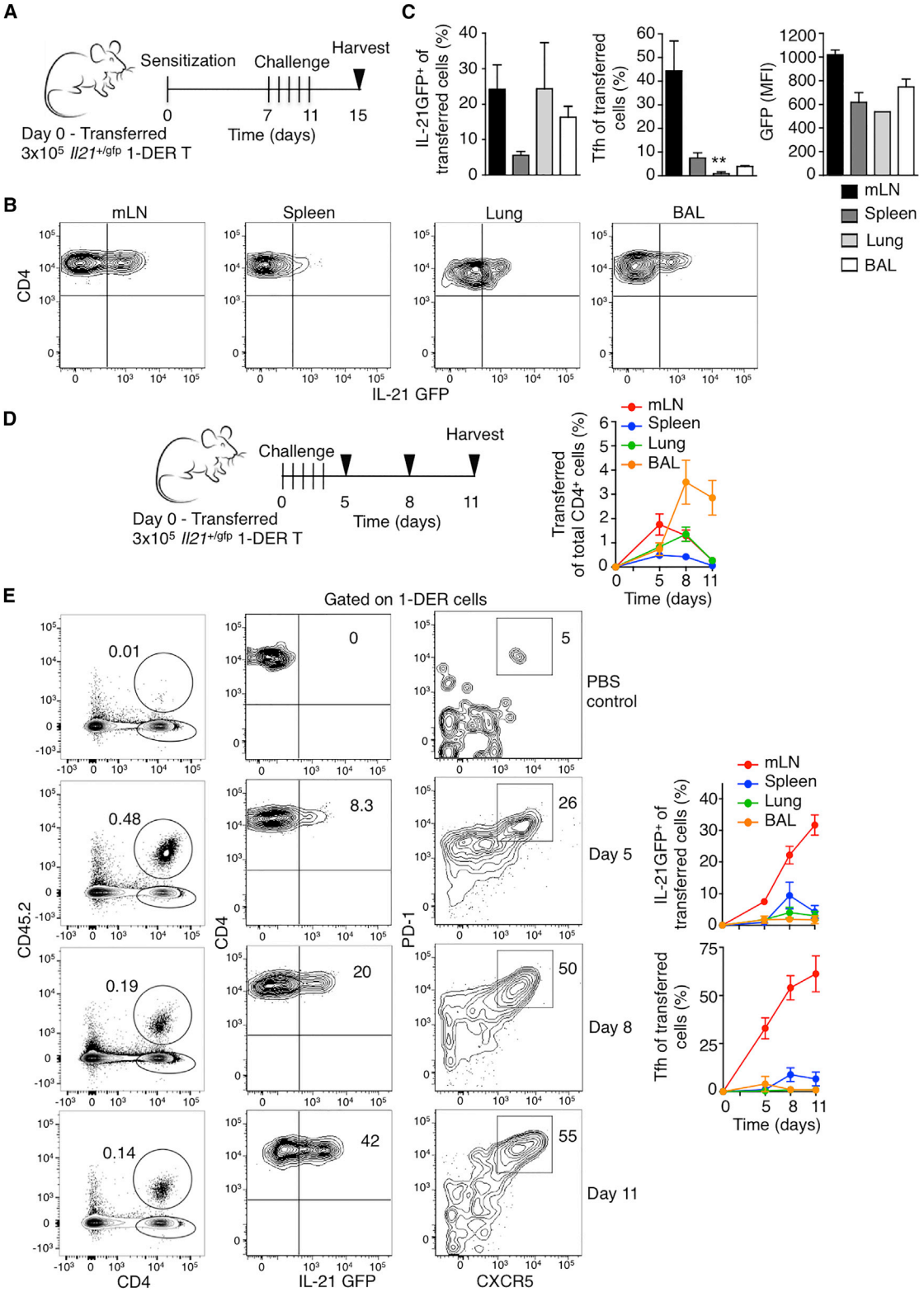

Figure 4. 1-DER TCR Transgenic Cells Rapidly Acquire Tfh Cell Characteristics

Spleens of *Il21*$^{+/gfp}$ 1-DER TCR transgenic mice were harvested and magnetically purified for CD4^{+} T cells. 3 × 10^{5} 1-DER T cells were transferred into naive CD45.1 congenic mice.

(A and B) Mice were sensitized with 100 μg HDM extract on the skin and challenged with 10 μg HDM extract i.n. between days 7 and 11.

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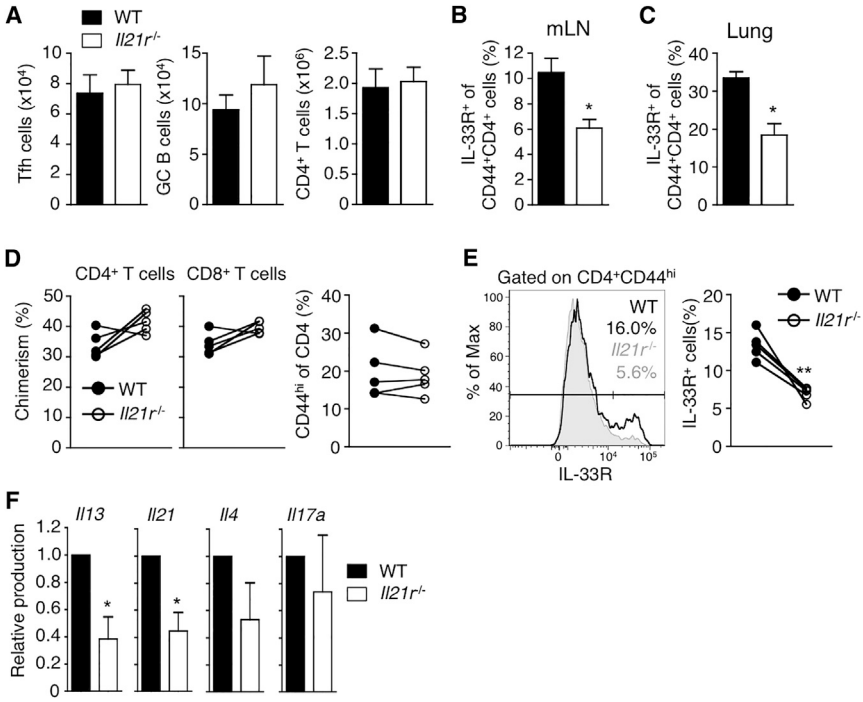

Figure 5. IL-21R Influences IL-33R, IL-13, and IL-21 Expression in a Cell-Intrinsic Manner

(A) WT and *Il21r*$^{-/-}$ mice sensitized and challenged with HDMs were analyzed by flow cytometry for the presence of Tfh (CD4$^+$CD44$^+$CXCR5$^+$PD-1$^+$), germinal center B (CD19$^+$Fas$^+$GL7$^+$), and total CD4$^+$ T (CD4$^+$CD3$^+$) cells. One representative experiment of four with four to five mice per group is shown.

(B and C) Gated CD4$^+$CD44$^+$ cells were analyzed for IL-33R expression in the mLN (B) and lung (C) by flow cytometry. Graphs depict the percentage of IL-33R$^+$ cells among gated cells in nine to ten mice from two independent experiments.

(D–F) CD45.1$^+$CD45.2$^+$ double congenic mice were lethally irradiated and reconstituted with a 1:1 mixture of WT (CD45.1$^+$) and *Il21r*$^{-/-}$ (CD45.2$^+$) bone marrow cells. 8 weeks later, mice were sensitized via the skin with 100 μg HDM extract and challenged with 10 μg HDM extract i.n. between days 7 and 11. At day 15, the contribution of WT and *Il21r*$^{-/-}$ cells to the immune response was analyzed.

(D) The percentage of WT and *Il21r*$^{-/-}$ cells within the CD4$^+$ (left) and CD8$^+$ T (middle) cell pools and the percentage of CD44$^+$ memory cells among gated WT or *Il21r*$^{-/-}$ CD4$^+$ T cells (right). Lines between circles indicate chimerism within the same mouse. One representative of four experiments is shown with three to five mice per experiment. Radio-resistant cells comprised approximately 20% of cells.

(E) Histogram and graph of IL-33R expression on gated memory CD44$^+$CD4$^+$ T cells from WT or *Il21r*$^{-/-}$ cells. Shown is one representative of two independent experiments.

Results from (A)–(E) were analyzed by Mann-Whitney U test. *$p < 0.05$, **$p < 0.01$.

(F) WT and *Il21r*$^{-/-}$ CD4$^+$ T cells from mLN were purified by flow cytometry and analyzed by quantitative PCR for the expression of the genes indicated. Values in *Il21r*$^{-/-}$ cells are shown relative to WT. Results are from seven separate mice from two independent experiments. Wilcoxon-Rank test was used to analyze normalized values. *$p < 0.05$.

can provide important cues specifically for Th2 cell differentiation. Although a role for IL-21 in enhancing type 2 immunity has previously been proposed (Fröhlich et al., 2007; Pesce et al., 2006) and IL-21 induces Th2-cell-associated cytokine production by NKT cells (Coquet et al., 2007, 2013), other reports have suggested that IL-21 is dispensable for Th2 cell differentiation (King et al., 2010). It is hard to reconcile this discrepancy because both King et al. (2010) and Fröhlich et al. (2007) analyzed IL-4 production in the *H. polygyrus* model. A pertinent point is that IL-4 is expressed by multiple T cell subsets and that the use of markers such as IL-5, IL-13, and IL-33R probably better delineate differentiated effector Th2 cells. Our findings in 1:1 chimeric mice strongly suggest that IL-21R promotes Th2 cell effector functions and these results are backed by findings from Stritesky et al. (2011), who have previously demonstrated a key cell-intrinsic role for STAT3 in stabilizing STAT6 at critical Th2-cell-associated gene loci.

It remains unclear in exactly what context IL-21R signals are transmitted to developing Th2 cells. Because Th2 cells are known to receive signals at the T cell-B cell border (León et al., 2012), it is possible that this brings them in close proximity with IL-21$^+$ Tfh cells in the follicle. However, the presence of IL-21$^+$CXCR5$^-$CD4$^+$ T cells in lung is also indicative of a role outside of primary lymphoid organs. The ability of rIL-21 to synergize with the innate stimulus rIL-25, to induce potent Th2 cell responses and airway eosinophilia is testament to this function. We sought to investigate the effect of co-administration of rIL-21 and rIL-33 on lung inflammation; however, the highly potent effects of i.n. rIL-33

(B and C) Cells from mLN, spleen, lung, and BAL were analyzed by flow cytometry at day 15.

(B) Contour plots depict the expression of IL-21GFP versus CD4 on gated CD45.2$^+$CD3$^+$CD4$^+$ cells.

(C) Graphs show the percentage IL-21GFP$^+$ (top) and CXCR5$^+$PD-1$^+$ (middle) among transferred cells, and the MFI of IL-21GFP on gated IL-21GFP$^+$CD4$^+$ cells (bottom) (n = 3 mice). Similar results were obtained after i.n. sensitization in three mice. Groups were compared by Kruskall-Wallis and post-Dunn's test. *$p < 0.05$.

(D and E) 1-DER T cells were transferred and recipient mice were challenged daily with 10 μg i.n. HDM extract until day 4. Spleen, mLN, lung, and BAL of mice were harvested at day 5, 8, and 11.

(D) The graph shows the percentage of transferred 1-DER T cells among total CD4$^+$ T cells.

(E) Plots depict the expression of CD4 versus CD45.2 (left), IL-21GFP versus CD4 (middle), and CXCR5 versus PD-1 (right) on transferred 1-DER T cells over time in mLN. A PBS control mouse at day 8 is shown. Graphs show the percentage of IL-21GFP$^+$ and CXCR5$^+$PD-1$^+$ cells of transferred 1-DER T cells (n = 6 mice per time point pooled from 2 independent experiments).

Also refer to Figure S3.

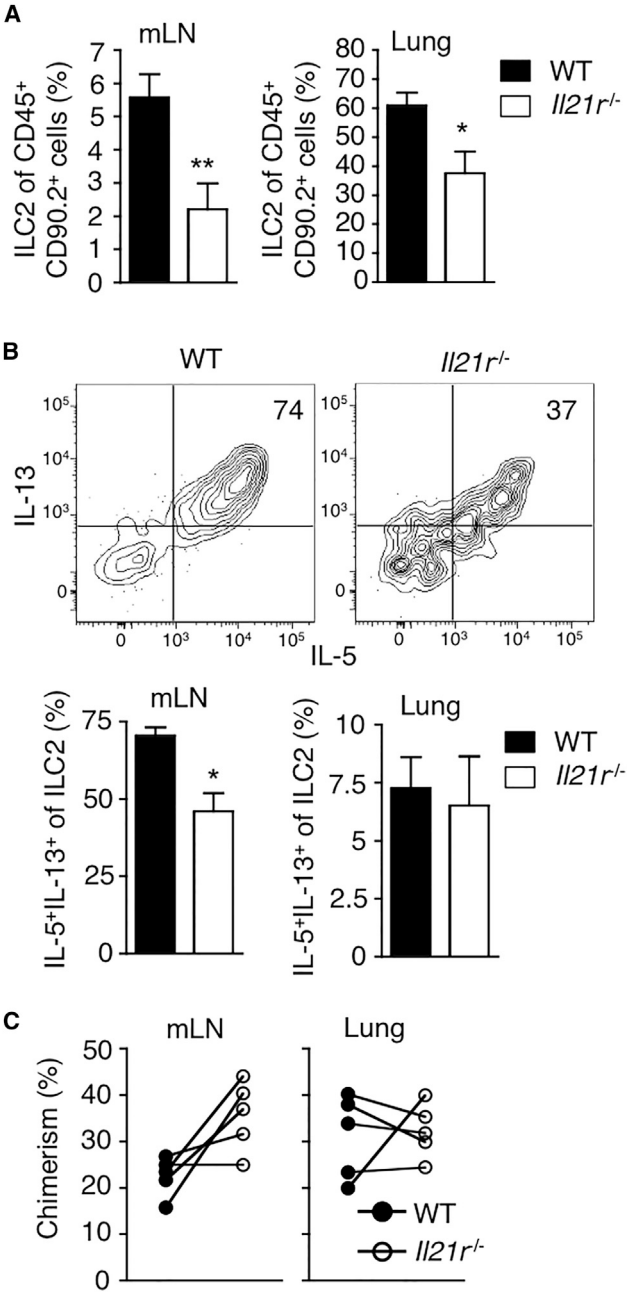

Figure 6. ILC2s Are Numerically and Functionally Aberrant in *Il21r*$^{-/-}$ Mice

WT and *Il21r*$^{-/-}$ mice sensitized and challenged with HDMs were analyzed by flow cytometry for ILC2s. ILC2s were defined as Lin^{-}CD45^{+}CD90.2^{+} CD127^{+}IL-33R^{+}.

(A) The graphs depict the percentage of IL-33R^{+}CD127^{+} cells among gated lineage^{-}CD45^{+}CD90.2^{+} cells in the mLN and lung.

(B) Restimulated mLN and lung cells were analyzed for intracellular IL-5 and IL-13. Contour plots depict the expression of IL-5 versus IL-13 in gated ILC2s. Graphs depict the percentage of IL-5^{+}IL-13^{+} cells of ILC2s in the mLN and lung of WT and *Il21r*$^{-/-}$ mice (n = 9–10 mice pooled from 2 independent experiments).

(C) The contribution of WT and *Il21r*$^{-/-}$ cells to the ILC2 population in 1:1 chimeras exposed to HDMs was analyzed. Graphs show the percentage of WT and *Il21r*$^{-/-}$ in gated ILC2s in one of two independent experiments.

Results were analyzed by Mann-Whitney U test. *$p < 0.05$, **$p < 0.01$.

administration alone made this difficult. The more subtle effects of rIL-25 administration on airway inflammation and the known ability of IL-25 to recruit immature ILC2s and induce IL-33 production from epithelial cells (Huang et al., 2015; Kang et al., 2012) provided a more physiological platform through which to study the impact of IL-21 on Th2-cell-mediated inflammation. We concluded that the effects of rIL-21 and rIL-25 co-administration proceeded largely through CD4^{+} T cells, probably resident memory CD4^{+} T cells of the lung. Memory CD4^{+} Th2 cells are highly responsive to cytokine stimulation with IL-25 and IL-33, even in the absence of TCR ligation (Meisel et al., 2001). This suggests an almost "innate" function of memory Th2 cells triggered by innate cytokines and boosted by IL-21 from recently generated CD4^{+} T cells that move to the lung. This does not rule out a potentially important role for IL-21 in promoting ILC2 function either. Certainly, the reduced frequency and function of ILC2s in *Il21r*$^{-/-}$ mice and the ability of the combination of rIL-21 and rIL-25 to induce mild eosinophilia even in *Rag2*$^{-/-}$ mice suggest that IL-21 can also play an amplificatory role in ILC2 function. The effects of IL-21 on ILC2s were, however, indirect in the HDM model. IL-21 might indirectly promote ILC2s through Th2 cells, which have the potential to stimulate ILC2s through cytokines such as IL-2 and IL-9 (Liu et al., 2015; Roediger et al., 2013). Regardless of the exact mechanism of action, the presence of IL-21^{+} cells in the lung and potent effects of rIL-21 suggest that neutralizing therapies of IL-21 in lung tissue might reduce pathogenic type-2-cell-mediated immune responses.

Taken together, these data highlight a prominent role for IL-21-producing CD4^{+} T cells in HDM-induced asthma and pinpoint an important amplificatory role for IL-21 in type-2-cell-mediated immunity via induction of IL-33R^{+} Th2 cells.

EXPERIMENTAL PROCEDURES

Mice and Generation of Bone Marrow Chimeras

WT C57BL/6, *Il21r*$^{-/-}$, IL-21GFP, IL-4GFP, and C57BL/6.CD45.1 congenic mice were bred at Ghent University in specific-pathogen-free conditions. 1-DER TCR transgenic mice were generated as described in Results. *Il21r*$^{-/-}$ mice were obtained originally from Dr. Warren Leonard. Experiments were approved by the animal ethics committees of the University of Ghent and the IRC, VIB.

To make 1-DER mice, antigen-specific T cell hybridomas were generated by fusion of the T cell lymphoma cell line BW5147 to in vitro restimulated CD4^{+} splenocytes of C57BL/6 mice that received the specific antigen in vivo. Antigen-specific clones were selected based on IL-2 production and CD69 upregulation upon stimulation with antigen-specific peptide-pulsed BM-DCs. The TCR (Vα8 and Vβ4 chain) was cloned into a mammalian expression vector pTarget (Promega) and tested for functionality in Jurkat cells. Subsequently, the Vα8 and Vβ4 cDNA were cloned into modified TCR expression vectors p428 and p783 (provided by Dr. Nigel Killeen), respectively, and co-injected in an equimolar concentration of 1 ng/μl into the pronuclei of fertilized C57BL/6 *Rag*$^{-/-}$ oocytes. Founders were screened for TCR functionality by flow cytometry and offspring was genotyped by PCR with primers specific for the Vα8 and Vβ4 transgene.

For generation of bone marrow chimeras, C57BL/6 CD45.1^{+}CD45.2^{+} mice were lethally irradiated with 8 Gy γ-irradiation. The following day, mice were reconstituted intravenously with a 1:1 mixture of WT CD45.1^{+} and *Il21r*$^{-/-}$ CD45.2^{+} bone marrow cells. Mice were kept on antibiotics for 4 weeks and used in experiments 8 weeks after irradiation.

Models of Asthma and Cytokine Instillation

Mice were anesthetized briefly with isoflurane. For i.n. sensitization, mice received 1 μg HDM extract in a volume of 40 μl PBS. For skin sensitization,

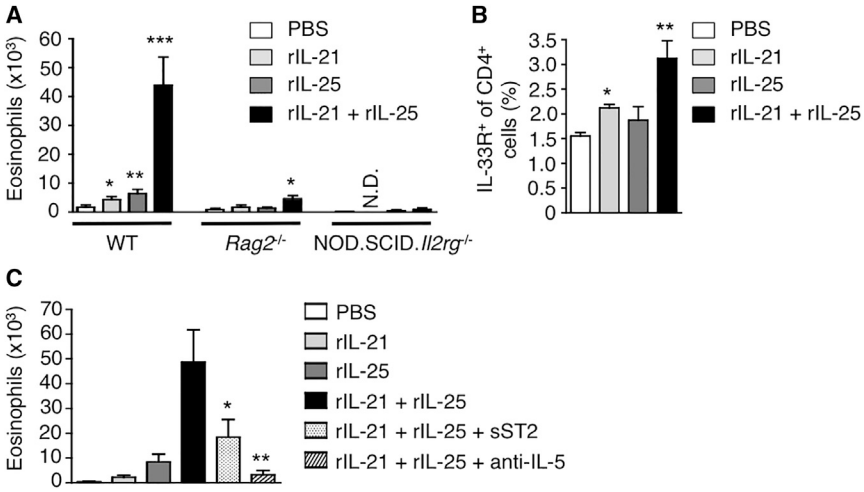

Figure 7. Recombinant IL-21 Induces Airway Eosinophilia in an IL-33R- and IL-5-Dependent Fashion

(A) WT, *Rag2*$^{-/-}$, or NOD.SCID.*Il2rg*$^{-/-}$ mice were administered i.n. PBS, 10 μg rIL-21, 125 ng rIL-25, or both rIL-21 and IL-25 daily for 3 days and BAL was harvested 24 hr after the last challenge. The graph depicts the number of eosinophils in the BAL of mice as determined by flow cytometry. Results were pooled from two to three experiments (WT, n = 10 mice; *Rag2*$^{-/-}$ and NOD.SCID.*Il2rg*$^{-/-}$, n = 5–7).

