Basic

European Journal of Immunology

Allergy and inflammation

Research Article

T cells and ILC2s are major effector cells in influenza-induced exacerbation of allergic airway inflammation in mice

Bobby W. S. Li[1], Marjolein J. W. de Bruijn[1], Melanie Lukkes[1], Menno van Nimwegen[1], Ingrid M. Bergen[1], Alex KleinJan[1], Corine H. GeurtsvanKessel[2], Arno Andeweg[2], Guus F. Rimmelzwaan[2] and Rudi W. Hendriks[1]

[1] Department of Pulmonary Medicine, Erasmus MC Rotterdam, Rotterdam, the Netherlands
[2] Department of Viroscience, Erasmus MC Rotterdam, Rotterdam, the Netherlands

Influenza virus infection is an important cause of severe asthma exacerbations, but it remains unclear how a Th1-mediated antiviral response triggers a prototypical Th2 disease. We investigated CD4$^+$ T cells and group 2 innate lymphoid cells (ILC2s) in influenza virus-infected mice. We found that ILC2s accumulated in the lung rapidly after influenza virus infection, but the induction of IL-5 and IL-13 secretion was delayed and concomitant with T cell activation. In an influenza-induced exacerbation of allergic airway inflammation model we noticed an initial reduction of ILC2 numbers and cytokine production in broncho-alveolar lavage compared to chronic house dust mite (HDM)-mediated airway inflammation alone. ILC2s phenotype was characterized by low T1/ST2, ICOS, KLRG1, and CD25 expression, resembling naïve ILC2s. The contribution of ILC2s to type 2 cytokine production in the early stage of the influenza-induced exacerbation was limited. In contrast, T cells showed increased IL-4 and IL-5 production when exposed to both HDM and influenza virus. Upon virus clearance, ILC2s regained an activated T1/ST2^{high}ICOS^{high}KLRG1^{high}CD25^{high} phenotype paired with cytokine production and were major contributors to the type 2 cytokine milieu. Collectively, our data indicate that both T cells and ILC2s contribute to influenza-induced exacerbation of allergic airway inflammation, but with different kinetics.

Keywords: Allergy · Allergic airway inflammation · Exacerbation · ILC2 · Influenza virus

Additional supporting information may be found online in the Supporting Information section at the end of the article.

Introduction

Asthma exacerbations are often provoked by respiratory viruses, particularly rhinovirus and RSV [1, 2]. While influenza virus infections occur less often, it is able to cause the most severe forms of asthma exacerbations [3, 4]. Adult asthma patients have a higher incidence of influenza virus infection than children, suggesting that adults are more susceptible [5]. Influenza virus causes severe epithelial cell damage [6] and induces NF-κB-mediated release of cytokines from these cells, such as IL-1β, IL-6, and IL-8 [7, 8]. This compromises the epithelial barrier function and additionally increases vascular endothelial permeability [9], resulting in

Correspondence: Dr. Rudi W. Hendriks
e-mail: r.hendriks@erasmusmc.nl

© 2018 The Authors. *European Journal of Immunology* published by WILEY-VCH Verlag GmbH & Co. KGaA, Weinheim.
This is an open access article under the terms of the Creative Commons Attribution License, which permits use, distribution and reproduction in any medium, provided the original work is properly cited.

a highly robust inflammatory response. In conjunction with the pre-existing inflammatory environment in asthma patients, loss of barrier function provides a partial explanation for the clinical observations that indicate an association between influenza virus infection and asthma exacerbation. However, the precise underlying pathways remain elusive.

Allergic airway inflammation is centrally mediated by Th2 cells that secrete the key effector cytokines IL-4, IL-5, and IL-13 resulting in characteristic asthma pathology that includes persistent eosinophilic inflammation, airway hyperresponsiveness and remodeling, smooth muscle cell hyperplasia, goblet cell metaplasia, and increased angiogenesis [10, 11]. Group 2 innate lymphoid cells (ILC2) also secrete IL-5 and IL-13 when stimulated by epithelium-derived IL-25, IL-33, and thymic stromal lymphopoietin (TSLP) as well as IL-4 under particular conditions [12]. Several studies have stressed the potency of ILC2s as a crucial early T cell-independent source of type 2 cytokines in papain- or *Alternaria*-induced allergic airway inflammation [13–16]. However, we and others have demonstrated that the contribution of T cells is critical in other allergen-based models of asthma, such as house dust mite (HDM), OVA, and *Aspergillus* [17–19]. Importantly, the groups of Chang et al. and Monticelli et al. have found increased ILC2 numbers in the lungs of influenza virus-infected mice. Hereby, ILC2s can contribute to airway hyperreactivity, but can also be protective because of their capacity to produce amphiregulin, a member of the epidermal growth factor family that restores epithelial cell integrity [20, 21].