(B) The graph depicts the percentage of IL-33R^{+} cells among gated total CD4^{+} T cells as determined by flow cytometry.

(C) Mice were administered cytokines as described in (A) in combination with 50 μg soluble ST2 or anti-IL-5 i.p. on days 0 and 1. The number of eosinophils in the BAL on day 4 is shown. n = 6–8 mice per group pooled from 2 experiments, except for mice receiving anti-IL-5 (n = 4).

In (A) and (B), cytokine-administered groups were compared to PBS controls and in (C), mice receiving sST2 and anti-IL-5 were compared to mice receiving the combination of rIL-21 and rIL-25. Results were analyzed by Mann-Whitney U test. *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$. Also refer to Figure S4.

mice were shaved on the hind flank and 100 μg HDM extract was applied to the skin in a volume of 50 μl. A small piece of absorbent gauze was applied and the area taped to prevent access. 1 week after sensitization, mice were challenged on 5 consecutive days with 10 μg HDM extract i.n. under light anesthesia with isoflurane. 4 days after the last administration, mice were sacrificed and organs were dissected for analysis. BAL was performed by two consecutive flushes of the lung with 1 ml PBS. Blood was taken to screen for serum antibodies. mLN and spleen were dissected through 100 μM sieves and analyzed by flow cytometry. Lung function was analyzed with the Flexivent apparatus.

Restimulation by ICS, ELISA, and PMA

Cell culture was performed in RPMI-1640 medium supplemented with gentamycin, glutamine, 2-mercaptoethanol (all from Invitrogen), and 10% heat-inactivated FCS (Sigma). To analyze cytokine production in WT and *Il21r*$^{-/-}$ mice, 1×10^{6} mLN cells were restimulated in a 48-well plate for 48 hr in the presence of 20 μg/ml HDM extract. Supernatants were analyzed for the presence of cytokines using the Read-Set-Go! ELISA sets (E Bioscience). To analyze cytokine production by ILC, 50 ng/ml PMA and 1 μM ionomycin (both from Sigma) were added to cultures of total mLN or lung for 3 hr in the presence of BD GolgiStop. To stain for intracellular IL-21, mLN cells were restimulated overnight with 20 μg/ml HDM extract in the presence of BD GolgiStop. The BD Fixation/Permeabilization kit was used for intracellular staining of cells.

Statistical Analysis

A non-parametric Mann-Whitney U or Kruskall-Wallis test was used except for normalized data, which was analyzed by log-rank test. In all graphs, the standard error of the mean is shown.

ACCESSION NUMBERS

Microarray data from affymetrix arrays were deposited in the NCBI GEO database under accession number GEO: GSE72005.

SUPPLEMENTAL INFORMATION

Supplemental Information includes four figures and Supplemental Experimental Procedures and can be found with this article online at http://dx.doi.org/10.1016/j.immuni.2015.07.015.

AUTHOR CONTRIBUTIONS

J.M.C., M.J. Schuijs, and K.D. performed experiments; M.J. Smyth, S.L.N., H.B., G.B.K.H., L.B., and R.B. contributed critical reagents and mice; J.M.C. wrote the paper; and J.M.C., H.H., and B.N.L. conceived the study.

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REFERENCES

Barlow, J.L., Bellosi, A., Hardman, C.S., Drynan, L.F., Wong, S.H., Cruickshank, J.P., and McKenzie, A.N. (2012). Innate IL-13-producing nuocytes arise during allergic lung inflammation and contribute to airways hyperreactivity. J. Allergy Clin. Immunol. *129*, 191–8.e1, 4.

Baumjohann, D., Okada, T., and Ansel, K.M. (2011). Cutting Edge: Distinct waves of BCL6 expression during T follicular helper cell development. J. Immunol. *187*, 2089–2092.

Birrell, M.A., Van Oosterhout, A.J., and Belvisi, M.G. (2010). Do the current house dust mite-driven models really mimic allergic asthma? Eur. Respir. J. *36*, 1220–1221.

Burrows, B., Martinez, F.D., Halonen, M., Barbee, R.A., and Cline, M.G. (1989). Association of asthma with serum IgE levels and skin-test reactivity to allergens. N. Engl. J. Med. *320*, 271–277.

Chtanova, T., Tangye, S.G., Newton, R., Frank, N., Hodge, M.R., Rolph, M.S., and Mackay, C.R. (2004). T follicular helper cells express a distinctive transcriptional profile, reflecting their role as non-Th1/Th2 effector cells that provide help for B cells. J. Immunol. *173*, 68–78.

Coquet, J.M., Kyparissoudis, K., Pellicci, D.G., Besra, G., Berzins, S.P., Smyth, M.J., and Godfrey, D.I. (2007). IL-21 is produced by NKT cells and modulates NKT cell activation and cytokine production. J. Immunol. *178*, 2827–2834.

Coquet, J.M., Skak, K., Davis, I.D., Smyth, M.J., and Godfrey, D.I. (2013). IL-21 modulates activation of NKT cells in patients with Stage IV malignant melanoma. Clin. Transl. Immunology *2*, e6, http://dx.doi.org/10.1038/cti.2013.1037.

Dullaers, M., De Bruyne, R., Ramadani, F., Gould, H.J., Gevaert, P., and Lambrecht, B.N. (2012). The who, where, and when of IgE in allergic airway disease. J. Allergy Clin. Immunol. *129*, 635–645.

Fazilleau, N., McHeyzer-Williams, L.J., Rosen, H., and McHeyzer-Williams, M.G. (2009). The function of follicular helper T cells is regulated by the strength of T cell antigen receptor binding. Nat. Immunol. *10*, 375–384.

Fort, M.M., Cheung, J., Yen, D., Li, J., Zurawski, S.M., Lo, S., Menon, S., Clifford, T., Hunte, B., Lesley, R., et al. (2001). IL-25 induces IL-4, IL-5, and IL-13 and Th2-associated pathologies in vivo. Immunity *15*, 985–995.

Fröhlich, A., Marsland, B.J., Sonderegger, I., Kurrer, M., Hodge, M.R., Harris, N.L., and Kopf, M. (2007). IL-21 receptor signaling is integral to the development of Th2 effector responses in vivo. Blood *109*, 2023–2031.

Gajewski, T.F., and Fitch, F.W. (1988). Anti-proliferative effect of IFN-gamma in immune regulation. I. IFN-gamma inhibits the proliferation of Th2 but not Th1 murine helper T lymphocyte clones. J. Immunol. *140*, 4245–4252.

Glatman Zaretsky, A., Taylor, J.J., King, I.L., Marshall, F.A., Mohrs, M., and Pearce, E.J. (2009). T follicular helper cells differentiate from Th2 cells in response to helminth antigens. J. Exp. Med. *206*, 991–999.

Halim, T.Y., Steer, C.A., Mathä, L., Gold, M.J., Martinez-Gonzalez, I., McNagny, K.M., McKenzie, A.N., and Takei, F. (2014). Group 2 innate lymphoid cells are critical for the initiation of adaptive T helper 2 cell-mediated allergic lung inflammation. Immunity *40*, 425–435.

Hammad, H., and Lambrecht, B.N. (2015). Barrier epithelial cells and the control of type 2 immunity. Immunity *43*, 29–40.

Hammad, H., Chieppa, M., Perros, F., Willart, M.A., Germain, R.N., and Lambrecht, B.N. (2009). House dust mite allergen induces asthma via Toll-like receptor 4 triggering of airway structural cells. Nat. Med. *15*, 410–416.

Harada, M., Magara-Koyanagi, K., Watarai, H., Nagata, Y., Ishii, Y., Kojo, S., Horiguchi, S., Okamoto, Y., Nakayama, T., Suzuki, N., et al. (2006). IL-21-induced Bepsilon cell apoptosis mediated by natural killer T cells suppresses IgE responses. J. Exp. Med. *203*, 2929–2937.

Huang, Y., Guo, L., Qiu, J., Chen, X., Hu-Li, J., Siebenlist, U., Williamson, P.R., Urban, J.F., Jr., and Paul, W.E. (2015). IL-25-responsive, lineage-negative KLRG1(hi) cells are multipotential 'inflammatory' type 2 innate lymphoid cells. Nat. Immunol. *16*, 161–169.

Johansson-Lindbom, B., Ingvarsson, S., and Borrebaeck, C.A. (2003). Germinal centers regulate human Th2 development. J. Immunol. *171*, 1657–1666.

Kang, Z., Swaidani, S., Yin, W., Wang, C., Barlow, J.L., Gulen, M.F., Bulek, K., Do, J.S., Aronica, M., McKenzie, A.N., et al. (2012). Epithelial cell-specific Act1 adaptor mediates interleukin-25-dependent helminth expulsion through expansion of Lin(-)c-Kit(+) innate cell population. Immunity *36*, 821–833.

Kaplan, M.H., Schindler, U., Smiley, S.T., and Grusby, M.J. (1996). Stat6 is required for mediating responses to IL-4 and for development of Th2 cells. Immunity *4*, 313–319.

King, I.L., and Mohrs, M. (2009). IL-4-producing CD4+ T cells in reactive lymph nodes during helminth infection are T follicular helper cells. J. Exp. Med. *206*, 1001–1007.

King, I.L., Mohrs, K., and Mohrs, M. (2010). A nonredundant role for IL-21 receptor signaling in plasma cell differentiation and protective type 2 immunity against gastrointestinal helminth infection. J. Immunol. *185*, 6138–6145.

Kudo, M., Melton, A.C., Chen, C., Engler, M.B., Huang, K.E., Ren, X., Wang, Y., Bernstein, X., Li, J.T., Atabai, K., et al. (2012). IL-17A produced by αβ T cells drives airway hyper-responsiveness in mice and enhances mouse and human airway smooth muscle contraction. Nat. Med. *18*, 547–554.

Lambrecht, B.N., and Hammad, H. (2015). The immunology of asthma. Nat. Immunol. *16*, 45–56.

León, B., Ballesteros-Tato, A., Browning, J.L., Dunn, R., Randall, T.D., and Lund, F.E. (2012). Regulation of T(H)2 development by CXCR5+ dendritic cells and lymphotoxin-expressing B cells. Nat. Immunol. *13*, 681–690.

Liang, H.E., Reinhardt, R.L., Bando, J.K., Sullivan, B.M., Ho, I.C., and Locksley, R.M. (2012). Divergent expression patterns of IL-4 and IL-13 define unique functions in allergic immunity. Nat. Immunol. *13*, 58–66.

Linterman, M.A., Beaton, L., Yu, D., Ramiscal, R.R., Srivastava, M., Hogan, J.J., Verma, N.K., Smyth, M.J., Rigby, R.J., and Vinuesa, C.G. (2010). IL-21 acts directly on B cells to regulate Bcl-6 expression and germinal center responses. J. Exp. Med. *207*, 353–363.

Liu, B., Lee, J.B., Chen, C.Y., Hershey, G.K., and Wang, Y.H. (2015). Collaborative interactions between type 2 innate lymphoid cells and antigen-specific CD4+ Th2 cells exacerbate murine allergic airway diseases with prominent eosinophilia. J. Immunol. *194*, 3583–3593.

Löhning, M., Stroehmann, A., Coyle, A.J., Grogan, J.L., Lin, S., Gutierrez-Ramos, J.C., Levinson, D., Radbruch, A., and Kamradt, T. (1998). T1/ST2 is preferentially expressed on murine Th2 cells, independent of interleukin 4, interleukin 5, and interleukin 10, and important for Th2 effector function. Proc. Natl. Acad. Sci. USA *95*, 6930–6935.

Lüthje, K., Kallies, A., Shimohakamada, Y., Belz, G.T., Light, A., Tarlinton, D.M., and Nutt, S.L. (2012). The development and fate of follicular helper T cells defined by an IL-21 reporter mouse. Nat. Immunol. *13*, 491–498.

Meisel, C., Bonhagen, K., Löhning, M., Coyle, A.J., Gutierrez-Ramos, J.C., Radbruch, A., and Kamradt, T. (2001). Regulation and function of T1/ST2 expression on CD4+ T cells: induction of type 2 cytokine production by T1/ST2 cross-linking. J. Immunol. *166*, 3143–3150.

Neill, D.R., Wong, S.H., Bellosi, A., Flynn, R.J., Daly, M., Langford, T.K., Bucks, C., Kane, C.M., Fallon, P.G., Pannell, R., et al. (2010). Nuocytes represent a new innate effector leukocyte that mediates type-2 immunity. Nature *464*, 1367–1370.

Ozaki, K., Spolski, R., Feng, C.G., Qi, C.F., Cheng, J., Sher, A., Morse, H.C., 3rd, Liu, C., Schwartzberg, P.L., and Leonard, W.J. (2002). A critical role for IL-21 in regulating immunoglobulin production. Science *298*, 1630–1634.

Parrish-Novak, J., Dillon, S.R., Nelson, A., Hammond, A., Sprecher, C., Gross, J.A., Johnston, J., Madden, K., Xu, W., West, J., et al. (2000). Interleukin 21 and its receptor are involved in NK cell expansion and regulation of lymphocyte function. Nature *408*, 57–63.

Paul, W.E., and Zhu, J. (2010). How are T(H)2-type immune responses initiated and amplified? Nat. Rev. Immunol. *10*, 225–235.

Pesce, J., Kaviratne, M., Ramalingam, T.R., Thompson, R.W., Urban, J.F., Jr., Cheever, A.W., Young, D.A., Collins, M., Grusby, M.J., and Wynn, T.A. (2006). The IL-21 receptor augments Th2 effector function and alternative macrophage activation. J. Clin. Invest. *116*, 2044–2055.

Plantinga, M., Guilliams, M., Vanheerswynghels, M., Deswarte, K., Branco-Madeira, F., Toussaint, W., Vanhoutte, L., Neyt, K., Killeen, N., Malissen, B., et al. (2013). Conventional and monocyte-derived CD11b(+) dendritic cells initiate and maintain T helper 2 cell-mediated immunity to house dust mite allergen. Immunity *38*, 322–335.

Reinhardt, R.L., Liang, H.E., and Locksley, R.M. (2009). Cytokine-secreting follicular T cells shape the antibody repertoire. Nat. Immunol. *10*, 385–393.

Roediger, B., Kyle, R., Yip, K.H., Sumaria, N., Guy, T.V., Kim, B.S., Mitchell, A.J., Tay, S.S., Jain, R., Forbes-Blom, E., et al. (2013). Cutaneous immunosurveillance and regulation of inflammation by group 2 innate lymphoid cells. Nat. Immunol. *14*, 564–573.

Ruedl, C., Bachmann, M.F., and Kopf, M. (2000). The antigen dose determines T helper subset development by regulation of CD40 ligand. Eur. J. Immunol. *30*, 2056–2064.

Schaerli, P., Willimann, K., Lang, A.B., Lipp, M., Loetscher, P., and Moser, B. (2000). CXC chemokine receptor 5 expression defines follicular homing T cells with B cell helper function. J. Exp. Med. *192*, 1553–1562.

Schmitz, J., Owyang, A., Oldham, E., Song, Y., Murphy, E., McClanahan, T.K., Zurawski, G., Moshrefi, M., Qin, J., Li, X., et al. (2005). IL-33, an interleukin-1-

like cytokine that signals via the IL-1 receptor-related protein ST2 and induces T helper type 2-associated cytokines. Immunity *23*, 479–490.

Schnyder-Candrian, S., Togbe, D., Couillin, I., Mercier, I., Brombacher, F., Quesniaux, V., Fossiez, F., Ryffel, B., and Schnyder, B. (2006). Interleukin-17 is a negative regulator of established allergic asthma. J. Exp. Med. *203*, 2715–2725.

Shulman, Z., Gitlin, A.D., Weinstein, J.S., Lainez, B., Esplugues, E., Flavell, R.A., Craft, J.E., and Nussenzweig, M.C. (2014). Dynamic signaling by T follicular helper cells during germinal center B cell selection. Science *345*, 1058–1062.

Spolski, R., and Leonard, W.J. (2008). Interleukin-21: basic biology and implications for cancer and autoimmunity. Annu. Rev. Immunol. *26*, 57–79.

Stritesky, G.L., Muthukrishnan, R., Sehra, S., Goswami, R., Pham, D., Travers, J., Nguyen, E.T., Levy, D.E., and Kaplan, M.H. (2011). The transcription factor STAT3 is required for T helper 2 cell development. Immunity *34*, 39–49.

Suto, A., Kashiwakuma, D., Kagami, S., Hirose, K., Watanabe, N., Yokote, K., Saito, Y., Nakayama, T., Grusby, M.J., Iwamoto, I., and Nakajima, H. (2008). Development and characterization of IL-21-producing CD4+ T cells. J. Exp. Med. *205*, 1369–1379.

Tubo, N.J., Pagán, A.J., Taylor, J.J., Nelson, R.W., Linehan, J.L., Ertelt, J.M., Huseby, E.S., Way, S.S., and Jenkins, M.K. (2013). Single naive CD4+ T cells from a diverse repertoire produce different effector cell types during infection. Cell *153*, 785–796.

Van Dyken, S.J., Mohapatra, A., Nussbaum, J.C., Molofsky, A.B., Thornton, E.E., Ziegler, S.F., McKenzie, A.N., Krummel, M.F., Liang, H.E., and Locksley, R.M. (2014). Chitin activates parallel immune modules that direct distinct inflammatory responses via innate lymphoid type 2 and $\gamma\delta$ T cells. Immunity *40*, 414–424.

van Panhuys, N., Klauschen, F., and Germain, R.N. (2014). T-cell-receptor-dependent signal intensity dominantly controls CD4(+) T cell polarization In Vivo. Immunity *41*, 63–74.

Vogelzang, A., McGuire, H.M., Yu, D., Sprent, J., Mackay, C.R., and King, C. (2008). A fundamental role for interleukin-21 in the generation of T follicular helper cells. Immunity *29*, 127–137.

Willart, M.A., Deswarte, K., Pouliot, P., Braun, H., Beyaert, R., Lambrecht, B.N., and Hammad, H. (2012). Interleukin-1α controls allergic sensitization to inhaled house dust mite via the epithelial release of GM-CSF and IL-33. J. Exp. Med. *209*, 1505–1517.

Xu, H., Li, X., Liu, D., Li, J., Zhang, X., Chen, X., Hou, S., Peng, L., Xu, C., Liu, W., et al. (2013). Follicular T-helper cell recruitment governed by bystander B cells and ICOS-driven motility. Nature *496*, 523–527.

Zheng, W., and Flavell, R.A. (1997). The transcription factor GATA-3 is necessary and sufficient for Th2 cytokine gene expression in CD4 T cells. Cell *89*, 587–596.

Zotos, D., Coquet, J.M., Zhang, Y., Light, A., D'Costa, K., Kallies, A., Corcoran, L.M., Godfrey, D.I., Toellner, K.M., Smyth, M.J., et al. (2010). IL-21 regulates germinal center B cell differentiation and proliferation through a B cell-intrinsic mechanism. J. Exp. Med. *207*, 365–378.

Immunity

Supplemental Information

Interleukin-21-Producing $CD4^+$ T Cells Promote Type 2 Immunity to House Dust Mites

Jonathan M. Coquet, Martijn J. Schuijs, Mark J. Smyth, Kim Deswarte, Rudi Beyaert, Harald Braun, Louis Boon, Gunilla B. Karlsson Hedestam, Steven L. Nutt, Hamida Hammad, and Bart N. Lambrecht

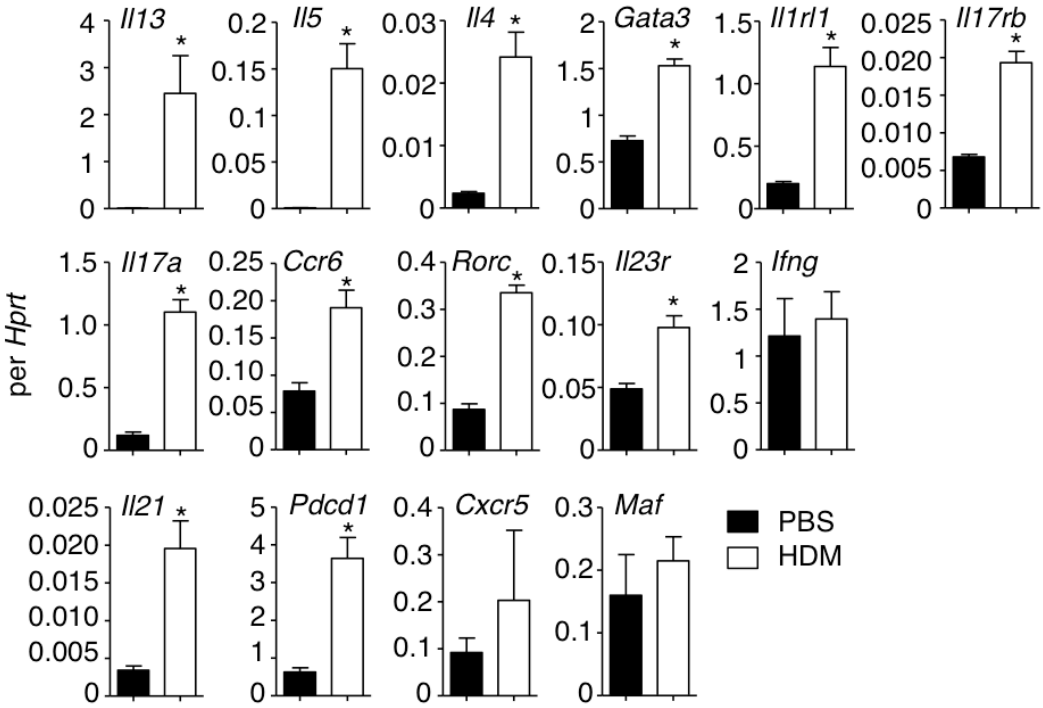


Figure S1. Quantitative PCR analysis of lung-infiltrating CD4$^+$ T cells (Related to Figure 1). In separate experiments, CD4$^+$ T cells from the lungs of PBS or HDM sensitized and challenged mice were analyzed. Cells were purified as described in Figure 1 and mRNA was quantified for each gene by real time PCR. Results depict 4 mice per group and were analyzed by Mann-Whitney U test. *$p<0.05$

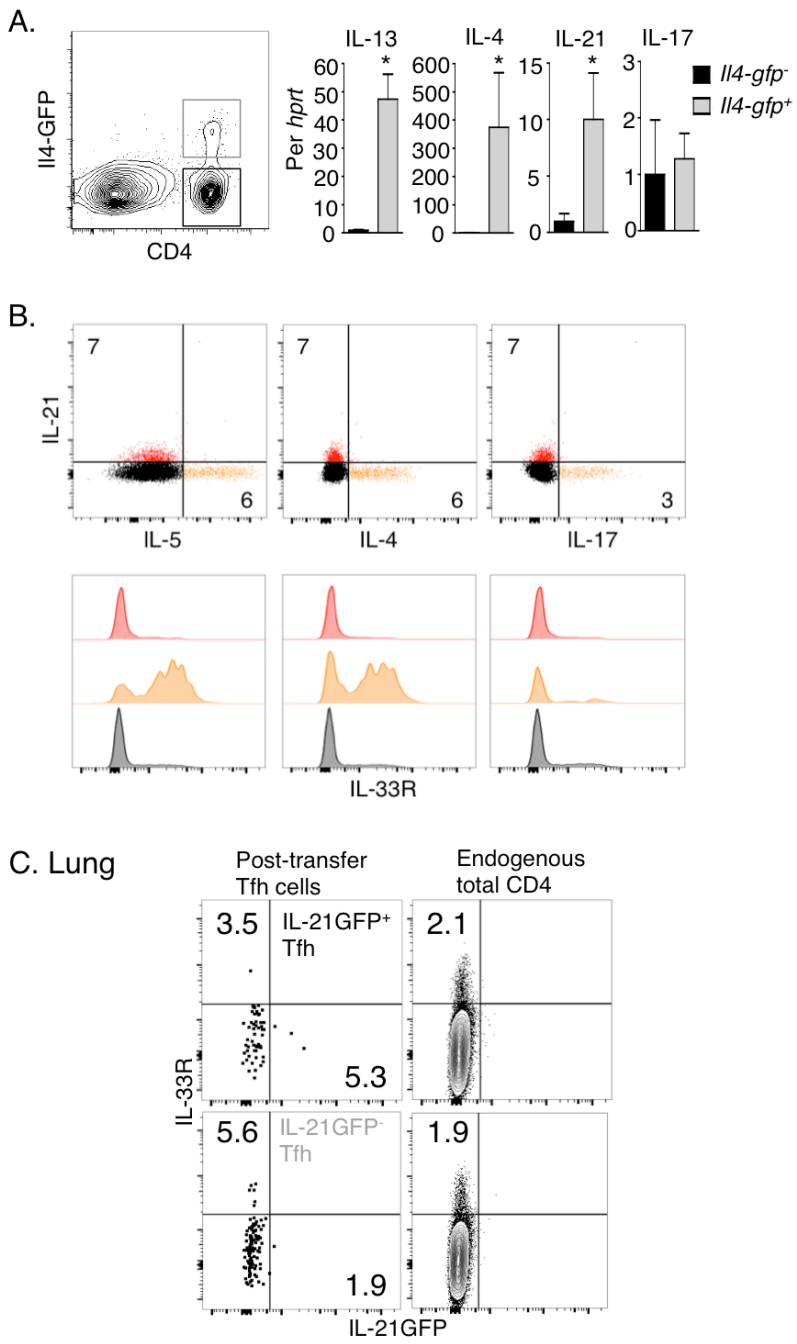


Figure S2. Distinguishing IL-21 expression from other effector cytokines (Related to Figure 3). A. IL-4GFP mice were sensitized and challenged with HDM as depicted in Figure 1 and at day 15 after sensitization, $CD4^{+}GFP^{-}$ and $CD4^{+}GFP^{+}$ cells were purified from the mLN. The contour plot depicts CD4 versus IL-4GFP expression in HDM sensitized and challenged mice. Bar graphs depict the expression of IL-13, IL-4, IL-21 and IL-17 mRNA in the two populations. Results are from 4 mice pooled from 2 experiments. **B.** Bronchoalveolar lavage cells from WT HDM-sensitized and –challenged mice were stimulated with PMA and

ionomycin in the presence of golgi transport blocking. Cells were surface stained for CD4 and IL-33R and subsequently for intracellular IL-4, IL-5, IL-17 and IL-21, which was detected by staining with the hIL-21R.Fc followed by a secondary goat anti-human PE antibody detecting the common Fc portion. The expression of IL-33R on the different color-coded populations is shown in histograms. Data is representative of 3 individual mice. **C.** Adoptive transfer of IL-21GFP^{+} and IL-21GFP^{-} Tfh cells was performed as described in Figure 3. Dot and contour plots show the expression of IL-21GFP and IL-33R on transferred cells (CD45.2^{+}CD4^{+}) and endogenous (CD45.2^{-}CD4^{+}) cells from the lung at day 8. Results were analyzed by Mann-Whitney U test. *$p<0.01$

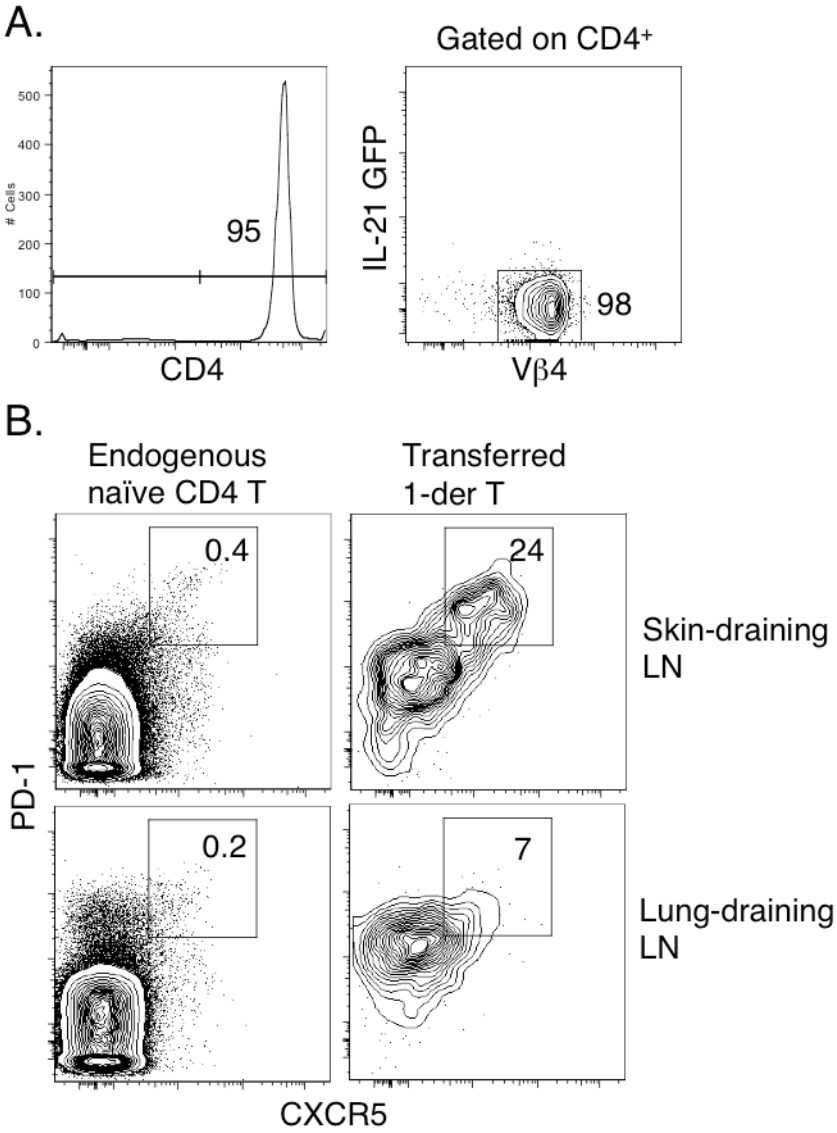


Figure S3. CD4$^+$ T cells from naïve *Il21*$^{+/gfp}$ 1-DER mice express very little IL-21GFP and differentiate effectively into Tfh after just one administration of HDM (Related to Figure 4). A. CD4$^+$ T cells were enriched and analyzed for CD4 and Vβ4 expression. These cells were used in adoptive transfer experiments described in Figure 4. **B.** 1-DER CD4$^+$ T cells (CD45.2$^+$) were transferred into CD45.1 congenic mice. Mice were then either sensitized with 100 μg HDM on the right flank or 10 μg HDM intranasal. Five days later, the skin-draining inguinal LN of mice sensitized via the skin (top row) or lung-draining mLN of mice sensitized via the lung (bottom row) was analyzed by flow cytometry. Plots depict the expression of CXCR5 versus PD-1 on gated endogenous naïve CD4$^+$ T (left-hand column) or gated 1-der T (right-hand column) cells. Plots are representative of 3 mice each receiving HDM either via the skin or lung.

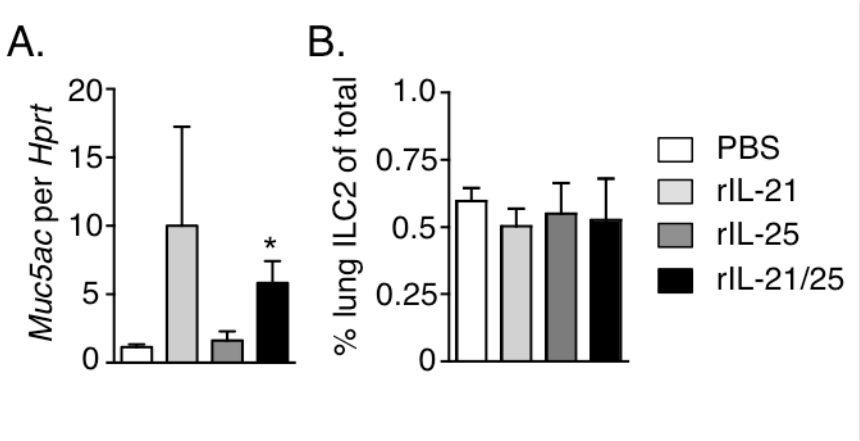


Figure S4. rIL-21 and rIL-25 induce *Muc5AC* expression but do not influence the frequency of ILC2 in the lung (Related to Figure 7). Mice were treated with recombinant cytokines on 3 consecutive days. **A.** *Muc5ac* mRNA expression was analyzed in whole lung tissue of cytokine and PBS treated mice. *p<0.01 **B.** The percentage of IL-33R^{+}CD127^{+}CD45^{+}CD90.2^{+}CD4^{-}lineage^{-} ILC2 among total cells in the lung is depicted. Results were pooled from 2 experiments with 6 mice per group total.

Supplemental Experimental Procedures

Reagents, antibodies and flow cytometry

HDM extracts were obtained from Greer Laboratories. rIL-25 was purchased from Biolegend (Cat # 579804) and rIL-21 was provided by Zymogenetics. Flurochrome-labelled and biotinylated antibodies from BD (CD3, CD8, SiglecF, Ly6C/G, CD127, CXCR5, IL-5), E Bioscience (CD4, CD11c, CD19, MHC-II, CD45, CD45.1, CD45.2, CD90.2, PD-1, IL-13, CD45), MBD Bioproducts (T1/ST2) and Biolegend (NK1.1) were used. Lineage$^-$ cells when gating for ILC were identified as CD3$^-$CD4$^-$CD11c$^-$CD19$^-$CD11b$^-$NK1.1$^-$. IL-13 and IL-5 ELISA kits were purchased from E Biosciences and IgG1, IgG2a, IgA and IgE kits were from BD. rhIL-21R.Fc (R&D Systems) and goat anti-human IgG Fc fragment PE (Jackson) were used for intracellular staining of IL-21. Recombinant mouse soluble ST2 (sST2) was PCR amplified from OriGene cDNA clone MC204735 (using forward and reverse primers: AGTTCTAGAATGATTGACAGACAGAGAATGG; TAATCTAGAAGCAATGTGTGAGGGACACTC) and ligated in the XbaI site of pEF6/myc-His A (Invitrogen), resulting in a mammalian expression vector (pEF-mST2s) for mouse sST2 with a C-terminal myc/His6 tag. pEF-mST2s was transiently transfected in HEK293T cells and secreted recombinant sST2 was purified in endotoxin free conditions from the supernatant using standard chromatographic methods (metal affinity chromatography using nickel-Sepharose and gelfiltration on a Superdex 200 column). Purified sST2 was stored in PBS at -80˚C. Protein concentration was determined by Micro BCA protein assay and purity of sST2 was >95% as assayed by SDS-PAGE and western blotting using anti-His antibody (Roche). Purification upscaling was done at VIB Protein Service Facility. Neutralizing activity of sST2 towards mouse IL-33 was tested in a biological assay measuring IL-33 induced NF-κB reporter activation in ST2:L1RAcP transfected HEK293T cells. sST2 and anti-IL-5 (TRFK5) were administered intra peritoneal on days 0 and 1 of recombinant cytokine administration.

Stained samples were analyzed BD LSRII or LSRFortessa and subsequently using FlowJo (TreeStar) software. Sorting was performed on a BD FACSAria III and sort purities were always in excess of 95%.

Adoptive transfer experiments

For adoptive transfer of IL-21GFP$^+$ and IL-21GFP$^-$ PD-1$^+$ Tfh cells, *Il21*$^{gfp/+}$ mice were sensitized and challenged with HDM. At day 15, mLN and spleen was harvested and pooled, and single cell suspensions were enriched for CD4$^+$ T cells with the CD4$^+$ T Cell Isolation Kit (Miltenyi). IL-21GFP$^+$ and IL-21GFP$^-$ PD-1$^+$ cells from the CD4-enriched fraction were purified by flow cytometry and transferred into recipient mice. For adoptive transfer of 1-DER CD4$^+$ T cells, spleens of naïve *Il21*$^{gfp/+}$ 1-DER mice were enriched using the CD4$^+$ T Cell Isolation Kit (Miltenyi). Enriched cells were injected into CD45.1 congenic recipients. Following transfer, mice were inoculated with HDM as described.

Quantitative PCR and array

RNA was obtained using the TriPure isolation reagent (Roche) and RNA was isolated according to manufacturers instructions. RNA was reverse transcribed with a Transcriptor High Fidelity cDNA Synthesis Kit (Roche) and samples were analyzed by SYBR green-based RT-PCR with a LightCycler 480 system (Roche). PCR primers for HPRT, IL-21, IL-13, IL-4 and IL-17 were as follows: HPRT 5' TGAAGAGCTACTGTAATGATCAGTCAAC, HPRT 3' AGCAAGCTTGCAACCTTAACCA, IL-21 5' AAGATTCCTGAGGATCCGAGAAG, IL-21 3' TGCATTCGTGAGCGTCTATAGTG, IL-13 5' TGACCAACATCTCCAATTGCA, IL-13 3' TTGTTATAAAGTGGGCTACTTCGATTT, IL-4 5' CTCATGGAGCTGCAGAGACTCTT, IL-4 3' CATTCATGGTGCAGCTTATCGA, IL-17 5' TGAAGGCAGCAGCGATCA, IL-17 3' GGAAGTCCTTGGCCTCAGTGT.

CD4$^+$ T cells from the lung were purified by flow cytometry by gating on CD4$^+$CD45$^+$F4/80$^-$ cells in 3 independent experiments from naïve PBS-treated or asthmatic HDM-treated mice. RNA from CD4$^+$ T cells was assessed with a Bioanalyser 2100 (Agilent). With a WT Expression Kit (Ambion), 50 ng of total RNA per sample, 'spiked' with bacterial poly(A) RNA positive control (Affymetrix) was converted to double-stranded cDNA in a reverse-transcription reaction. Samples were fragmented and labeled with biotin in a terminal labeling reaction according to the Affymetrix WT Terminal Labeling Kit. A mixture of fragmented biotinylated cDNA and hybridization controls (Affymetrix) was hybridized on a GeneChip

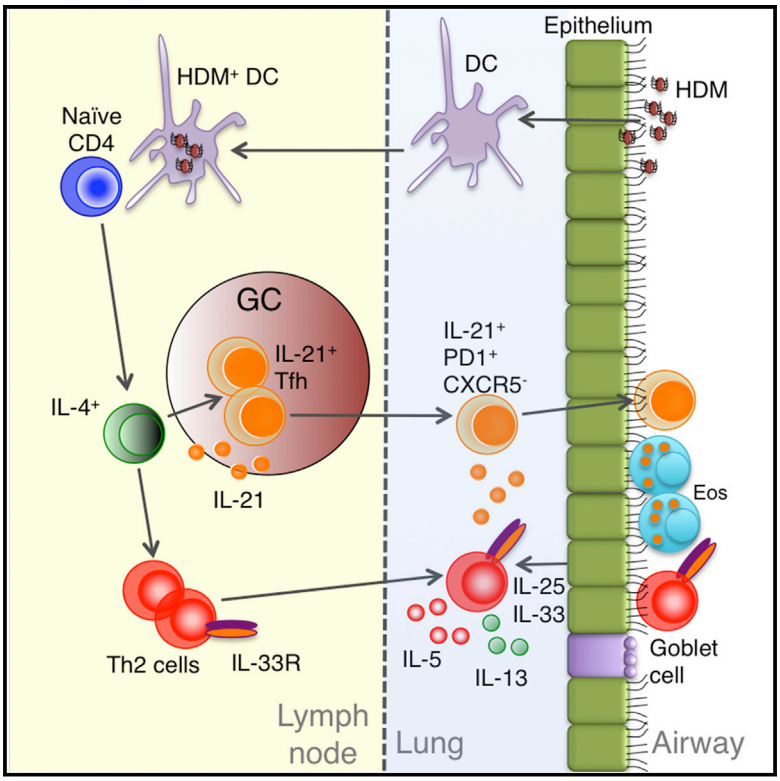
Mouse Gene 2.0 ST Array (Affymetrix), followed by staining and washing in a GeneChip fluidics station 450 according to the manufacturer's procedures (Affymetrix). For analysis of raw probe signal intensities, chips were scanned with a GeneChip scanner 3000 (Affymetrix). Samples were subsequently analyzed and clustered in ‘R’ software. Graphs of normalized data from each experiment were made for T helper cell specific genes.

Article

Immunity

Interleukin-21-Producing CD4+ T Cells Promote Type 2 Immunity to House Dust Mites

Graphical Abstract



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In Brief

Asthma is a well-described Th2-cell-mediated disease. Coquet, Lambrecht, and colleagues demonstrate that the allergen house dust mite induces pronounced IL-21 production in lymph nodes draining the lung, and also in lung tissue and in the airways. IL-21 and its receptor promote type 2 responses from adaptive CD4+ T cells.