These findings—together with evidence that during influenza virus infection NKT cells and alveolar macrophages can be important endogenous sources of IL-33 that enhance IL-5 production from ILC2s [22]—highlight a possible role for ILC2s as the causative agent between influenza virus infection and exacerbation of allergic airway inflammation. Moreover, they open up new avenues of research into treatment of severe asthma exacerbations, as the current standard treatment with high doses of inhaled or systemic corticosteroids is not always effective and has a high risk of side effects [23, 24].

When the effect of influenza virus infection on an acute model of HDM-driven allergic airway inflammation was studied in mice, simultaneous activation of eosinophils and neutrophils and a synergistic effect of influenza and HDM on airway resistance and cell influx into the BALF was found [25]. In addition, a marked innate response followed by an accumulation of eosinophils, neutrophils, dendritic cells, and T cells was reported in an X31 H3N2 influenza virus infection model preceded by chronic exposure to HDM [26]. In these experiments, anti-IL-5 treatment prevented eosinophil influx and influenza-induced exacerbation of airway inflammation. However, the involvement of ILC2s in the influenza-mediated exacerbations remains unknown.

Here, we studied the kinetics and function of ILC2s in influenza virus infection and their contribution to influenza-mediated exacerbation in a robust, physiologically relevant, chronic model of HDM-induced allergic airway inflammation. We found that although ILC2s accumulated earlier in the lungs than T cells after influenza virus infection, their cytokine production was delayed and only initiated after the peak of T cell activation. The contribution of ILC2s to the total type 2 cytokine milieu in influenza-mediated exacerbation was modest in the early stages of infection but increased to significant levels at day 11 post infection when the virus was cleared. Because in these experiments also Th2 cytokine production by $CD4^+$ T cells was increased, we conclude that both T cells and ILC2s play a role in influenza-induced exacerbation of allergic airway inflammation, but with different kinetics.

Results

Influenza virus infection induces major changes in expressed genes in the lungs

We aimed to identify the activation patterns of T cells and ILC2s in mice infected with the X31 H3N2 strain of influenza virus. Gene expression profiling of total lung cells over time revealed distinct patterns for genes associated with innate and adaptive immunity (Fig. 1). Compared to PBS-treated animals, several pro-inflammatory mediators exhibited a response that peaked at day 2–4 post infection, indicating initiation of viral immunity. Notably, IL-1β and IL-6 were increased ~four- and ~12-fold, respectively. Chemokines such as CCL11 and CXCL1 were also upregulated, which attract eosinophils and neutrophils, respectively. Likewise, factors secreted by innate immune cells, including IFN-β and TNF-α were elevated early after infection (Fig. 1A). As expected, upregulation of genes that signify an adaptive immune response, such as IgM and T cell-associated markers (CD4, CD8α, CD28, and CTLA-4), occurred later in the infection at day 8 (Fig. 1A and B). Hereby, genes associated with Th1 immunity, IFN-γ, T-bet, and IL-27Rα also showed maximal induction at day 8.

Chang et al. have demonstrated rapid increase of IL-33, a potent activator of ILC2s, in the lungs after influenza virus infection and have shown alveolar macrophages as a potential source [20]. Our gene expression analysis confirmed this and we also found an upregulation of IL-33 at day 4 after inoculation. However, other genes implicated in ILC2 activation including IL-25, TSLP, IL-2, and IL-7, did not follow this pattern. Amphiregulin and arginase-1, cytokines known to be produced by ILC2s [21, 27], arose concomitantly with the increase in IL-33 expression. Several signature ILC2 genes, including IL-5 and IL-13 cytokines, and the transcription factors GATA3 and RORα remained remarkably unaltered (Fig. 1C). Although these findings may suggest that expression changes of these genes are difficult to detect in total lung, alternatively IL-33-driven Th2 cytokine production may be suppressed during influenza infection.

Taken together, this expression analysis shows that influenza virus infection induces major changes in gene expression within the lungs, reflecting induction of innate and adaptive immune responses. Changes in ILC2-associated genes were not readily detected, but several cytokines that were reported to suppress ILC2s, including type I IFNs and IFN-γ [28–32], were clearly induced.