Highlights

- House dust mites induce potent IL-21 production by Tfh cells in lymphoid organs
- IL-21+ cells are distinct from Th2 cells and migrate to the lung tissue and airway
- IL-21R provides cell-intrinsic cues for Th2 cell functions
- IL-21 synergizes with innate signals to promote adaptive Th2 cell responses

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Interleukin-21-Producing CD4+ T Cells Promote Type 2 Immunity to House Dust Mites

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SUMMARY

Asthma is a T helper 2 (Th2)-cell-mediated disease; however, recent findings implicate Th17 and innate lymphoid cells also in regulating airway inflammation. Herein, we have demonstrated profound interleukin-21 (IL-21) production after house dust mite (HDM)-driven asthma by using T cell receptor (TCR) transgenic mice reactive to *Dermatophagoides pteronyssinus 1* and an IL-21GFP reporter mouse. IL-21-producing cells in the mediastinal lymph node (mLN) bore characteristics of T follicular helper (Tfh) cells, whereas IL-21$^+$ cells in the lung did not express CXCR5 (a chemokine receptor expressed by Tfh cells) and were distinct from effector Th2 or Th17 cells. *Il21r*$^{-/-}$ mice developed reduced type 2 responses and the IL-21 receptor (IL-21R) enhanced Th2 cell function in a cell-intrinsic manner. Finally, administration of recombinant IL-21 and IL-25 synergistically promoted airway eosinophilia primarily via effects on CD4$^+$ lymphocytes. This highlights an important Th2-cell-amplifying function of IL-21-producing CD4$^+$ T cells in allergic airway inflammation.

INTRODUCTION

Asthma is an ever-increasing global health problem characterized by the development of robust T helper 2 (Th2)-cell-mediated immunity (Lambrecht and Hammad, 2015). Th2 cells secrete cytokines including interleukin-4 (IL-4), IL-5, IL-9, and IL-13, which promote airway inflammation, stimulate mucus production by lung epithelial cells, and modulate antibody secretion by B cells (Paul and Zhu, 2010). Critical for the differentiation of Th2 cells from naive CD4$^+$ T cells are the transcription factors signal transducer and activator of transcription (STAT) 6 and GATA-3 (Kaplan et al., 1996; Zheng and Flavell, 1997), which remodel chromatin at critical Th2 cell gene loci and can directly influence Th2-cell-associated cytokine gene expression (Paul and Zhu, 2010). A number of soluble factors such as IL-4 can activate and induce the expression of STAT-6 and GATA-3, whereas IL-25 and IL-33 directly induce secretion of IL-5 and IL-13 from differentiated Th2 cells (Fort et al., 2001; Schmitz et al., 2005). Studies in mice and humans have provided conclusive evidence that Th2 cells are the major cell population driving asthma.

Other T helper cell subsets also regulate the development of asthma (Lambrecht and Hammad, 2015). Th1 cells that secrete interferon-γ (IFN-γ) are thought to prevent airway inflammation and can impede the differentiation of Th2 cells (Gajewski and Fitch, 1988). IL-17 secreted by Th17 cells has a potentially dual function, on the one hand enhancing T cell priming in lung-draining lymph nodes (Schnyder-Candrian et al., 2006) and triggering bronchial smooth muscle cell contraction (Kudo et al., 2012), but on the other hand reducing eosinophilic infiltration of the airways (Schnyder-Candrian et al., 2006), a hallmark of asthma. More recently, the innate lymphoid cell type 2 (ILC2) population, which shares many characteristics of Th2 cells including pronounced secretion of IL-5 and IL-13, has also been demonstrated to promote many features of asthma (Barlow et al., 2012; Neill et al., 2010). Because ILC2s lack a T cell receptor (TCR), they react to allergen challenge in an antigen non-specific, yet cytokine-driven, manner, mainly responding to the cytokines IL-25 and IL-33 made by barrier epithelial cells (Hammad and Lambrecht, 2015). Hence, although Th2 cells remain central to the asthmatic response, a range of cell types might influence their activity.

The role of follicular T helper (Tfh) cells in the development of asthma remains more ambiguous. Tfh cells are characterized by the expression of the chemokine receptor CXCR5 as well as PD-1 (Chtanova et al., 2004; Schaerli et al., 2000). An important feature of Tfh cells is their ability to secrete high amounts of IL-21 (Vogelzang et al., 2008). IL-21, originally identified as a CD4$^+$ T-cell- (Parrish-Novak et al., 2000) and NKT-cell- (Coquet et al., 2007; Harada et al., 2006) derived cytokine, has broad immune-stimulatory properties on a range of cell types including

T and B cells (Spolski and Leonard, 2008). The expression of CXCR5 on the surface of Tfh cells allows for their migration to B cell areas in primary lymphoid organs, where IL-21 promotes antibody affinity maturation in germinal center (GC) B cells (Linterman et al., 2010; Zotos et al., 2010). The important role for IgE production by plasma cells in some forms of asthma (Burrows et al., 1989) has led to the presumption that Tfh cells might play an important role in allergic diseases, even if IgE GC reactions are very short lived (Dullaers et al., 2012).

A number of experimental mouse models have alluded to close co-operation between Th2 and Tfh cells. The experimental antigen chicken ovalbumin (OVA) in combination with alum induces potent Th2 and Tfh cell responses (Baumjohann et al., 2011). Furthermore, in models of nematode infection, robust Th2-cell-mediated immunity is known to coincide with Tfh cell responses (King and Mohrs, 2009; León et al., 2012; Reinhardt et al., 2009). In these models, effector IL-4-producing Th2 cells are known to develop at the T cell-B cell border of the priming lymph node and this has been shown to depend on CXCR5 expression on DCs (León et al., 2012; Liang et al., 2012). These studies imply that Th2 and Tfh cells develop in close co-operation during Th2-cell-mediated immune responses. However, the potential role(s) of Tfh cells in clinically relevant models of asthma has not been systematically examined.

Herein, we investigated the role of IL-21 in a mouse model of asthma induced by house dust mites (HDMs). By using IL-21GFP reporter mice (Lüthje et al., 2012) and T cell receptor (TCR) transgenic mice expressing a TCR reactive to the Der p1 peptide of HDM, we demonstrate that IL-21 was highly expressed in mice sensitized and challenged with HDMs. IL-21$^+$CD4$^+$ T cells were prominent in the airways, lung tissue, and lung-draining lymph nodes and IL-21R promoted Th2 cell function in a cell-intrinsic manner. These findings suggest that IL-21-producing cells are crucial for promoting type-2-cell-mediated immune responses in asthma.

RESULTS

Th2-, Th17-, and Tfh-Cell-Associated Gene Expression Is Increased in Lung CD4$^+$ T Cells during House-Dust-Mite-Induced Asthma

Recent evidence has implicated a variety of cell types in the asthmatic response (Lambrecht and Hammad, 2015). To gain greater insight into the role of other T helper cell subsets during asthma, we conducted gene expression profiling of lung CD4$^+$ T cells in naive and asthmatic mice sensitized to HDMs on day 0 and challenged daily intranasally (i.n.) between days 7 and 11. Lung CD4$^+$ T cells from HDM-exposed asthmatic mice had upregulated the expression of numerous Th2-cell-associated factors such as *Il13*, *Il4*, *Il5*, *Il1rl1* (part of IL-33R), and *Il17rb* (part of IL-25R) (Figures 1A, 1B, and S1). Classical Th17-cell-associated genes were also upregulated in lung CD4$^+$ T cells from asthmatic mice. Flow cytometric analysis of CD4$^+$ T cells infiltrating the airways showed that Th17 cells were largely distinct from IL-4$^+$ or IL-5$^+$ cells and did not express the classical Th2 cell marker, IL-33R (Figure 1C). In addition to Th2 and Th17 cells, *Il21* and *Pdcd1* (PD-1) were increased in lung CD4$^+$ T cells (Figure 1B), suggesting that Tfh cell responses might be involved in the development of asthma. Gene expression changes in lung CD4$^+$ T cells were confirmed with limited quantitative PCR in separate experiments (Figure S1). Th1-cell-associated gene expression was generally unchanged or decreased in mice receiving HDMs. These data imply that CD4$^+$ T cells from asthmatic mice acquire features not only of Th2 cells, but also of the Th17 and Tfh cell lineages.

IL-21R Is Necessary for the Development of Eosinophilic Airway Inflammation

To investigate the role of Tfh cells and IL-21 in HDM-induced asthma, we analyzed the development of asthmatic features in IL-21R-deficient mice. WT or *Il21r*$^{-/-}$ mice were sensitized either via the airways or skin, and subsequently challenged with HDMs i.n. on days 7–11. At day 15, inflammation of the airways was analyzed by flow cytometry. Actively sensitized mice mounted robust airway responses characterized by an influx of eosinophils and lymphocytes to the airways, accessible by bronchoalveolar lavage (BAL). The degree of eosinophilia and lymphocytosis was identical irrespective of the route of sensitization. *Il21r*$^{-/-}$ mice sensitized via the skin or airways showed a significant reduction in airway eosinophilia (Figure 2A), and skin-sensitized *Il21r*$^{-/-}$ mice also had a significant reduction in T cell infiltration of the airways. Of note, *Il21r*$^{-/-}$ mice even showed a slight reduction in airway eosinophilia in the non-sensitized setting, suggesting that IL-21 can promote airway eosinophilia via effects on innate immunity. The induction of BAL fluid eosinophilia is a reflection of adaptive Th2 cell immunity to HDMs (Lambrecht and Hammad, 2015). To address this further, we measured cytokine production by lymphocytes in the mediastinal lymph nodes (mLNs) at day 15. IL-5 and IL-13 production by mLN cells restimulated in the presence of HDMs was greatly reduced in the absence of IL-21R (Figure 2B). Compared with WT mice, *Il21r*$^{-/-}$ mice also had higher amounts of total serum IgG2c and lower IgG1 (Figure 2C). As previously reported, serum IgE concentrations were higher in *Il21r*$^{-/-}$ compared with WT mice (Ozaki et al., 2002). Allergen-specific IgG1 was increased in WT but not *Il21r*$^{-/-}$ mice administered HDMs, but HDM-specific IgG2c, IgA, and IgE were not significantly elevated at day 15. Lung function was measured by assessing bronchial hyperreactivity to methacholine. HDM-sensitized and -challenged WT mice developed considerably more hyperreactivity than *Il21r*$^{-/-}$ mice (Figure 2D). Together, these results indicate that Th2 cell responses are impaired in the absence of IL-21R.

HDMs Induce IL-21 Expression from CD4$^+$ T Cells that Are Distinct from Effector Th2 Cells in the Lung

To more clearly characterize the response to HDMs, IL-21GFP reporter mice were analyzed (Lüthje et al., 2012). Very little IL-21 was detected in naive *Il21*$^{gfp/+}$ mice (Figure 3A). After exposure of mice to HDMs, IL-21GFP was exclusively expressed by CD4$^+$ cells in the mLN, spleen, lung, and BAL (Figure 3A). Invariant NKT cells (identified with PBS57-loaded CD1d tetramers) showed no signs of IL-21 expression (data not shown), suggesting that HDMs induced IL-21 production in conventional CD4$^+$ T cells. The expression of IL-21GFP in mLNs correlated highly with the expression of the Tfh-cell-associated markers CXCR5 and PD-1 (Figures 3A and 3B). This was also true of IL-21GFP$^+$ cells from the spleen, although to a lesser extent. IL-21GFP$^+$ cells in the lung and BAL expressed high amounts of PD-1 in accord with the findings of gene expression from lung

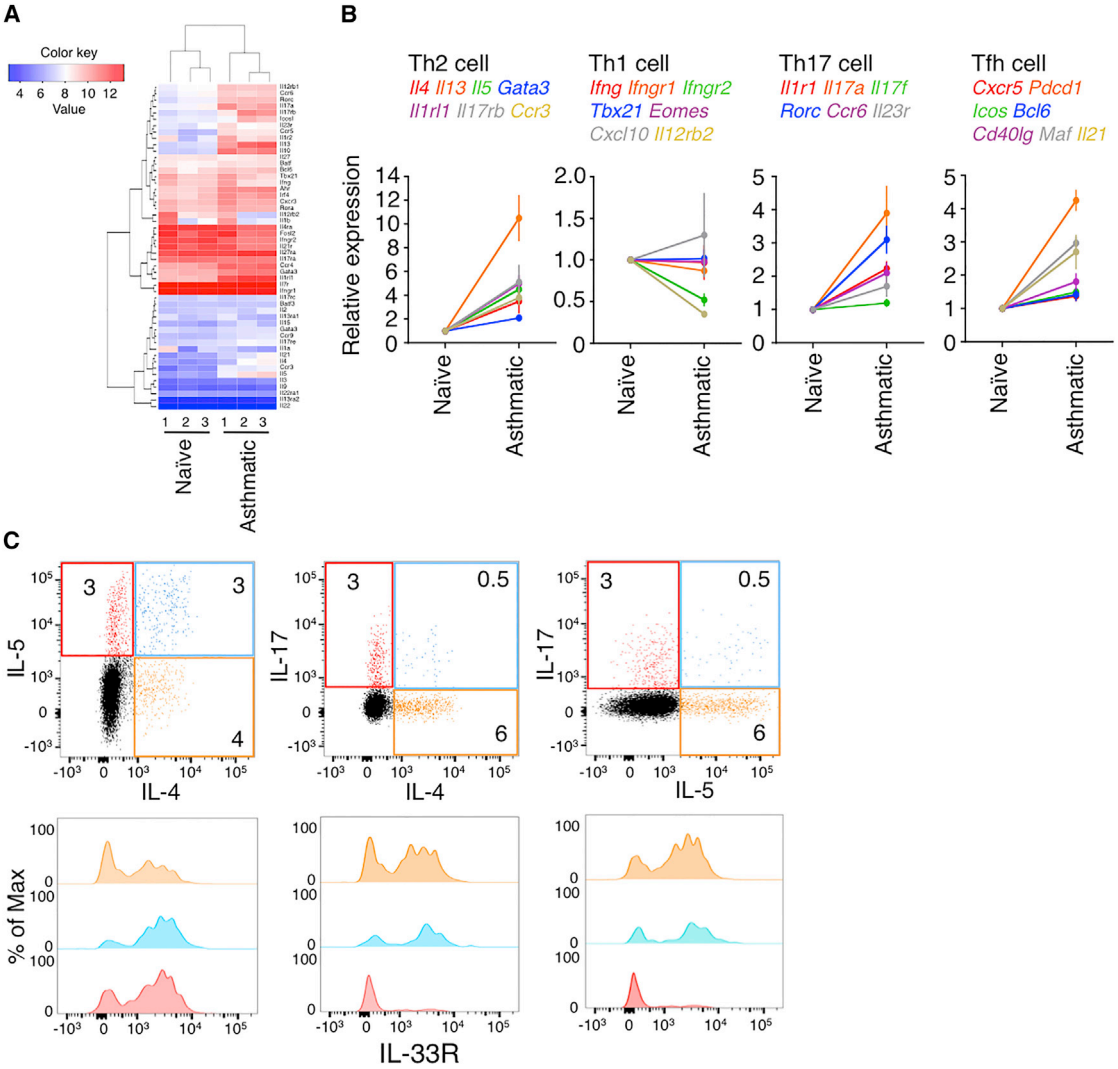

Figure 1. Upregulation of Genes Associated with Th2, Th17, and Tfh Cells in Asthmatic Mice

WT mice were sensitized i.n. with PBS or HDM extract (1 μg) on day 0 and challenged i.n. with PBS or HDM extract (10 μg) daily between days 7 and 11 to induce signs of asthma. On day 15, lungs were isolated and $CD4^+CD45^+CD11b^-$ cells were purified by flow cytometry. RNA was extracted and analyzed on the Affymetrix platform.

(A) Clustering analysis on a restricted set of Th-cell-associated genes.

(B) Normalized gene expression of samples isolated from three independent experiments. Graphs depict the expression of genes typically associated with Th1, Th2, Th17, or Tfh cells.

(C) Intracellular staining for IL-4, IL-5, and IL-17 in combination with IL-33R on $CD4^+$ cells in the BAL of mice. Expression of IL-33R on different color-coded populations is shown in histograms.

Data are representative of four individual mice. Also refer to Figure S1.

$CD4^+$ T cells (Figures 1 and S1); however, they expressed little CXCR5, indicating that these were extrafollicular cells. The intensity of IL-21GFP expression from the lung and BAL was reduced compared with the intensity of those from the mLN (Figure 3C), suggesting that IL-21 protein expression might be reduced in peripheral organs compared to in mLN follicles.

Previous studies have suggested that Th2 and Tfh cells develop along closely related lines (Baumjohann et al., 2011) and that IL-21 might promote Th2 cell responses (Fröhlich et al., 2007; Pesce et al., 2006). Furthermore, IL-21 can be co-expressed by Th2 and Th17 cells in vitro (Suto et al., 2008), although whether this is the case in a physiological disease setting is unknown. In mice sensitized and challenged with HDMs, IL-21 mRNA was enriched in IL-4GFP$^+$CD4$^+$ T cells (Figure S2A), suggesting that IL-21 was Th2 cell product. However, IL-4 expression alone is not an accurate measure of effector Th2 cells because IL-5$^+$ and IL-13$^+$ effector cells in the airways might not always express IL-4 (Figure 1C; Liang et al., 2012). To more accurately elucidate the relationship between IL-21$^+$ cells and effector Th2 cells, we analyzed IL-33R expression on IL-21GFP$^+$ and IL-21GFP$^-$ CD4$^+$ T cells because IL-33R is a reliable marker of differentiated effector Th2 cells (Löhning et al., 1998).

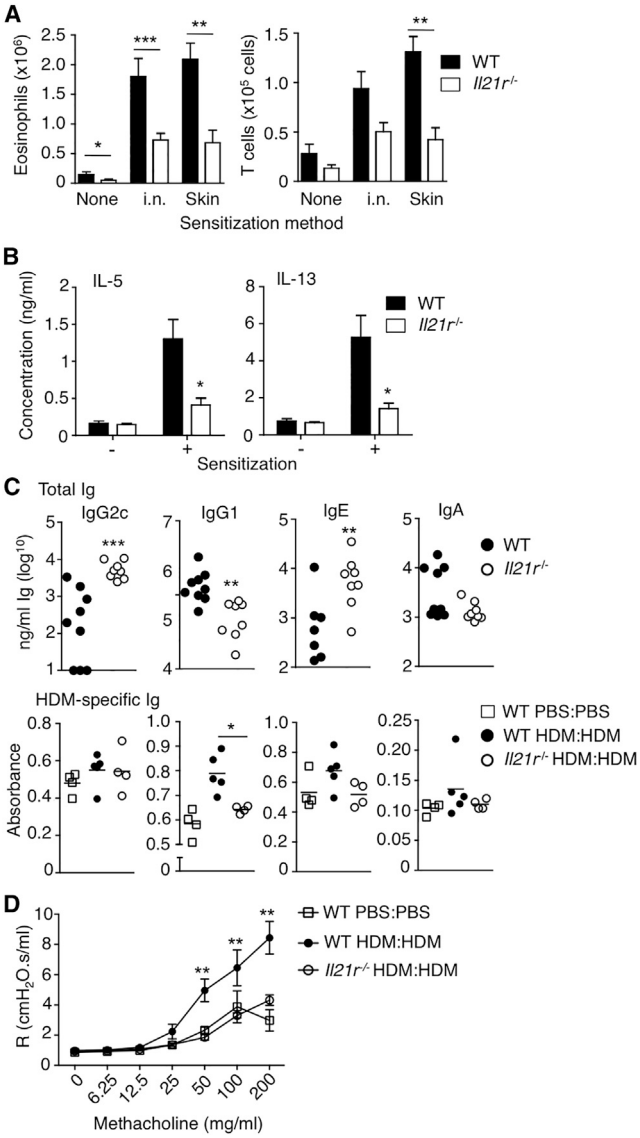

Figure 2. Mice Deficient in IL-21R Develop Reduced Airway Eosinophilia and Poor Th2 Cell Responses

WT and *Il21r*$^{-/-}$ mice were sensitized via the skin (100 μg) or i.n. (1 μg) with HDM extract or PBS on day 0. Mice were then challenged i.n. with 10 μg HDM extract daily between days 7 and 11, and on day 15 bronchoalveolar lavage (BAL) was performed.

(A) Shown are the numbers of eosinophils (SiglecF$^+$CD11c$^-$) and T cells (SSC^{lo}FSC^{lo}CD3$^+$MHCII$^-$) as assessed by flow cytometry. Pooled results from 2–4 experiments with 9–20 mice per group.

(B) 1 × 10^6 lung-draining mLN cells from skin-sensitized or non-sensitized mice at day 15 were cultured for 48 hr in the presence of 20 μg/ml HDM extract and cytokines in the supernatant were measured by ELISA. Graphs depict the amount of IL-5 and IL-13 in supernatants. One representative of three experiments is shown.

(C) Serum total (pooled data from two experiments) and HDM-specific (representative data from one of two experiments) Ig concentrations in WT and *Il21r*$^{-/-}$ mice sensitized and challenged with HDM extract or PBS.

(D) Lung function test of WT and *Il21r*$^{-/-}$ mice that were sensitized via the skin and challenged via the airways. 24 hr prior to lung function testing, mice were administered 10 μg HDM extract i.n.

WT and *Il21r*$^{-/-}$ mice were compared by Mann-Whitney U test. *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$.

The expression of IL-33R was not enhanced on IL-21GFP$^+$ CD4$^+$ T cells in the lung, BAL, or spleen, but was moderately enhanced on IL-21GFP$^+$ CD4$^+$ T cells in the mLN, compared with IL-21GFP$^-$ CD4$^+$ T cells (Figure 3A). IL-21 protein expression was also verified by intracellular staining with chimeric IL-21R.Fc. IL-21$^+$ cells in the BAL were distinct from effector IL-4- and IL-5-producing cells and appeared distinct from Th17 cells (Figure S2B). Moreover, IL-21$^+$ cells did not express IL-33R (Figure S2B). Thus, HDMs induce the production of IL-21 by Tfh cells in the lung-draining mLN and lead to the development of an IL-21-producing CD4$^+$ T cell population in the lung that is distinct from Th2 or Th17 cells.

Tfh Cells Induce Eosinophilia but Do Not Productively Develop into Effector Th2 Cells

Previous studies have demonstrated that Tfh cells transferred into mice inoculated with influenza can efficiently produce IFN-γ and contribute to the effector Th1 cell pool (Lüthje et al., 2012). To analyze whether Tfh cells can contribute to the effector Th2 cell pool, IL-21$^+$ and IL-21$^-$ Tfh cells were purified from *Il21*$^{gfp/+}$ mice that had been exposed to HDMs. 5 × 10^4 cells were transferred into naive CD45.1$^+$ mice, which were subsequently challenged daily with HDMs between days 0 and 4. Cellular infiltration into the airways was assessed on day 8. Mice that received no cell transfer developed very little eosinophilic infiltration of the airways, whereas mice that received IL-21$^+$ Tfh cells developed noticeable eosinophilia (Figure 3D). IL-21$^-$ Tfh cell transfer did not significantly enhance airway eosinophilia. Phenotypic analysis of the transferred Tfh cells revealed that only approximately 25% of transferred IL-21$^+$ Tfh cells remained IL-21GFP$^+$ and around 20% of transferred IL-21$^-$ Tfh cells had acquired IL-21GFP expression (Figure 3E) in the mLN. This is in line with the notion that IL-21$^-$ Tfh cells are precursors for IL-21$^+$ Tfh cells (Lüthje et al., 2012). Thus, although Tfh cells effectively develop into Th1 cells after influenza infection (Lüthje et al., 2012), transferred Tfh cells did not productively upregulate IL-33R (Figures 3E and S2C), nor express IL-5 or IL-13 (data not shown) following challenge with HDMs. These data indicate that IL-21$^+$ Tfh cells amplify type 2 responses to HDMs, but do not themselves readily differentiate into IL-33R$^+$ effector cells.

Der p1-Reactive CD4$^+$ T Cells in the mLN Strongly Acquire Tfh Cell Characteristics

To explore the kinetics and antigen specificity of the CD4$^+$ T cell response to HDMs, we crossed IL-21GFP reporter mice to the recently constructed "1-DER" mouse, which expresses a TCR specific to the Der p1 peptide of HDM (Plantinga et al., 2013). In their initial description, 1-DERβ mice expressed only the TCR Vβ chain of a selected Der p1-reactive hybridoma, leading to a precursor frequency of 5%–7% of Ag-reactive CD4$^+$ T cells. However, after new rounds of microinjection, we obtained mice TCR Tg for both the Vβ4 and Vα8 chain of the hybridoma leading to a precursor frequency of 60%–70% of Der p1-reactive CD4$^+$ T cells (unpublished data). *Il21*$^{gfp/+}$ 1-DER CD4$^+$ T cells were transferred into naive CD45.1 congenic mice. Purified CD4$^+$ T cells did not express GFP before transfer (Figure S3A). In the first instance, the expression of IL-21GFP by transferred 1-DER T cells was analyzed at day 15, subsequent

to skin sensitization and intranasal challenge with HDMs (Figure 4A). Around 20% of transferred 1-DER cells in the mLN, lung, and BAL expressed GFP (Figures 4B and 4C). This frequency was lower in the spleen of recipient mice (Figures 4B and 4C). In addition, the proportion of transferred 1-DER T cells expressing PD-1 and CXCR5 (bona fide Tfh cells) was close to 50% in the mLN, compared to fewer than 10% in the spleen, lung, and BAL (Figure 4C). The intensity of IL-21GFP was greater in 1-DER T cells from the mLN compared with other organs, similar to our findings in IL-21GFP reporter mice expressing heterogeneous TCRs (Figure 3). Hence, Der p1-reactive $CD4^+$ T cells exhibit a high capacity to upregulate the expression of IL-21 after HDM administration and this population is prevalent in the lung tissue and airspace.

To gain further insight into the kinetics of IL-21 expression, *Il21*$^{gfp/+}$ 1-DER $CD4^+$ T cells were transferred into naive mice and recipients were challenged daily with HDMs for 5 days. The expression of IL-21GFP among transferred cells was analyzed at days 5, 8, and 11 (Figures 4D and 4E). The frequency of 1-DER T cells peaked around 5 days after transfer and declined thereafter in the mLN and spleen whereas the frequency of transferred cells in the lung and BAL was highest at day 8 (Figures 4D and 4E). Around 8% of 1-DER T cells in the mLN expressed IL-21GFP at day 5 after transfer and this proportion increased to more than 30% by day 11 (Figure 4E). Virtually all IL-21GFP$^+$ cells in the mLN expressed PD-1 and CXCR5 at these time points (data not shown). Furthermore, more than 30% of transferred 1-DER T cells in the mLN already expressed PD-1 and CXCR5 at day 5. This proportion increased over time such that more than 60% of 1-DER T cells in the mLN at day 11 were PD-1$^+$CXCR5$^+$. Very few 1-DER T cells recovered from lung tissue or BAL expressed IL-21GFP or markers consistent with Tfh cells using this HDM administration protocol (Figure 4E). This suggests that the migration of IL-21-producing $CD4^+$ T cells to the lung and BAL requires additional time or (more likely) multiple rounds of antigen stimulation.

Cell-Intrinsic IL-21R Signaling Supports the Acquisition of Th2 Cell Functions

Given that HDM challenge induced potent Tfh cell responses and because *Il21r*$^{-/-}$ mice developed weak Th2 cell responses, we analyzed whether this might be due to a defect in Tfh cell differentiation. WT and *Il21r*$^{-/-}$ mice that had been sensitized and challenged with HDMs had similar amounts of total $CD4^+$ T, germinal center (GC) B, and Tfh cells in the mLN (Figure 5A), suggesting that the impaired Th2 cell response in *Il21r*$^{-/-}$ mice was not due to a deficiency in Tfh cells. Despite the normal frequency of $CD4^+$ T cells in the mLN, the proportion of IL-33R$^+$ cells among CD44$^+$ memory $CD4^+$ T cells was significantly greater in WT compared with *Il21r*$^{-/-}$ mice (Figures 5B and 5C), again indicative of a defect in effector Th2 cell differentiation.

To investigate whether IL-21R provided cell-intrinsic signals to $CD4^+$ T cells responding to HDMs, CD45.1$^+$CD45.2$^+$ double congenic mice were lethally irradiated and reconstituted 1:1 with WT (CD45.1) and *Il21r*$^{-/-}$ (CD45.2) bone marrow cells. Eight weeks after reconstitution, mice were sensitized and challenged with HDMs and the $CD4^+$ T cell response was analyzed. WT and *Il21r*$^{-/-}$ cells accounted for a roughly equal proportion of $CD4^+$ and $CD8^+$ T cells and the percentage of memory CD44$^+$ cells among $CD4^+$ T cells from each fraction was similar (Figure 5D). However, a greater proportion of WT cells expressed IL-33R compared with *Il21r*$^{-/-}$ cells within the same mouse (Figure 5E). In addition, purified WT $CD4^+$ T cells from the mLN expressed significantly more IL-13 and IL-21 and moderately more IL-4 mRNA than *Il21r*$^{-/-}$ cells from the same mouse (Figure 5F). IL-17 expression was similar in WT and *Il21r*$^{-/-}$ cells suggesting that IL-21R preferentially supports the acquisition of Th2, rather than of Th17, cell effector functions. These results indicate that IL-21R provides cell-intrinsic signals that induce the acquisition of Th2 cell functions.

IL21R Signaling Supports ILC2 Function and Number in a Non-Cell-Intrinsic Manner

ILC2s are an innate population of cells, which share many features of conventional Th2 cells and have been proposed to play an important role in controlling eosinophilic lung inflammation (Halim et al., 2014; Neill et al., 2010; Van Dyken et al., 2014). As such, the frequency and function of ILC2s was analyzed in WT and *Il21r*$^{-/-}$ mice. In the mLN and lung of HDM-sensitized and challenged mice, the frequency of ILC2s among gated lineage$^-$CD45$^+$CD90.2$^+$ cells was reduced in *Il21r*$^{-/-}$ compared with WT mice (Figure 6A). In addition, the ability of ILC2s in the mLN to produce IL-5 and IL-13 was significantly reduced in *Il21r*$^{-/-}$ mice (Figure 6B), although the cytokine-producing capacity of the lung ILC2 population was similar between WT and *Il21r*$^{-/-}$ mice. This suggested that IL-21R might numerically and functionally support the ILC2 population. To test whether IL-21R also provided cell-intrinsic inputs to developing ILC2s, we analyzed the frequency of ILC2s in 1:1 chimeras as described in Figure 5. WT and *Il21r*$^{-/-}$ cells evenly contributed to the ILC2 pool in the mLN and lung of 1:1 chimeras (Figure 6C), suggesting that although IL-21R can augment ILC2 responses, it does not provide cell-intrinsic inputs as it does for Th2 cells.

Recombinant IL-21 and rIL-25 Synergize to Promote Lung Eosinophilia

The presence of IL-21$^+$ cells that were distinct from Th2 or Th17 cells and were not precursors of Th2 cells in the lung was indicative of an extra-follicular function for IL-21. Because IL-21R promoted the acquisition of Th2 cell function in a cell-intrinsic manner and because transferred $CD4^+$IL-21-GFP$^+$ cells induced lung eosinophilia in a short-term challenge model (Figure 3), we hypothesized that IL-21 might directly promote type-2-cell-mediated immunity in the lungs. Recombinant IL-21 was administered to mice in isolation or in combination with the innate cytokine IL-25, because the receptor for IL-25 (*Il17rb*) was upregulated on Th2 cells in mice immunized with HDMs (Figures 1A and S1). IL-25 is made by epithelial cells in response to HDMs or protease allergens and can potentiate ILC2 precursors in the lung (Hammad et al., 2009; Huang et al., 2015; Willart et al., 2012). Cytokines were administered i.n. for 3 consecutive days and eosinophilia of the airways was assessed on day 4. rIL-21 and rIL-25 both induced little eosinophilia in the airways of WT mice, but the combination of rIL-21 and rIL-25 synergistically promoted airway eosinophilia (Figure 7A). Expression of *Muc5ac* mRNA in lung tissue, which is indicative of IL-13-induced mucus production by lung epithelial cells, was also induced in response to rIL-21 and rIL-25 co-administration (Figure S4A). In *Rag2*$^{-/-}$

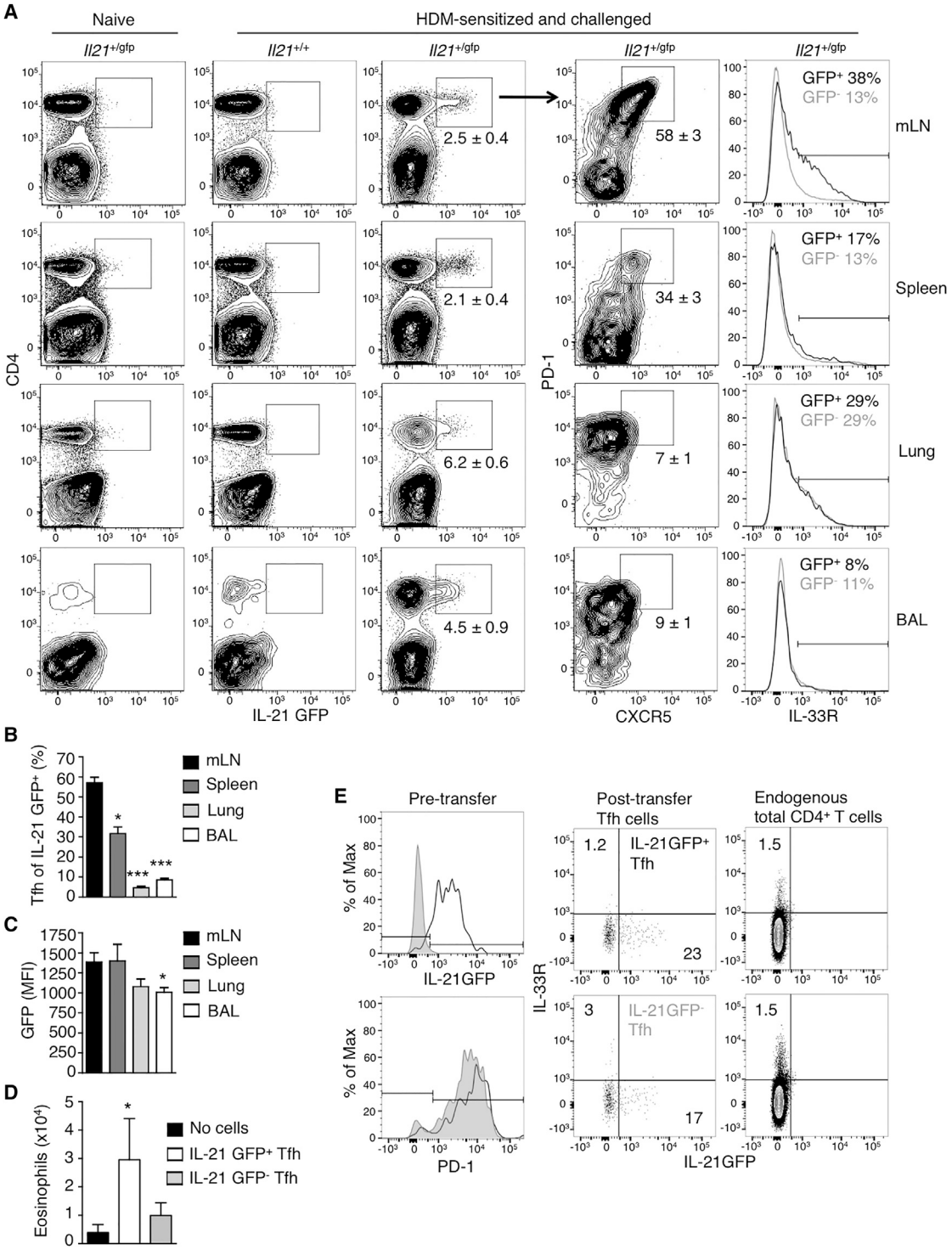

Figure 3. HDMs Induce IL-21 Expression by Tfh Cells in the mLN and Adoptive Transfer of IL-21$^+$ Cells Mediates Airway Eosinophilia

The mLN, spleen, lung, and BAL of naive or HDM-sensitized and challenged *Il21*$^{+/+}$ and *Il21*$^{+/gfp}$ mice was analyzed by flow cytometry.

(A) Contour plots depict IL-21GFP versus CD4 expression in naive or HDM-exposed mice and CXCR5 versus PD-1 expression on gated IL-21GFP$^+$CD4$^+$ cells. Histograms (right-hand) depict IL-33R expression on gated IL-21GFP$^+$CD4$^+$ and IL-21GFP$^-$CD4$^+$ cells. Numbers show the mean percentage of cells in the gated region ± SEM.

(B) The graph represents the percentage of IL-21GFP$^+$CD4$^+$ cells that express CXCR5 and PD-1. Results are representative of 6–15 mice pooled over 2–4 experiments.

(C) The graph represents the mean fluorescence intensity (MFI) of IL-21GFP expression in IL-21GFP$^+$CD4$^+$ cells where n = 6 mice from 2 experiments.

(legend continued on next page)

mice lacking T and B lymphocytes, the number of eosinophils in the BAL of cytokine-administered mice was reduced by an order of magnitude compared with WT mice, yet there was still a significant trend for IL-21 and IL-25 to cooperate in driving eosinophilia (Figure 7A). No eosinophilia was observed in NOD.SCID.*Il2rg*$^{-/-}$ mice. These results suggested that combined rIL-21 and rIL-25 induced airway eosinophilia primarily via RAG-dependent CD4$^+$ T cells. Although IL-25 can promote ILC2 responses (Huang et al., 2015), administration of recombinant cytokines at these doses and for only 3 days had no numerical effect on the lung ILC2 population (Figure S4B). In support of a role for IL-21 in directly promoting Th2 cell functions in lung CD4$^+$ T cells, a significant increase in the frequency of IL-33R$^+$CD4$^+$ T cells was observed in mice administered either rIL-21 alone or both rIL-21 and rIL-25 (Figure 7B). To test whether rIL-21 and rIL-25 induced airway eosinophilia via a Th2-cell- and IL-33R-dependent mechanism, mice were administered anti-IL-5 or soluble ST2 (to block IL-33:L-33R) (Willart et al., 2012). The ability of combined rIL-21 and rIL-25 administration to induce airway eosinophilia was indeed dependent on both IL-5 and the IL-33:IL-33R pathway (Figure 7C). The source of IL-33 in this system is likely to be epithelial, because IL-25 promotes IL-33 production by epithelial cells (Kang et al., 2012).

Collectively, these data illustrate that the allergen HDM potently induces IL-21 production that promotes the acquisition of Th2 cell effector functions. IL-21-producing cells in the mLN are primarily Tfh cells, but IL-21-producing CD4$^+$ T cells are present in large numbers in the lungs of asthmatic mice and appear capable of potentiating type 2 immunity in this organ directly.

DISCUSSION

Asthma is a complex disease comprising of diverse clinical and biological symptoms, which are triggered by the activity of cytokines produced by Th2 cells (Lambrecht and Hammad, 2015). Although the majority of research has centered on the role of Th2 cells in asthma, roles for Th17 cells and ILCs have also recently been described, both in the pathogenesis and potentially the alleviation of typical signs of asthma (Halim et al., 2014; Neill et al., 2010; Schnyder-Candrian et al., 2006; Van Dyken et al., 2014). Herein, we have persuasively demonstrated that HDMs potently induced IL-21$^+$ Tfh cells in the lung-draining mLN and promoted the emergence of a distinct CXCR5$^-$PD1$^+$IL-21$^+$ cell population in the lung tissue and airway.

Because Tfh cells are generally regarded to provide help to B cells, it has been logical to assume that Tfh cells play an amplificatory role in allergic asthma. However, there is little evidence that HDMs induce robust IgE production in mice in short-term models (Birrell et al., 2010). Furthermore, mice deficient in IL-21R developed normal GC B cell responses and displayed higher concentrations of circulating total IgE as previously reported (Ozaki et al., 2002), arguing that IgE production from B cells plays a limited role in this model. Reduced HDM-specific IgG1 in *Il21r*$^{-/-}$ mice and the ability of transferred IL-21$^+$ Tfh cells or soluble rIL-21 to rapidly induce IL-5-dependent airway eosinophilia, also strongly supports a role for IL-21-producing T cells in promoting type 2 immunity, rather than in promoting B cell function to HDMs.

A number of studies have described Tfh cell responses under various Th2-cell-inducing conditions (Baumjohann et al., 2011; King and Mohrs, 2009; León et al., 2012; Reinhardt et al., 2009), suggesting that Th2 and Tfh cells share common developmental pathways. Furthermore, IL-21-producing Tfh cells can acquire effector functions associated with other T helper cell lineages (Johansson-Lindbom et al., 2003; Lüthje et al., 2012; Zotos et al., 2010) and vice versa, Th2 cells have been shown to take on Tfh cell characteristics (Glatman Zaretsky et al., 2009). Herein, we observed that even a single dose of HDMs administered through the skin or i.n. could induce the expression of PD-1 and CXCR5 in 1-DER T cells within 5 days (Figure S3B). The relatively rapid induction of IL-21 in CD4$^+$ T cells after HDM administration and the requirement for IL-21R in the acquisition of Th2 effector cell functions suggests that IL-21 expression precedes, or is at least temporally co-regulated, with Th2 cell differentiation.