© 2018 The Authors. *European Journal of Immunology* published by WILEY-VCH Verlag GmbH & Co. KGaA, Weinheim.

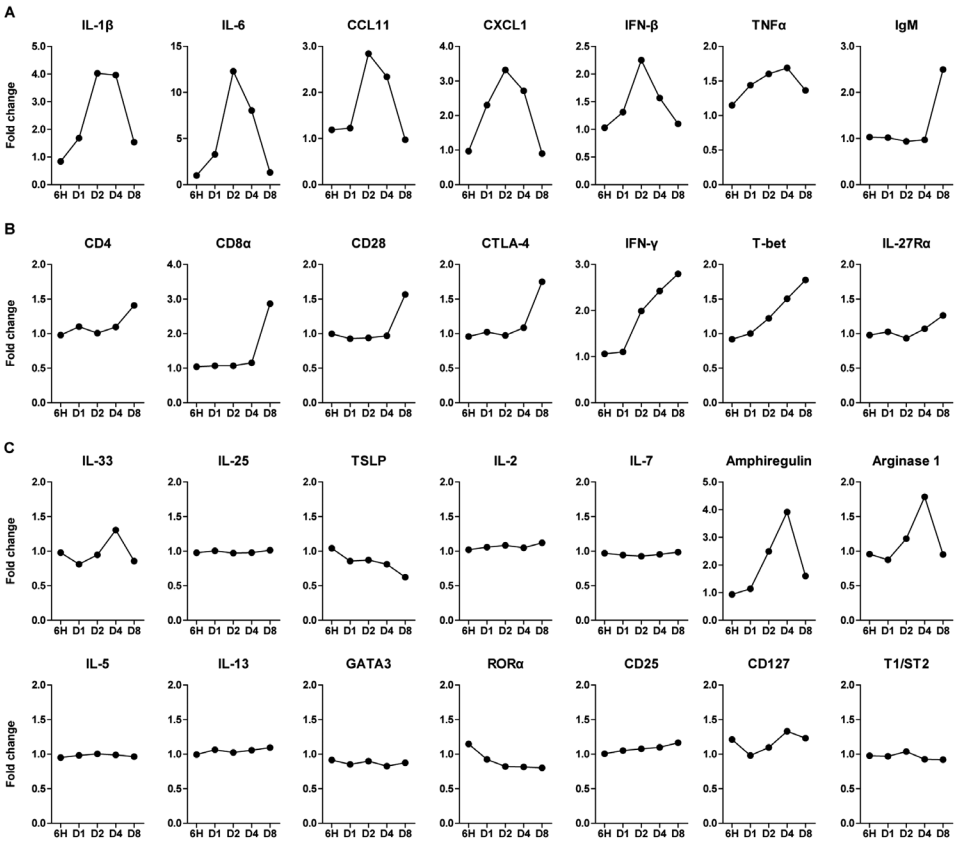

Figure 1. Expression profiles of genes associated with ILC2s and T cells show distinct temporal patterns in influenza virus infection. Quantification of the expression levels of (A) inflammatory mediators, (B) T cell-related genes, and (C) ILC2-related genes at 6 h, 1 day, 2, 4, and 8 days after X31 influenza virus inoculation, expressed as fold change compared to PBS-treated mice ($n = 5$) from a single experiment. Expression was determined by microarray analysis.

In influenza virus infection T cells and ILC2s display distinct activation kinetics

To study the various innate and adaptive immune cell populations during influenza virus infection in detail, we infected mice with 10^4 PFU viral particles and followed the composition of the immune response over a period of 35 days. Mice rapidly lost weight in the first 4 days, after which their weight stabilized and gradually recovered from day 7 onward (Fig. 2A). Virus titers significantly decreased by day 7 and were almost undetectable by day 10 (Fig. 2A). ILC2s were characterized by flow cytometry as Lineage$^-$ lymphocytes that expressed intracellular GATA3 and Sca-1, as described previously (Fig. 2B) [17]. Although expression of the ILC2-associated markers IL-7R (CD127), ICOS, KLRG1, and IL-33R (T1/ST2) was heterogeneous [33], the majority of these cells were positive for these surface markers (Fig. 2B). In agreement with the gene expression data in Fig. 1, increased numbers of CD4$^+$ and CD8$^+$ T cells were present in the lungs at day 7, after which a slow decline to baseline was reached at day 17. In contrast, ILC2 accumulation was already evident at day 4 post infection and remained significantly elevated in the lungs at day 7 and day 10, and returned to baseline values by day 17 (Fig. 2C). Subsequently, we zoomed in on the initiation phase of the response to influenza virus infection and found that whereas the apex of ILC2 influx into the lungs occurred at day 3, T cell numbers remained unchanged. We observed a slight, but significant reduction in B cell numbers in the lung (Fig. 2D). By employing a *Gata3* reporter mouse, which exhibits concomitant expression of GATA3 and yellow fluorescent protein (YFP) [33] [T.N.R and H.J.F., manuscript in preparation], we were able to visualize the location of ILC2s in the lung during influenza virus infection. ILC2s appeared underneath the airway epithelium, often in close proximity to CD3$^+$ T cells (Fig. 2E), also in severe cases where a strong influx of B220$^+$ cells (plasmacytoid dendritic cells or B cells) was present (Supporting Information Fig. 1).

Taken together these data indicate that upon influenza virus infection ILC2s in the lung increase more rapidly in number than CD4$^+$ or CD8$^+$ T cells.

ILC2 cytokine response to influenza virus follows accumulation in BALF and CD25 upregulation

Given that Th1-associated cytokines may drive plasticity of ILC2s [34–37], we examined the expression of cytokines as well as a number of cell surface markers of ILC2s over time in influenza virus-infected mice.

While the numbers of IL-5$^+$ and IL-13$^+$ T cells present in BALF significantly increased at day 7 and 10, only a small proportion of T cells produced these Th2 cytokines (Fig. 3A and B). The numbers of ILC2s in BALF increased as early as day 4 after infection and reached their highest values at day 7, however, type 2 cytokine production by this wave of ILC2s was remarkably limited. Rather,

© 2018 The Authors. *European Journal of Immunology* published by WILEY-VCH Verlag GmbH & Co. KGaA, Weinheim.