Although considerable in vitro and in vivo evidence suggested that IL-21-expressing cells would co-express effector Th2 cytokines, we found very little evidence of this in response to HDMs. Recently activated IL-4$^+$ T cells migrate to the T cell-B cell border where they have been shown to receive further Th2 cell priming inputs or enter the follicle to differentiate into IL-21$^+$ Tfh cells (León et al., 2012; Xu et al., 2013). Our results suggest that the entry of cells into the follicle and the expression of IL-21 therein stifles the later acquisition of Th2 effector cell functions in response to HDMs. In line with this, Tfh cells transferred into naive recipients and subsequently challenged with influenza acquired Th1 cell effector functions but lost any ability to produce IL-4 (Lüthje et al., 2012). One possibility is that the nature of CD4$^+$ T cell priming makes it more difficult for cells that have already developed into Tfh cells, to acquire Th2-cell- rather than Th1-cell-associated functions. Studies proposing that Tfh and Th1 cells share overlapping T cell antigen receptors corroborate this idea (Fazilleau et al., 2009; Tubo et al., 2013). High-affinity TCR signals are believed to predispose cells toward the Tfh or Th1 cell fate and away from the Th2 cell fate, which are favored in the presence of lower affinity signals (Ruedl et al., 2000; van Panhuys et al., 2014). Hence, it is possible that after contact of Tfh cells with germinal center B cells expressing high amounts of cognate antigen (Shulman et al., 2014), Tfh cells can no longer acquire functions associated with Th2 cells.

The cell-intrinsic function of IL-21R in promoting IL-13, IL-21, and IL-33R expression but not that of IL-17 suggests that IL-21

(D and E) mLN and spleen was harvested on day 15 from *Il21*$^{+/gfp}$ mice previously sensitized and challenged with HDMs. IL-21GFP$^+$ and IL-21GFP$^-$ PD-1$^+$ cells were purified. Naive CD45.1 mice received 5×10^4 IL-21GFP$^+$ Tfh cells, 5×10^4 IL-21GFP$^-$ Tfh cells, or no cells and were challenged with 10 μg HDM extract i.n. daily from the day of transfer until day 4. On day 8, eosinophilia in the BAL was assessed.

(D) The number of eosinophils in the BAL (n = 4–7 mice). Kruskall-Wallis with post-Dunn's test was used to compare groups. *$p < 0.05$.

(E) Histograms show the expression of IL-21 and PD-1 in IL-21GFP$^+$ Tfh (black unfilled) and IL-21GFP$^-$ Tfh (gray shaded) cells before transfer. Dot and contour plots show the expression of IL-21GFP and IL-33R on transferred cells (CD45.2$^+$CD4$^+$) and endogenous (CD45.2$^-$CD4$^+$) cells from mLN at day 8.

Also refer to Figure S2.

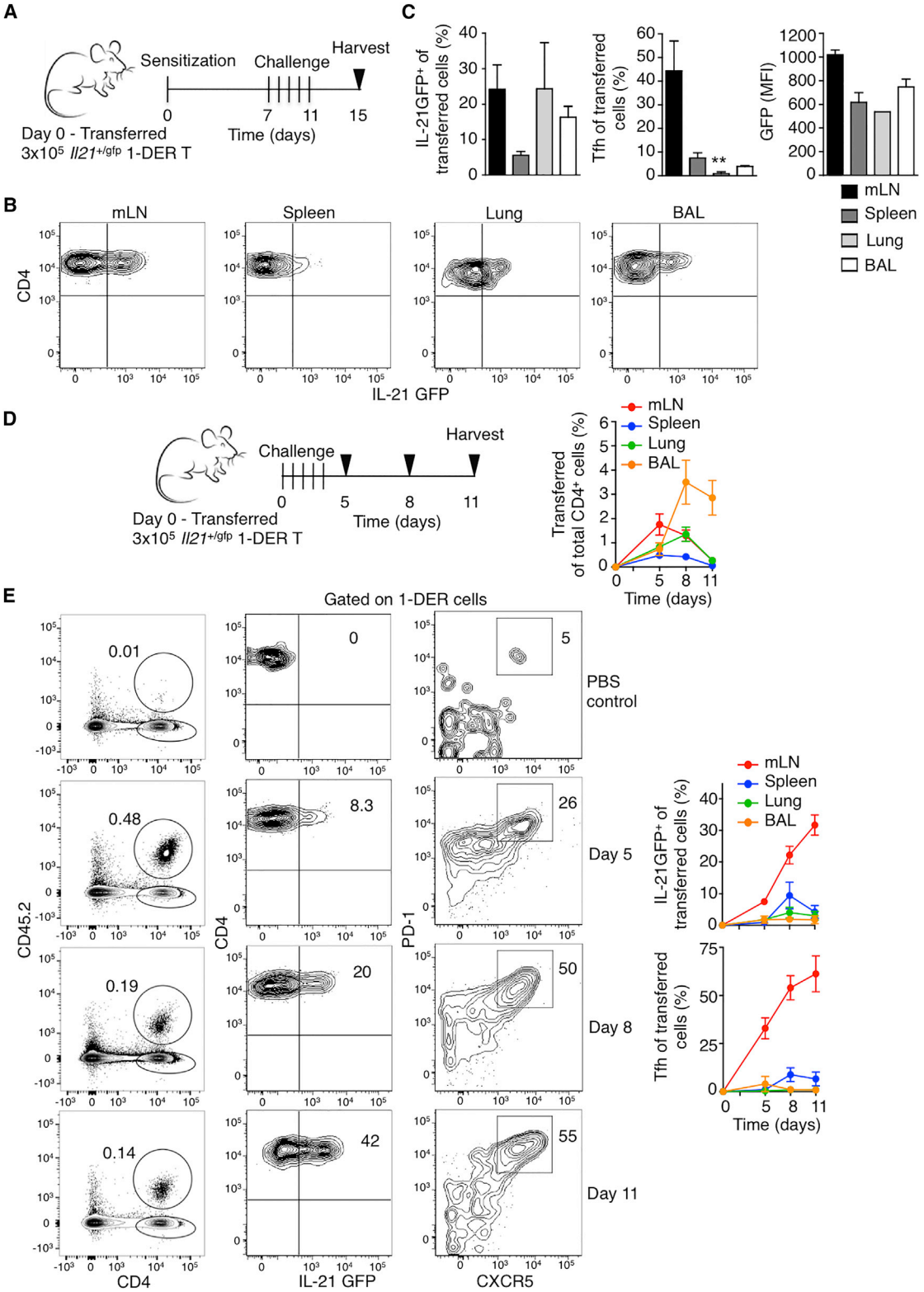

Figure 4. 1-DER TCR Transgenic Cells Rapidly Acquire Tfh Cell Characteristics

Spleens of *Il21*$^{+/gfp}$ 1-DER TCR transgenic mice were harvested and magnetically purified for CD4^{+} T cells. 3 × 10^{5} 1-DER T cells were transferred into naive CD45.1 congenic mice.

(A and B) Mice were sensitized with 100 μg HDM extract on the skin and challenged with 10 μg HDM extract i.n. between days 7 and 11.

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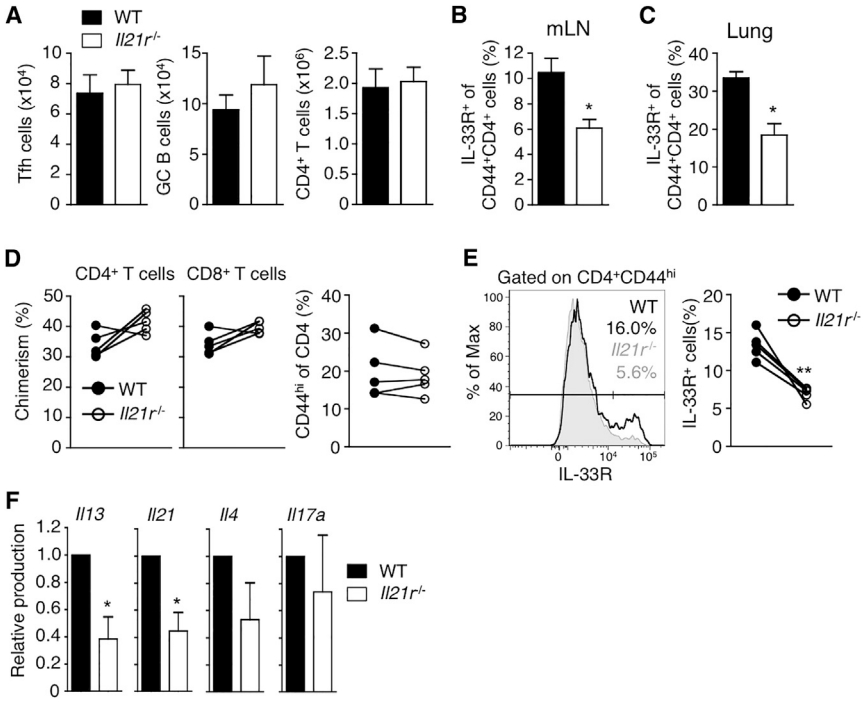

Figure 5. IL-21R Influences IL-33R, IL-13, and IL-21 Expression in a Cell-Intrinsic Manner

(A) WT and *Il21r*$^{-/-}$ mice sensitized and challenged with HDMs were analyzed by flow cytometry for the presence of Tfh (CD4$^+$CD44$^+$CXCR5$^+$PD-1$^+$), germinal center B (CD19$^+$Fas$^+$GL7$^+$), and total CD4$^+$ T (CD4$^+$CD3$^+$) cells. One representative experiment of four with four to five mice per group is shown.

(B and C) Gated CD4$^+$CD44$^+$ cells were analyzed for IL-33R expression in the mLN (B) and lung (C) by flow cytometry. Graphs depict the percentage of IL-33R$^+$ cells among gated cells in nine to ten mice from two independent experiments.

(D–F) CD45.1$^+$CD45.2$^+$ double congenic mice were lethally irradiated and reconstituted with a 1:1 mixture of WT (CD45.1$^+$) and *Il21r*$^{-/-}$ (CD45.2$^+$) bone marrow cells. 8 weeks later, mice were sensitized via the skin with 100 μg HDM extract and challenged with 10 μg HDM extract i.n. between days 7 and 11. At day 15, the contribution of WT and *Il21r*$^{-/-}$ cells to the immune response was analyzed.

(D) The percentage of WT and *Il21r*$^{-/-}$ cells within the CD4$^+$ (left) and CD8$^+$ T (middle) cell pools and the percentage of CD44$^+$ memory cells among gated WT or *Il21r*$^{-/-}$ CD4$^+$ T cells (right). Lines between circles indicate chimerism within the same mouse. One representative of four experiments is shown with three to five mice per experiment. Radio-resistant cells comprised approximately 20% of cells.

(E) Histogram and graph of IL-33R expression on gated memory CD44$^+$CD4$^+$ T cells from WT or *Il21r*$^{-/-}$ cells. Shown is one representative of two independent experiments.

Results from (A)–(E) were analyzed by Mann-Whitney U test. *$p < 0.05$, **$p < 0.01$.

(F) WT and *Il21r*$^{-/-}$ CD4$^+$ T cells from mLN were purified by flow cytometry and analyzed by quantitative PCR for the expression of the genes indicated. Values in *Il21r*$^{-/-}$ cells are shown relative to WT. Results are from seven separate mice from two independent experiments. Wilcoxon-Rank test was used to analyze normalized values. *$p < 0.05$.

can provide important cues specifically for Th2 cell differentiation. Although a role for IL-21 in enhancing type 2 immunity has previously been proposed (Fröhlich et al., 2007; Pesce et al., 2006) and IL-21 induces Th2-cell-associated cytokine production by NKT cells (Coquet et al., 2007, 2013), other reports have suggested that IL-21 is dispensable for Th2 cell differentiation (King et al., 2010). It is hard to reconcile this discrepancy because both King et al. (2010) and Fröhlich et al. (2007) analyzed IL-4 production in the *H. polygyrus* model. A pertinent point is that IL-4 is expressed by multiple T cell subsets and that the use of markers such as IL-5, IL-13, and IL-33R probably better delineate differentiated effector Th2 cells. Our findings in 1:1 chimeric mice strongly suggest that IL-21R promotes Th2 cell effector functions and these results are backed by findings from Stritesky et al. (2011), who have previously demonstrated a key cell-intrinsic role for STAT3 in stabilizing STAT6 at critical Th2-cell-associated gene loci.

It remains unclear in exactly what context IL-21R signals are transmitted to developing Th2 cells. Because Th2 cells are known to receive signals at the T cell-B cell border (León et al., 2012), it is possible that this brings them in close proximity with IL-21$^+$ Tfh cells in the follicle. However, the presence of IL-21$^+$CXCR5$^-$CD4$^+$ T cells in lung is also indicative of a role outside of primary lymphoid organs. The ability of rIL-21 to synergize with the innate stimulus rIL-25, to induce potent Th2 cell responses and airway eosinophilia is testament to this function. We sought to investigate the effect of co-administration of rIL-21 and rIL-33 on lung inflammation; however, the highly potent effects of i.n. rIL-33

(B and C) Cells from mLN, spleen, lung, and BAL were analyzed by flow cytometry at day 15.

(B) Contour plots depict the expression of IL-21GFP versus CD4 on gated CD45.2$^+$CD3$^+$CD4$^+$ cells.

(C) Graphs show the percentage IL-21GFP$^+$ (top) and CXCR5$^+$PD-1$^+$ (middle) among transferred cells, and the MFI of IL-21GFP on gated IL-21GFP$^+$CD4$^+$ cells (bottom) (n = 3 mice). Similar results were obtained after i.n. sensitization in three mice. Groups were compared by Kruskall-Wallis and post-Dunn's test. *$p < 0.05$.

(D and E) 1-DER T cells were transferred and recipient mice were challenged daily with 10 μg i.n. HDM extract until day 4. Spleen, mLN, lung, and BAL of mice were harvested at day 5, 8, and 11.

(D) The graph shows the percentage of transferred 1-DER T cells among total CD4$^+$ T cells.

(E) Plots depict the expression of CD4 versus CD45.2 (left), IL-21GFP versus CD4 (middle), and CXCR5 versus PD-1 (right) on transferred 1-DER T cells over time in mLN. A PBS control mouse at day 8 is shown. Graphs show the percentage of IL-21GFP$^+$ and CXCR5$^+$PD-1$^+$ cells of transferred 1-DER T cells (n = 6 mice per time point pooled from 2 independent experiments).

Also refer to Figure S3.

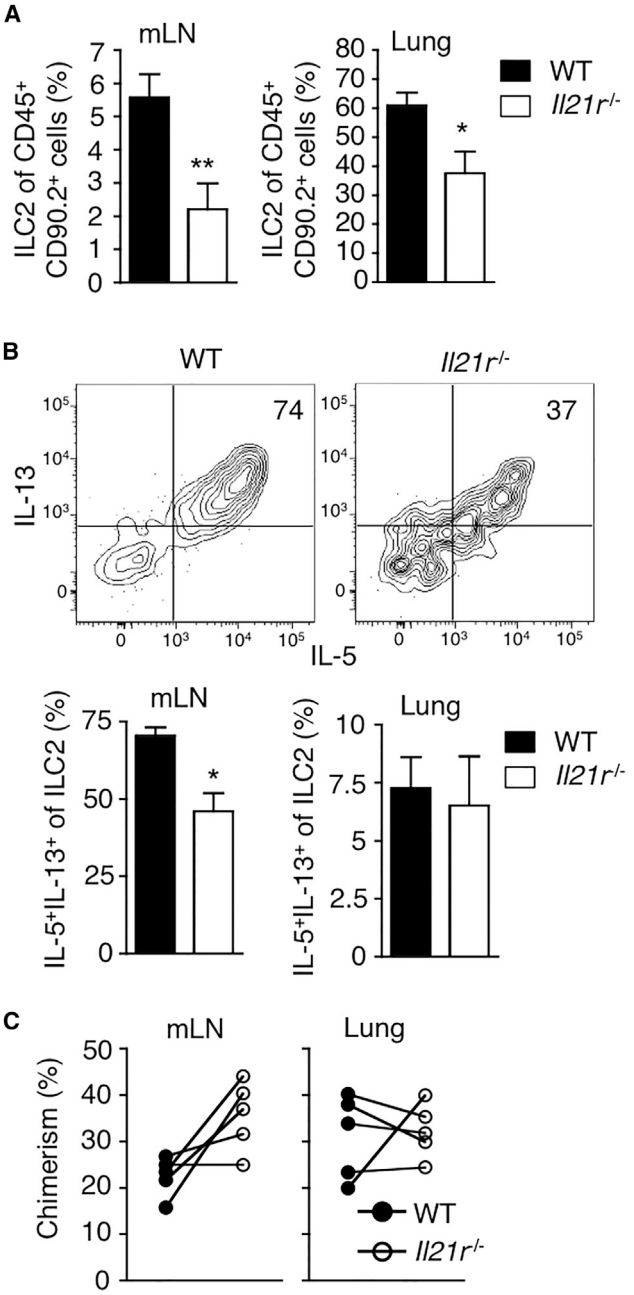

Figure 6. ILC2s Are Numerically and Functionally Aberrant in *Il21r*$^{-/-}$ Mice

WT and *Il21r*$^{-/-}$ mice sensitized and challenged with HDMs were analyzed by flow cytometry for ILC2s. ILC2s were defined as Lin^{-}CD45^{+}CD90.2^{+} CD127^{+}IL-33R^{+}.

(A) The graphs depict the percentage of IL-33R^{+}CD127^{+} cells among gated lineage^{-}CD45^{+}CD90.2^{+} cells in the mLN and lung.

(B) Restimulated mLN and lung cells were analyzed for intracellular IL-5 and IL-13. Contour plots depict the expression of IL-5 versus IL-13 in gated ILC2s. Graphs depict the percentage of IL-5^{+}IL-13^{+} cells of ILC2s in the mLN and lung of WT and *Il21r*$^{-/-}$ mice (n = 9–10 mice pooled from 2 independent experiments).

(C) The contribution of WT and *Il21r*$^{-/-}$ cells to the ILC2 population in 1:1 chimeras exposed to HDMs was analyzed. Graphs show the percentage of WT and *Il21r*$^{-/-}$ in gated ILC2s in one of two independent experiments.

Results were analyzed by Mann-Whitney U test. *$p < 0.05$, **$p < 0.01$.

administration alone made this difficult. The more subtle effects of rIL-25 administration on airway inflammation and the known ability of IL-25 to recruit immature ILC2s and induce IL-33 production from epithelial cells (Huang et al., 2015; Kang et al., 2012) provided a more physiological platform through which to study the impact of IL-21 on Th2-cell-mediated inflammation. We concluded that the effects of rIL-21 and rIL-25 co-administration proceeded largely through CD4^{+} T cells, probably resident memory CD4^{+} T cells of the lung. Memory CD4^{+} Th2 cells are highly responsive to cytokine stimulation with IL-25 and IL-33, even in the absence of TCR ligation (Meisel et al., 2001). This suggests an almost "innate" function of memory Th2 cells triggered by innate cytokines and boosted by IL-21 from recently generated CD4^{+} T cells that move to the lung. This does not rule out a potentially important role for IL-21 in promoting ILC2 function either. Certainly, the reduced frequency and function of ILC2s in *Il21r*$^{-/-}$ mice and the ability of the combination of rIL-21 and rIL-25 to induce mild eosinophilia even in *Rag2*$^{-/-}$ mice suggest that IL-21 can also play an amplificatory role in ILC2 function. The effects of IL-21 on ILC2s were, however, indirect in the HDM model. IL-21 might indirectly promote ILC2s through Th2 cells, which have the potential to stimulate ILC2s through cytokines such as IL-2 and IL-9 (Liu et al., 2015; Roediger et al., 2013). Regardless of the exact mechanism of action, the presence of IL-21^{+} cells in the lung and potent effects of rIL-21 suggest that neutralizing therapies of IL-21 in lung tissue might reduce pathogenic type-2-cell-mediated immune responses.

Taken together, these data highlight a prominent role for IL-21-producing CD4^{+} T cells in HDM-induced asthma and pinpoint an important amplificatory role for IL-21 in type-2-cell-mediated immunity via induction of IL-33R^{+} Th2 cells.

EXPERIMENTAL PROCEDURES

Mice and Generation of Bone Marrow Chimeras

WT C57BL/6, *Il21r*$^{-/-}$, IL-21GFP, IL-4GFP, and C57BL/6.CD45.1 congenic mice were bred at Ghent University in specific-pathogen-free conditions. 1-DER TCR transgenic mice were generated as described in Results. *Il21r*$^{-/-}$ mice were obtained originally from Dr. Warren Leonard. Experiments were approved by the animal ethics committees of the University of Ghent and the IRC, VIB.

To make 1-DER mice, antigen-specific T cell hybridomas were generated by fusion of the T cell lymphoma cell line BW5147 to in vitro restimulated CD4^{+} splenocytes of C57BL/6 mice that received the specific antigen in vivo. Antigen-specific clones were selected based on IL-2 production and CD69 upregulation upon stimulation with antigen-specific peptide-pulsed BM-DCs. The TCR (Vα8 and Vβ4 chain) was cloned into a mammalian expression vector pTarget (Promega) and tested for functionality in Jurkat cells. Subsequently, the Vα8 and Vβ4 cDNA were cloned into modified TCR expression vectors p428 and p783 (provided by Dr. Nigel Killeen), respectively, and co-injected in an equimolar concentration of 1 ng/μl into the pronuclei of fertilized C57BL/6 *Rag*$^{-/-}$ oocytes. Founders were screened for TCR functionality by flow cytometry and offspring was genotyped by PCR with primers specific for the Vα8 and Vβ4 transgene.

For generation of bone marrow chimeras, C57BL/6 CD45.1^{+}CD45.2^{+} mice were lethally irradiated with 8 Gy γ-irradiation. The following day, mice were reconstituted intravenously with a 1:1 mixture of WT CD45.1^{+} and *Il21r*$^{-/-}$ CD45.2^{+} bone marrow cells. Mice were kept on antibiotics for 4 weeks and used in experiments 8 weeks after irradiation.

Models of Asthma and Cytokine Instillation

Mice were anesthetized briefly with isoflurane. For i.n. sensitization, mice received 1 μg HDM extract in a volume of 40 μl PBS. For skin sensitization,

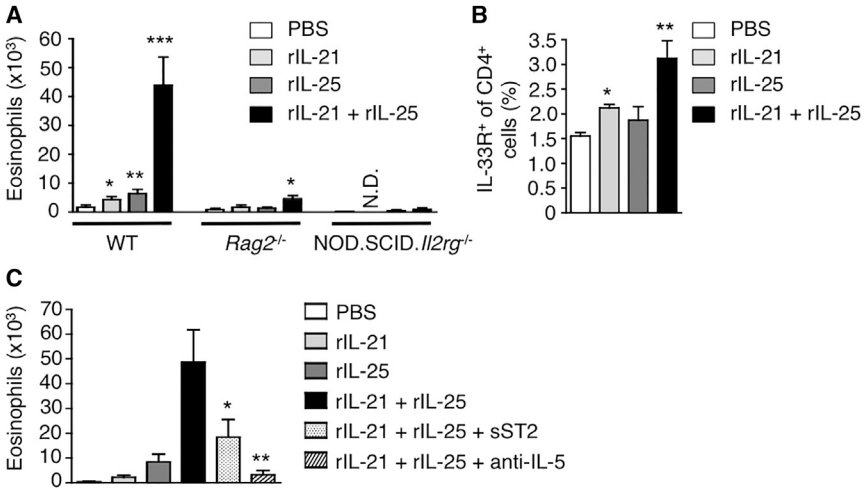

Figure 7. Recombinant IL-21 Induces Airway Eosinophilia in an IL-33R- and IL-5-Dependent Fashion

(A) WT, *Rag2*$^{-/-}$, or NOD.SCID.*Il2rg*$^{-/-}$ mice were administered i.n. PBS, 10 μg rIL-21, 125 ng rIL-25, or both rIL-21 and IL-25 daily for 3 days and BAL was harvested 24 hr after the last challenge. The graph depicts the number of eosinophils in the BAL of mice as determined by flow cytometry. Results were pooled from two to three experiments (WT, n = 10 mice; *Rag2*$^{-/-}$ and NOD.SCID.*Il2rg*$^{-/-}$, n = 5–7).

(B) The graph depicts the percentage of IL-33R^{+} cells among gated total CD4^{+} T cells as determined by flow cytometry.

(C) Mice were administered cytokines as described in (A) in combination with 50 μg soluble ST2 or anti-IL-5 i.p. on days 0 and 1. The number of eosinophils in the BAL on day 4 is shown. n = 6–8 mice per group pooled from 2 experiments, except for mice receiving anti-IL-5 (n = 4).

In (A) and (B), cytokine-administered groups were compared to PBS controls and in (C), mice receiving sST2 and anti-IL-5 were compared to mice receiving the combination of rIL-21 and rIL-25. Results were analyzed by Mann-Whitney U test. *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$. Also refer to Figure S4.

mice were shaved on the hind flank and 100 μg HDM extract was applied to the skin in a volume of 50 μl. A small piece of absorbent gauze was applied and the area taped to prevent access. 1 week after sensitization, mice were challenged on 5 consecutive days with 10 μg HDM extract i.n. under light anesthesia with isoflurane. 4 days after the last administration, mice were sacrificed and organs were dissected for analysis. BAL was performed by two consecutive flushes of the lung with 1 ml PBS. Blood was taken to screen for serum antibodies. mLN and spleen were dissected through 100 μM sieves and analyzed by flow cytometry. Lung function was analyzed with the Flexivent apparatus.

Restimulation by ICS, ELISA, and PMA

Cell culture was performed in RPMI-1640 medium supplemented with gentamycin, glutamine, 2-mercaptoethanol (all from Invitrogen), and 10% heat-inactivated FCS (Sigma). To analyze cytokine production in WT and *Il21r*$^{-/-}$ mice, 1×10^{6} mLN cells were restimulated in a 48-well plate for 48 hr in the presence of 20 μg/ml HDM extract. Supernatants were analyzed for the presence of cytokines using the Read-Set-Go! ELISA sets (E Bioscience). To analyze cytokine production by ILC, 50 ng/ml PMA and 1 μM ionomycin (both from Sigma) were added to cultures of total mLN or lung for 3 hr in the presence of BD GolgiStop. To stain for intracellular IL-21, mLN cells were restimulated overnight with 20 μg/ml HDM extract in the presence of BD GolgiStop. The BD Fixation/Permeabilization kit was used for intracellular staining of cells.

Statistical Analysis

A non-parametric Mann-Whitney U or Kruskall-Wallis test was used except for normalized data, which was analyzed by log-rank test. In all graphs, the standard error of the mean is shown.

ACCESSION NUMBERS

Microarray data from affymetrix arrays were deposited in the NCBI GEO database under accession number GEO: GSE72005.

SUPPLEMENTAL INFORMATION

Supplemental Information includes four figures and Supplemental Experimental Procedures and can be found with this article online at http://dx.doi.org/10.1016/j.immuni.2015.07.015.

AUTHOR CONTRIBUTIONS

J.M.C., M.J. Schuijs, and K.D. performed experiments; M.J. Smyth, S.L.N., H.B., G.B.K.H., L.B., and R.B. contributed critical reagents and mice; J.M.C. wrote the paper; and J.M.C., H.H., and B.N.L. conceived the study.

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REFERENCES

Barlow, J.L., Bellosi, A., Hardman, C.S., Drynan, L.F., Wong, S.H., Cruickshank, J.P., and McKenzie, A.N. (2012). Innate IL-13-producing nuocytes arise during allergic lung inflammation and contribute to airways hyperreactivity. J. Allergy Clin. Immunol. *129*, 191–8.e1, 4.

Baumjohann, D., Okada, T., and Ansel, K.M. (2011). Cutting Edge: Distinct waves of BCL6 expression during T follicular helper cell development. J. Immunol. *187*, 2089–2092.

Birrell, M.A., Van Oosterhout, A.J., and Belvisi, M.G. (2010). Do the current house dust mite-driven models really mimic allergic asthma? Eur. Respir. J. *36*, 1220–1221.

Burrows, B., Martinez, F.D., Halonen, M., Barbee, R.A., and Cline, M.G. (1989). Association of asthma with serum IgE levels and skin-test reactivity to allergens. N. Engl. J. Med. *320*, 271–277.

Chtanova, T., Tangye, S.G., Newton, R., Frank, N., Hodge, M.R., Rolph, M.S., and Mackay, C.R. (2004). T follicular helper cells express a distinctive transcriptional profile, reflecting their role as non-Th1/Th2 effector cells that provide help for B cells. J. Immunol. *173*, 68–78.

Coquet, J.M., Kyparissoudis, K., Pellicci, D.G., Besra, G., Berzins, S.P., Smyth, M.J., and Godfrey, D.I. (2007). IL-21 is produced by NKT cells and modulates NKT cell activation and cytokine production. J. Immunol. *178*, 2827–2834.

Coquet, J.M., Skak, K., Davis, I.D., Smyth, M.J., and Godfrey, D.I. (2013). IL-21 modulates activation of NKT cells in patients with Stage IV malignant melanoma. Clin. Transl. Immunology *2*, e6, http://dx.doi.org/10.1038/cti.2013.1037.

Dullaers, M., De Bruyne, R., Ramadani, F., Gould, H.J., Gevaert, P., and Lambrecht, B.N. (2012). The who, where, and when of IgE in allergic airway disease. J. Allergy Clin. Immunol. *129*, 635–645.

Fazilleau, N., McHeyzer-Williams, L.J., Rosen, H., and McHeyzer-Williams, M.G. (2009). The function of follicular helper T cells is regulated by the strength of T cell antigen receptor binding. Nat. Immunol. *10*, 375–384.

Fort, M.M., Cheung, J., Yen, D., Li, J., Zurawski, S.M., Lo, S., Menon, S., Clifford, T., Hunte, B., Lesley, R., et al. (2001). IL-25 induces IL-4, IL-5, and IL-13 and Th2-associated pathologies in vivo. Immunity *15*, 985–995.

Fröhlich, A., Marsland, B.J., Sonderegger, I., Kurrer, M., Hodge, M.R., Harris, N.L., and Kopf, M. (2007). IL-21 receptor signaling is integral to the development of Th2 effector responses in vivo. Blood *109*, 2023–2031.

Gajewski, T.F., and Fitch, F.W. (1988). Anti-proliferative effect of IFN-gamma in immune regulation. I. IFN-gamma inhibits the proliferation of Th2 but not Th1 murine helper T lymphocyte clones. J. Immunol. *140*, 4245–4252.

Glatman Zaretsky, A., Taylor, J.J., King, I.L., Marshall, F.A., Mohrs, M., and Pearce, E.J. (2009). T follicular helper cells differentiate from Th2 cells in response to helminth antigens. J. Exp. Med. *206*, 991–999.

Halim, T.Y., Steer, C.A., Mathä, L., Gold, M.J., Martinez-Gonzalez, I., McNagny, K.M., McKenzie, A.N., and Takei, F. (2014). Group 2 innate lymphoid cells are critical for the initiation of adaptive T helper 2 cell-mediated allergic lung inflammation. Immunity *40*, 425–435.

Hammad, H., and Lambrecht, B.N. (2015). Barrier epithelial cells and the control of type 2 immunity. Immunity *43*, 29–40.

Hammad, H., Chieppa, M., Perros, F., Willart, M.A., Germain, R.N., and Lambrecht, B.N. (2009). House dust mite allergen induces asthma via Toll-like receptor 4 triggering of airway structural cells. Nat. Med. *15*, 410–416.

Harada, M., Magara-Koyanagi, K., Watarai, H., Nagata, Y., Ishii, Y., Kojo, S., Horiguchi, S., Okamoto, Y., Nakayama, T., Suzuki, N., et al. (2006). IL-21-induced Bepsilon cell apoptosis mediated by natural killer T cells suppresses IgE responses. J. Exp. Med. *203*, 2929–2937.

Huang, Y., Guo, L., Qiu, J., Chen, X., Hu-Li, J., Siebenlist, U., Williamson, P.R., Urban, J.F., Jr., and Paul, W.E. (2015). IL-25-responsive, lineage-negative KLRG1(hi) cells are multipotential 'inflammatory' type 2 innate lymphoid cells. Nat. Immunol. *16*, 161–169.

Johansson-Lindbom, B., Ingvarsson, S., and Borrebaeck, C.A. (2003). Germinal centers regulate human Th2 development. J. Immunol. *171*, 1657–1666.

Kang, Z., Swaidani, S., Yin, W., Wang, C., Barlow, J.L., Gulen, M.F., Bulek, K., Do, J.S., Aronica, M., McKenzie, A.N., et al. (2012). Epithelial cell-specific Act1 adaptor mediates interleukin-25-dependent helminth expulsion through expansion of Lin(-)c-Kit(+) innate cell population. Immunity *36*, 821–833.

Kaplan, M.H., Schindler, U., Smiley, S.T., and Grusby, M.J. (1996). Stat6 is required for mediating responses to IL-4 and for development of Th2 cells. Immunity *4*, 313–319.

King, I.L., and Mohrs, M. (2009). IL-4-producing CD4+ T cells in reactive lymph nodes during helminth infection are T follicular helper cells. J. Exp. Med. *206*, 1001–1007.

King, I.L., Mohrs, K., and Mohrs, M. (2010). A nonredundant role for IL-21 receptor signaling in plasma cell differentiation and protective type 2 immunity against gastrointestinal helminth infection. J. Immunol. *185*, 6138–6145.

Kudo, M., Melton, A.C., Chen, C., Engler, M.B., Huang, K.E., Ren, X., Wang, Y., Bernstein, X., Li, J.T., Atabai, K., et al. (2012). IL-17A produced by αβ T cells drives airway hyper-responsiveness in mice and enhances mouse and human airway smooth muscle contraction. Nat. Med. *18*, 547–554.

Lambrecht, B.N., and Hammad, H. (2015). The immunology of asthma. Nat. Immunol. *16*, 45–56.

León, B., Ballesteros-Tato, A., Browning, J.L., Dunn, R., Randall, T.D., and Lund, F.E. (2012). Regulation of T(H)2 development by CXCR5+ dendritic cells and lymphotoxin-expressing B cells. Nat. Immunol. *13*, 681–690.

Liang, H.E., Reinhardt, R.L., Bando, J.K., Sullivan, B.M., Ho, I.C., and Locksley, R.M. (2012). Divergent expression patterns of IL-4 and IL-13 define unique functions in allergic immunity. Nat. Immunol. *13*, 58–66.

Linterman, M.A., Beaton, L., Yu, D., Ramiscal, R.R., Srivastava, M., Hogan, J.J., Verma, N.K., Smyth, M.J., Rigby, R.J., and Vinuesa, C.G. (2010). IL-21 acts directly on B cells to regulate Bcl-6 expression and germinal center responses. J. Exp. Med. *207*, 353–363.

Liu, B., Lee, J.B., Chen, C.Y., Hershey, G.K., and Wang, Y.H. (2015). Collaborative interactions between type 2 innate lymphoid cells and antigen-specific CD4+ Th2 cells exacerbate murine allergic airway diseases with prominent eosinophilia. J. Immunol. *194*, 3583–3593.

Löhning, M., Stroehmann, A., Coyle, A.J., Grogan, J.L., Lin, S., Gutierrez-Ramos, J.C., Levinson, D., Radbruch, A., and Kamradt, T. (1998). T1/ST2 is preferentially expressed on murine Th2 cells, independent of interleukin 4, interleukin 5, and interleukin 10, and important for Th2 effector function. Proc. Natl. Acad. Sci. USA *95*, 6930–6935.

Lüthje, K., Kallies, A., Shimohakamada, Y., Belz, G.T., Light, A., Tarlinton, D.M., and Nutt, S.L. (2012). The development and fate of follicular helper T cells defined by an IL-21 reporter mouse. Nat. Immunol. *13*, 491–498.

Meisel, C., Bonhagen, K., Löhning, M., Coyle, A.J., Gutierrez-Ramos, J.C., Radbruch, A., and Kamradt, T. (2001). Regulation and function of T1/ST2 expression on CD4+ T cells: induction of type 2 cytokine production by T1/ST2 cross-linking. J. Immunol. *166*, 3143–3150.

Neill, D.R., Wong, S.H., Bellosi, A., Flynn, R.J., Daly, M., Langford, T.K., Bucks, C., Kane, C.M., Fallon, P.G., Pannell, R., et al. (2010). Nuocytes represent a new innate effector leukocyte that mediates type-2 immunity. Nature *464*, 1367–1370.

Ozaki, K., Spolski, R., Feng, C.G., Qi, C.F., Cheng, J., Sher, A., Morse, H.C., 3rd, Liu, C., Schwartzberg, P.L., and Leonard, W.J. (2002). A critical role for IL-21 in regulating immunoglobulin production. Science *298*, 1630–1634.

Parrish-Novak, J., Dillon, S.R., Nelson, A., Hammond, A., Sprecher, C., Gross, J.A., Johnston, J., Madden, K., Xu, W., West, J., et al. (2000). Interleukin 21 and its receptor are involved in NK cell expansion and regulation of lymphocyte function. Nature *408*, 57–63.

Paul, W.E., and Zhu, J. (2010). How are T(H)2-type immune responses initiated and amplified? Nat. Rev. Immunol. *10*, 225–235.

Pesce, J., Kaviratne, M., Ramalingam, T.R., Thompson, R.W., Urban, J.F., Jr., Cheever, A.W., Young, D.A., Collins, M., Grusby, M.J., and Wynn, T.A. (2006). The IL-21 receptor augments Th2 effector function and alternative macrophage activation. J. Clin. Invest. *116*, 2044–2055.

Plantinga, M., Guilliams, M., Vanheerswynghels, M., Deswarte, K., Branco-Madeira, F., Toussaint, W., Vanhoutte, L., Neyt, K., Killeen, N., Malissen, B., et al. (2013). Conventional and monocyte-derived CD11b(+) dendritic cells initiate and maintain T helper 2 cell-mediated immunity to house dust mite allergen. Immunity *38*, 322–335.

Reinhardt, R.L., Liang, H.E., and Locksley, R.M. (2009). Cytokine-secreting follicular T cells shape the antibody repertoire. Nat. Immunol. *10*, 385–393.

Roediger, B., Kyle, R., Yip, K.H., Sumaria, N., Guy, T.V., Kim, B.S., Mitchell, A.J., Tay, S.S., Jain, R., Forbes-Blom, E., et al. (2013). Cutaneous immunosurveillance and regulation of inflammation by group 2 innate lymphoid cells. Nat. Immunol. *14*, 564–573.

Ruedl, C., Bachmann, M.F., and Kopf, M. (2000). The antigen dose determines T helper subset development by regulation of CD40 ligand. Eur. J. Immunol. *30*, 2056–2064.

Schaerli, P., Willimann, K., Lang, A.B., Lipp, M., Loetscher, P., and Moser, B. (2000). CXC chemokine receptor 5 expression defines follicular homing T cells with B cell helper function. J. Exp. Med. *192*, 1553–1562.

Schmitz, J., Owyang, A., Oldham, E., Song, Y., Murphy, E., McClanahan, T.K., Zurawski, G., Moshrefi, M., Qin, J., Li, X., et al. (2005). IL-33, an interleukin-1-

like cytokine that signals via the IL-1 receptor-related protein ST2 and induces T helper type 2-associated cytokines. Immunity *23*, 479–490.

Schnyder-Candrian, S., Togbe, D., Couillin, I., Mercier, I., Brombacher, F., Quesniaux, V., Fossiez, F., Ryffel, B., and Schnyder, B. (2006). Interleukin-17 is a negative regulator of established allergic asthma. J. Exp. Med. *203*, 2715–2725.

Shulman, Z., Gitlin, A.D., Weinstein, J.S., Lainez, B., Esplugues, E., Flavell, R.A., Craft, J.E., and Nussenzweig, M.C. (2014). Dynamic signaling by T follicular helper cells during germinal center B cell selection. Science *345*, 1058–1062.

Spolski, R., and Leonard, W.J. (2008). Interleukin-21: basic biology and implications for cancer and autoimmunity. Annu. Rev. Immunol. *26*, 57–79.

Stritesky, G.L., Muthukrishnan, R., Sehra, S., Goswami, R., Pham, D., Travers, J., Nguyen, E.T., Levy, D.E., and Kaplan, M.H. (2011). The transcription factor STAT3 is required for T helper 2 cell development. Immunity *34*, 39–49.

Suto, A., Kashiwakuma, D., Kagami, S., Hirose, K., Watanabe, N., Yokote, K., Saito, Y., Nakayama, T., Grusby, M.J., Iwamoto, I., and Nakajima, H. (2008). Development and characterization of IL-21-producing CD4+ T cells. J. Exp. Med. *205*, 1369–1379.

Tubo, N.J., Pagán, A.J., Taylor, J.J., Nelson, R.W., Linehan, J.L., Ertelt, J.M., Huseby, E.S., Way, S.S., and Jenkins, M.K. (2013). Single naive CD4+ T cells from a diverse repertoire produce different effector cell types during infection. Cell *153*, 785–796.

Van Dyken, S.J., Mohapatra, A., Nussbaum, J.C., Molofsky, A.B., Thornton, E.E., Ziegler, S.F., McKenzie, A.N., Krummel, M.F., Liang, H.E., and Locksley, R.M. (2014). Chitin activates parallel immune modules that direct distinct inflammatory responses via innate lymphoid type 2 and $\gamma\delta$ T cells. Immunity *40*, 414–424.

van Panhuys, N., Klauschen, F., and Germain, R.N. (2014). T-cell-receptor-dependent signal intensity dominantly controls CD4(+) T cell polarization In Vivo. Immunity *41*, 63–74.

Vogelzang, A., McGuire, H.M., Yu, D., Sprent, J., Mackay, C.R., and King, C. (2008). A fundamental role for interleukin-21 in the generation of T follicular helper cells. Immunity *29*, 127–137.

Willart, M.A., Deswarte, K., Pouliot, P., Braun, H., Beyaert, R., Lambrecht, B.N., and Hammad, H. (2012). Interleukin-1α controls allergic sensitization to inhaled house dust mite via the epithelial release of GM-CSF and IL-33. J. Exp. Med. *209*, 1505–1517.

Xu, H., Li, X., Liu, D., Li, J., Zhang, X., Chen, X., Hou, S., Peng, L., Xu, C., Liu, W., et al. (2013). Follicular T-helper cell recruitment governed by bystander B cells and ICOS-driven motility. Nature *496*, 523–527.

Zheng, W., and Flavell, R.A. (1997). The transcription factor GATA-3 is necessary and sufficient for Th2 cytokine gene expression in CD4 T cells. Cell *89*, 587–596.

Zotos, D., Coquet, J.M., Zhang, Y., Light, A., D'Costa, K., Kallies, A., Corcoran, L.M., Godfrey, D.I., Toellner, K.M., Smyth, M.J., et al. (2010). IL-21 regulates germinal center B cell differentiation and proliferation through a B cell-intrinsic mechanism. J. Exp. Med. *207*, 365–378.

Immunity

Supplemental Information

Interleukin-21-Producing $CD4^+$ T Cells Promote Type 2 Immunity to House Dust Mites

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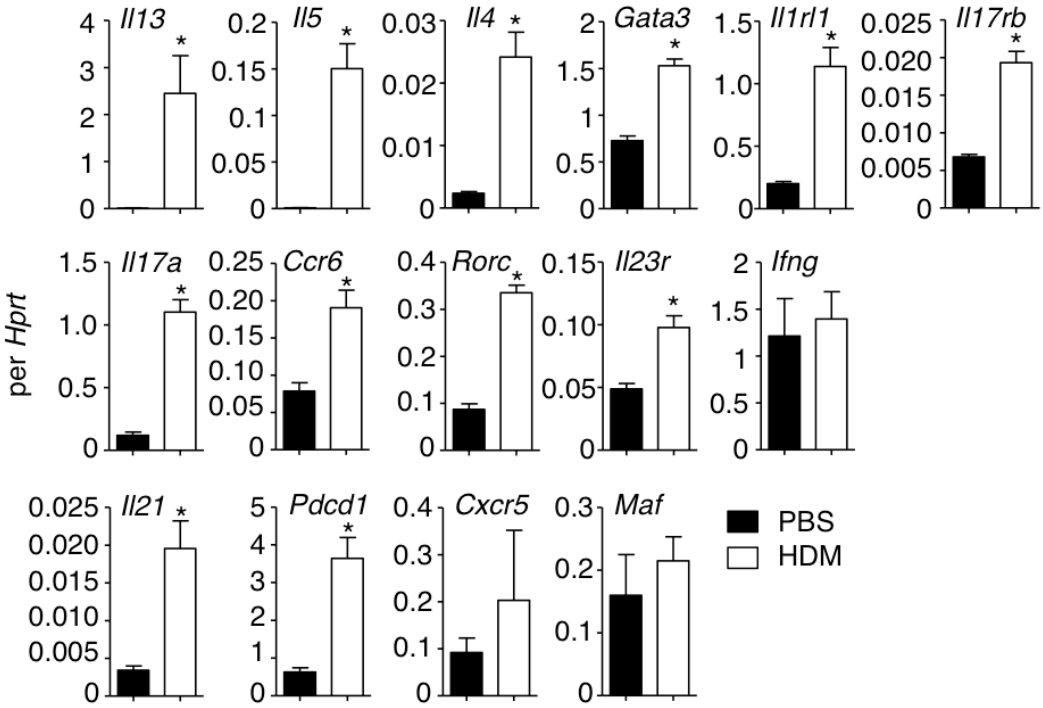

Figure S1. Quantitative PCR analysis of lung-infiltrating CD4$^+$ T cells (Related to Figure 1). In separate experiments, CD4$^+$ T cells from the lungs of PBS or HDM sensitized and challenged mice were analyzed. Cells were purified as described in Figure 1 and mRNA was quantified for each gene by real time PCR. Results depict 4 mice per group and were analyzed by Mann-Whitney U test. *$p<0.05$

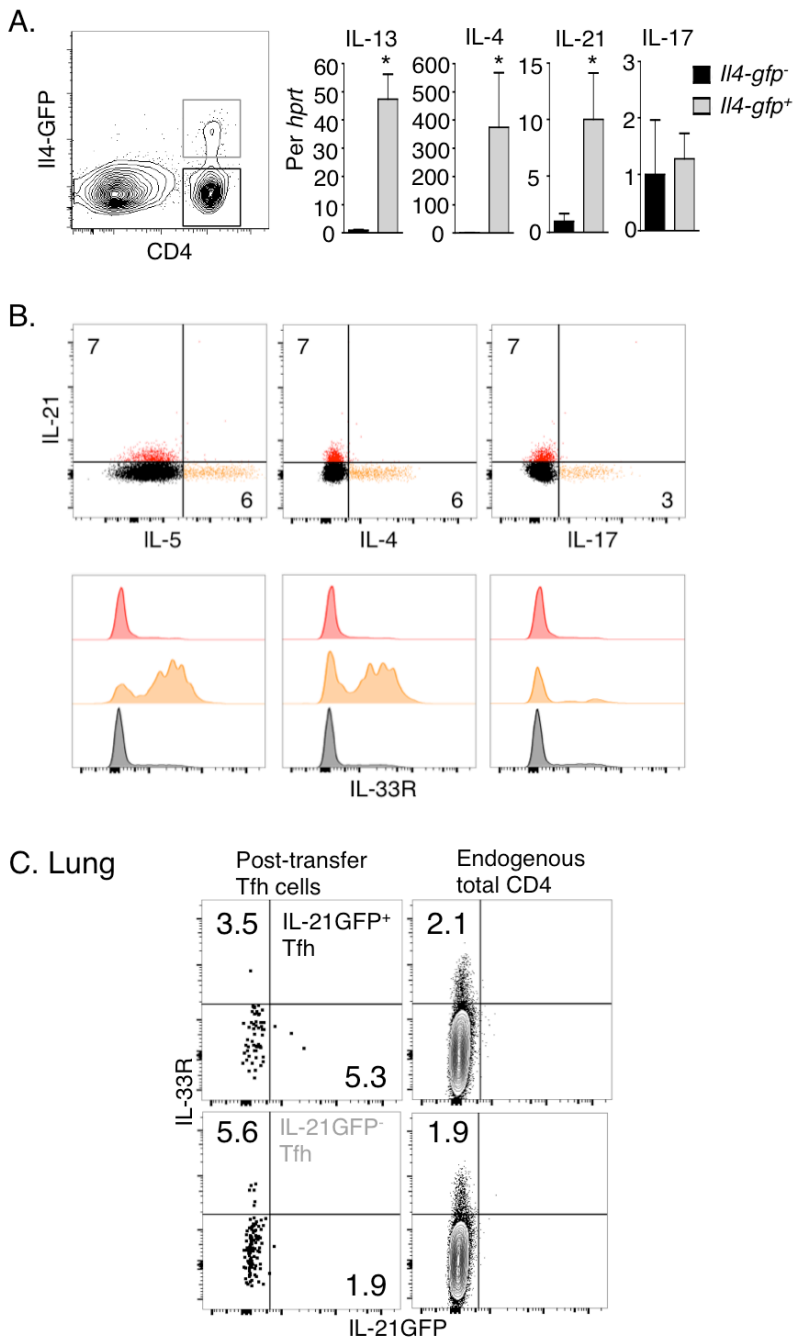

Figure S2. Distinguishing IL-21 expression from other effector cytokines (Related to Figure 3). A. IL-4GFP mice were sensitized and challenged with HDM as depicted in Figure 1 and at day 15 after sensitization, $CD4^{+}GFP^{-}$ and $CD4^{+}GFP^{+}$ cells were purified from the mLN. The contour plot depicts CD4 versus IL-4GFP expression in HDM sensitized and challenged mice. Bar graphs depict the expression of IL-13, IL-4, IL-21 and IL-17 mRNA in the two populations. Results are from 4 mice pooled from 2 experiments. **B.** Bronchoalveolar lavage cells from WT HDM-sensitized and –challenged mice were stimulated with PMA and

ionomycin in the presence of golgi transport blocking. Cells were surface stained for CD4 and IL-33R and subsequently for intracellular IL-4, IL-5, IL-17 and IL-21, which was detected by staining with the hIL-21R.Fc followed by a secondary goat anti-human PE antibody detecting the common Fc portion. The expression of IL-33R on the different color-coded populations is shown in histograms. Data is representative of 3 individual mice. **C.** Adoptive transfer of IL-21GFP^{+} and IL-21GFP^{-} Tfh cells was performed as described in Figure 3. Dot and contour plots show the expression of IL-21GFP and IL-33R on transferred cells (CD45.2^{+}CD4^{+}) and endogenous (CD45.2^{-}CD4^{+}) cells from the lung at day 8. Results were analyzed by Mann-Whitney U test. *$p<0.01$

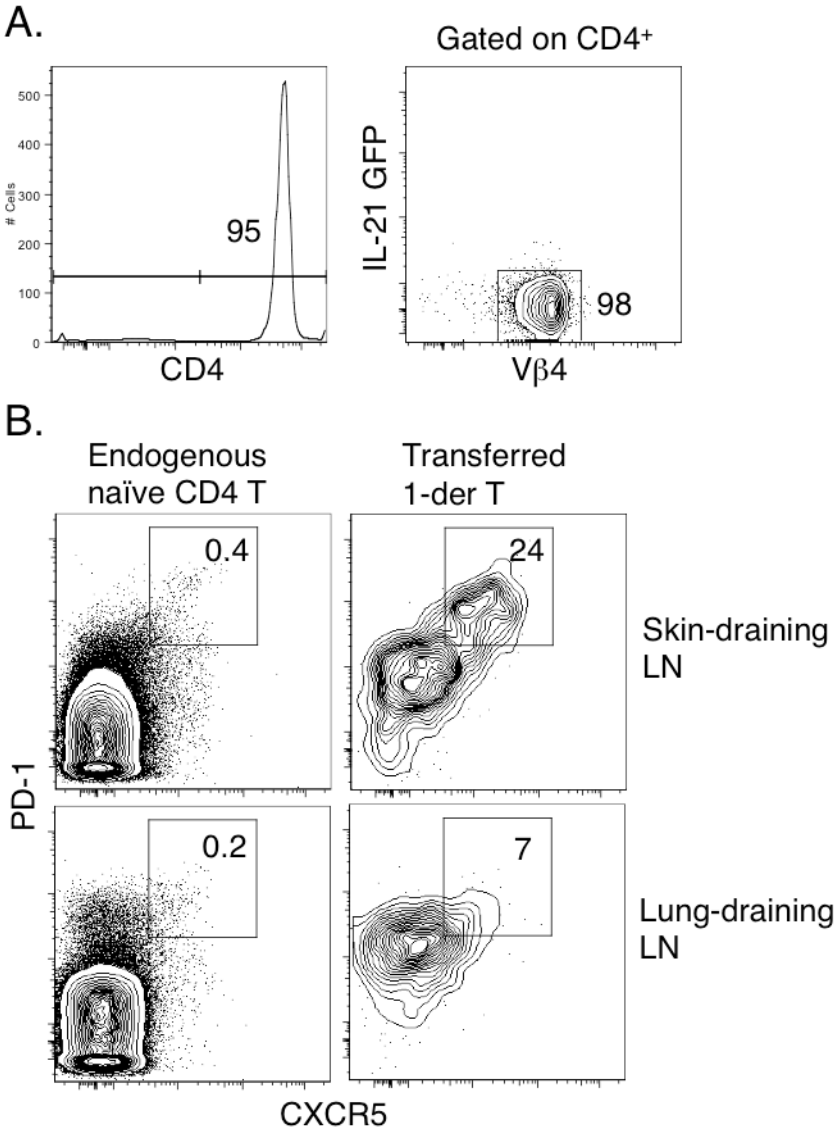

Figure S3. CD4$^+$ T cells from naïve *Il21*$^{+/gfp}$ 1-DER mice express very little IL-21GFP and differentiate effectively into Tfh after just one administration of HDM (Related to Figure 4). A. CD4$^+$ T cells were enriched and analyzed for CD4 and Vβ4 expression. These cells were used in adoptive transfer experiments described in Figure 4. **B.** 1-DER CD4$^+$ T cells (CD45.2$^+$) were transferred into CD45.1 congenic mice. Mice were then either sensitized with 100 μg HDM on the right flank or 10 μg HDM intranasal. Five days later, the skin-draining inguinal LN of mice sensitized via the skin (top row) or lung-draining mLN of mice sensitized via the lung (bottom row) was analyzed by flow cytometry. Plots depict the expression of CXCR5 versus PD-1 on gated endogenous naïve CD4$^+$ T (left-hand column) or gated 1-der T (right-hand column) cells. Plots are representative of 3 mice each receiving HDM either via the skin or lung.

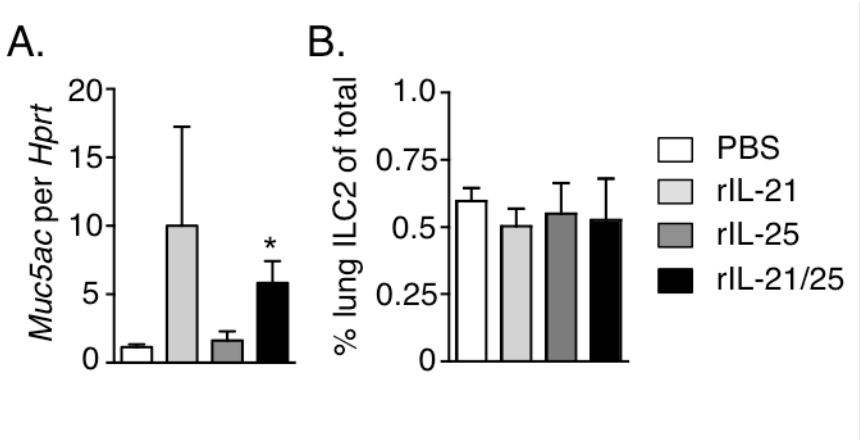

Figure S4. rIL-21 and rIL-25 induce *Muc5AC* expression but do not influence the frequency of ILC2 in the lung (Related to Figure 7). Mice were treated with recombinant cytokines on 3 consecutive days. **A.** *Muc5ac* mRNA expression was analyzed in whole lung tissue of cytokine and PBS treated mice. *p<0.01 **B.** The percentage of IL-33R^{+}CD127^{+}CD45^{+}CD90.2^{+}CD4^{-}lineage^{-} ILC2 among total cells in the lung is depicted. Results were pooled from 2 experiments with 6 mice per group total.

Supplemental Experimental Procedures

Reagents, antibodies and flow cytometry

HDM extracts were obtained from Greer Laboratories. rIL-25 was purchased from Biolegend (Cat # 579804) and rIL-21 was provided by Zymogenetics. Flurochrome-labelled and biotinylated antibodies from BD (CD3, CD8, SiglecF, Ly6C/G, CD127, CXCR5, IL-5), E Bioscience (CD4, CD11c, CD19, MHC-II, CD45, CD45.1, CD45.2, CD90.2, PD-1, IL-13, CD45), MBD Bioproducts (T1/ST2) and Biolegend (NK1.1) were used. Lineage$^-$ cells when gating for ILC were identified as CD3$^-$CD4$^-$CD11c$^-$CD19$^-$CD11b$^-$NK1.1$^-$. IL-13 and IL-5 ELISA kits were purchased from E Biosciences and IgG1, IgG2a, IgA and IgE kits were from BD. rhIL-21R.Fc (R&D Systems) and goat anti-human IgG Fc fragment PE (Jackson) were used for intracellular staining of IL-21. Recombinant mouse soluble ST2 (sST2) was PCR amplified from OriGene cDNA clone MC204735 (using forward and reverse primers: AGTTCTAGAATGATTGACAGACAGAGAATGG; TAATCTAGAAGCAATGTGTGAGGGACACTC) and ligated in the XbaI site of pEF6/myc-His A (Invitrogen), resulting in a mammalian expression vector (pEF-mST2s) for mouse sST2 with a C-terminal myc/His6 tag. pEF-mST2s was transiently transfected in HEK293T cells and secreted recombinant sST2 was purified in endotoxin free conditions from the supernatant using standard chromatographic methods (metal affinity chromatography using nickel-Sepharose and gelfiltration on a Superdex 200 column). Purified sST2 was stored in PBS at -80˚C. Protein concentration was determined by Micro BCA protein assay and purity of sST2 was >95% as assayed by SDS-PAGE and western blotting using anti-His antibody (Roche). Purification upscaling was done at VIB Protein Service Facility. Neutralizing activity of sST2 towards mouse IL-33 was tested in a biological assay measuring IL-33 induced NF-κB reporter activation in ST2:L1RAcP transfected HEK293T cells. sST2 and anti-IL-5 (TRFK5) were administered intra peritoneal on days 0 and 1 of recombinant cytokine administration.

Stained samples were analyzed BD LSRII or LSRFortessa and subsequently using FlowJo (TreeStar) software. Sorting was performed on a BD FACSAria III and sort purities were always in excess of 95%.

Adoptive transfer experiments

For adoptive transfer of IL-21GFP$^+$ and IL-21GFP$^-$ PD-1$^+$ Tfh cells, *Il21*$^{gfp/+}$ mice were sensitized and challenged with HDM. At day 15, mLN and spleen was harvested and pooled, and single cell suspensions were enriched for CD4$^+$ T cells with the CD4$^+$ T Cell Isolation Kit (Miltenyi). IL-21GFP$^+$ and IL-21GFP$^-$ PD-1$^+$ cells from the CD4-enriched fraction were purified by flow cytometry and transferred into recipient mice. For adoptive transfer of 1-DER CD4$^+$ T cells, spleens of naïve *Il21*$^{gfp/+}$ 1-DER mice were enriched using the CD4$^+$ T Cell Isolation Kit (Miltenyi). Enriched cells were injected into CD45.1 congenic recipients. Following transfer, mice were inoculated with HDM as described.

Quantitative PCR and array

RNA was obtained using the TriPure isolation reagent (Roche) and RNA was isolated according to manufacturers instructions. RNA was reverse transcribed with a Transcriptor High Fidelity cDNA Synthesis Kit (Roche) and samples were analyzed by SYBR green-based RT-PCR with a LightCycler 480 system (Roche). PCR primers for HPRT, IL-21, IL-13, IL-4 and IL-17 were as follows: HPRT 5' TGAAGAGCTACTGTAATGATCAGTCAAC, HPRT 3' AGCAAGCTTGCAACCTTAACCA, IL-21 5' AAGATTCCTGAGGATCCGAGAAG, IL-21 3' TGCATTCGTGAGCGTCTATAGTG, IL-13 5' TGACCAACATCTCCAATTGCA, IL-13 3' TTGTTATAAAGTGGGCTACTTCGATTT, IL-4 5' CTCATGGAGCTGCAGAGACTCTT, IL-4 3' CATTCATGGTGCAGCTTATCGA, IL-17 5' TGAAGGCAGCAGCGATCA, IL-17 3' GGAAGTCCTTGGCCTCAGTGT.

CD4$^+$ T cells from the lung were purified by flow cytometry by gating on CD4$^+$CD45$^+$F4/80$^-$ cells in 3 independent experiments from naïve PBS-treated or asthmatic HDM-treated mice. RNA from CD4$^+$ T cells was assessed with a Bioanalyser 2100 (Agilent). With a WT Expression Kit (Ambion), 50 ng of total RNA per sample, 'spiked' with bacterial poly(A) RNA positive control (Affymetrix) was converted to double-stranded cDNA in a reverse-transcription reaction. Samples were fragmented and labeled with biotin in a terminal labeling reaction according to the Affymetrix WT Terminal Labeling Kit. A mixture of fragmented biotinylated cDNA and hybridization controls (Affymetrix) was hybridized on a GeneChip

Mouse Gene 2.0 ST Array (Affymetrix), followed by staining and washing in a GeneChip fluidics station 450 according to the manufacturer's procedures (Affymetrix). For analysis of raw probe signal intensities, chips were scanned with a GeneChip scanner 3000 (Affymetrix). Samples were subsequently analyzed and clustered in ‘R’ software. Graphs of normalized data from each experiment were made for T helper cell specific genes.