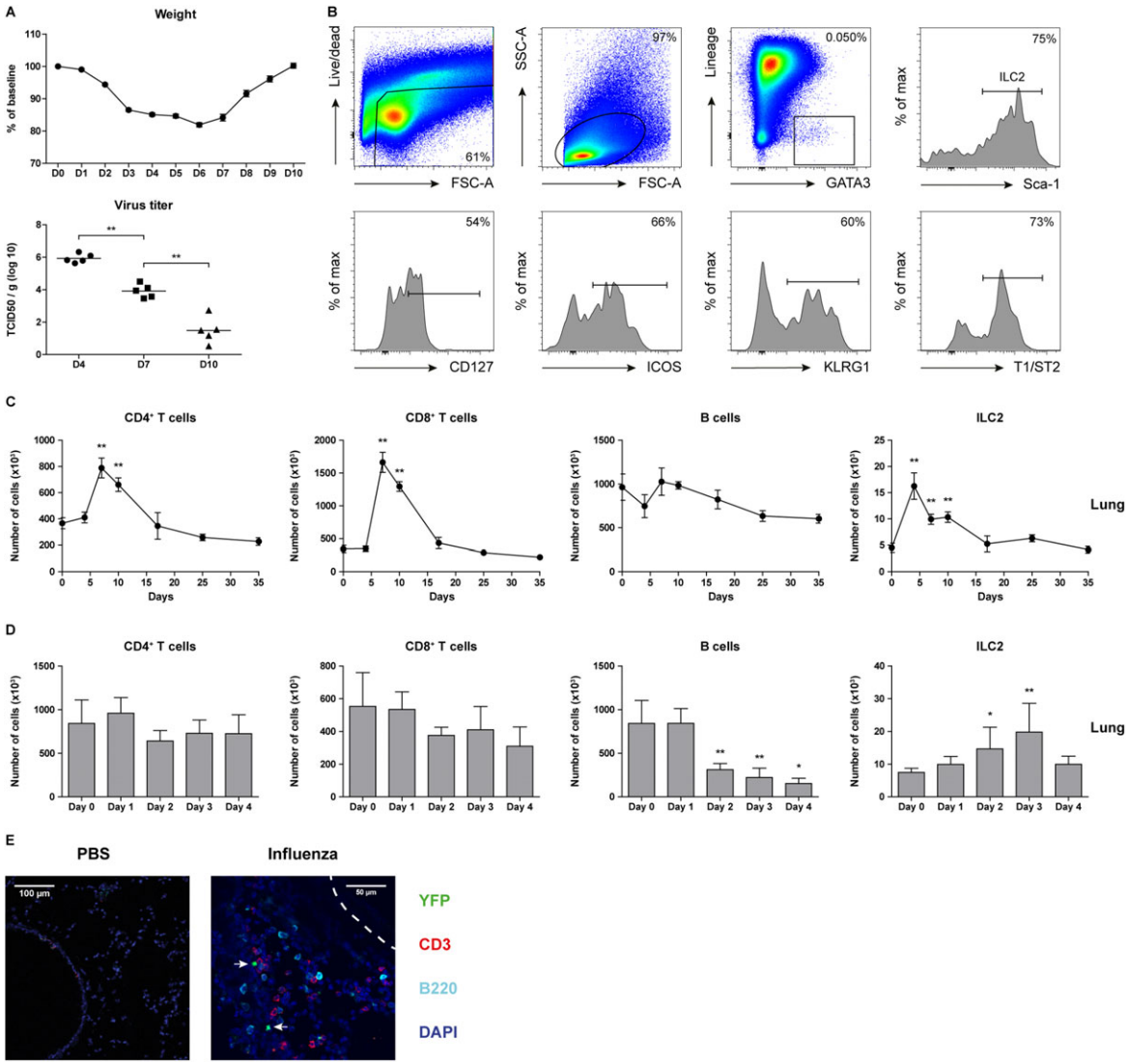

Figure 2. Accumulation of ILC2s in the lungs precedes T cell recruitment after inoculation with influenza virus. (A) Percentage of weight loss in mice infected with 10^4 PFU X31 influenza virus particles and measurement of virus titers in the lungs at the indicated days post infection. (B) Flow cytometric identification of ILC2s in BALF of X31 virus-infected mice and surface expression of CD127, ICOS, KLRG1, and T1/ST2. Dot plots showing subsequent gating represent combined data using the concatenate option in FlowJo ($n = 7$) of a single experiment, representative of two independent experiments. (C) Kinetics of $CD4^+$ T cells, $CD8^+$ T cells, B cells, and ILC2s in the lungs after X31 influenza virus infection. (D) Detailed kinetics of $CD4^+$ T cells, $CD8^+$ T cells, B cells, and ILC2s in the lungs up to day 4 post infection. (E) Lung cryosection from PBS and X31 virus-infected *Gata3* reporter (GATIR) mice, counterstained with CD3 (T cells) and B220 (B cells and plasmacytoid dendritic cells). Airway epithelium is outlined by the dashed line. ILC2s, indicated with arrows, are identified as $CD3^-YFP^+$ cells. In the confocal microscopy conditions, $GATA3^{low}$ cells (such as non-Th2 $CD4^+$ T cells, $CD8^+$ T cells, and other ILC lineages) are not detectable as YFP^+ cells. Representative image of $n = 3$ mice per group from three independent experiments. (A, C – D) Data are shown as mean values ± SEM ($n = 5$) of a single experiment from two independent experiments. $^*p \leq 0.05$, $^{**}p \leq 0.01$.

cytokine production by ILC2s was delayed and only initiated in a later phase of influenza virus infection at day 10, when the total number of ILC2s in the BALF was declining (Fig. 3A and B). A comparable pattern, reflecting a peak of IL-5/IL-13-expressing T cells at day 7 and IL-5/IL-13-expressing ILC2s at day 10, was also observed in the lungs and the mediastinal LNs (Supporting Information Fig. S2).

We observed that on the cell surface of ILC2s in the BALF, T1/ST2, KLRG1, and CD117 were upregulated at day 4 and 7 and remained highly expressed at day 11 (Fig. 3C). Interestingly, high IL-2R (CD25) expression on ILC2s was only observed on day 7, coinciding with the peak of T cell activation, after which it was downregulated. Compared with naïve mice, ICOS expression was downregulated during influenza infection, particularly at day 4 after inoculation (Fig. 3C).

Taken together, these data show that during influenza virus infection the capacity of ILC2s to produce IL-5/IL-13 is delayed and is only acquired after their accumulation in the BALF, after upregulation of T1/ST2 and CD25 and after the accumulation of activated T cells.

© 2018 The Authors. *European Journal of Immunology* published by WILEY-VCH Verlag GmbH & Co. KGaA, Weinheim.

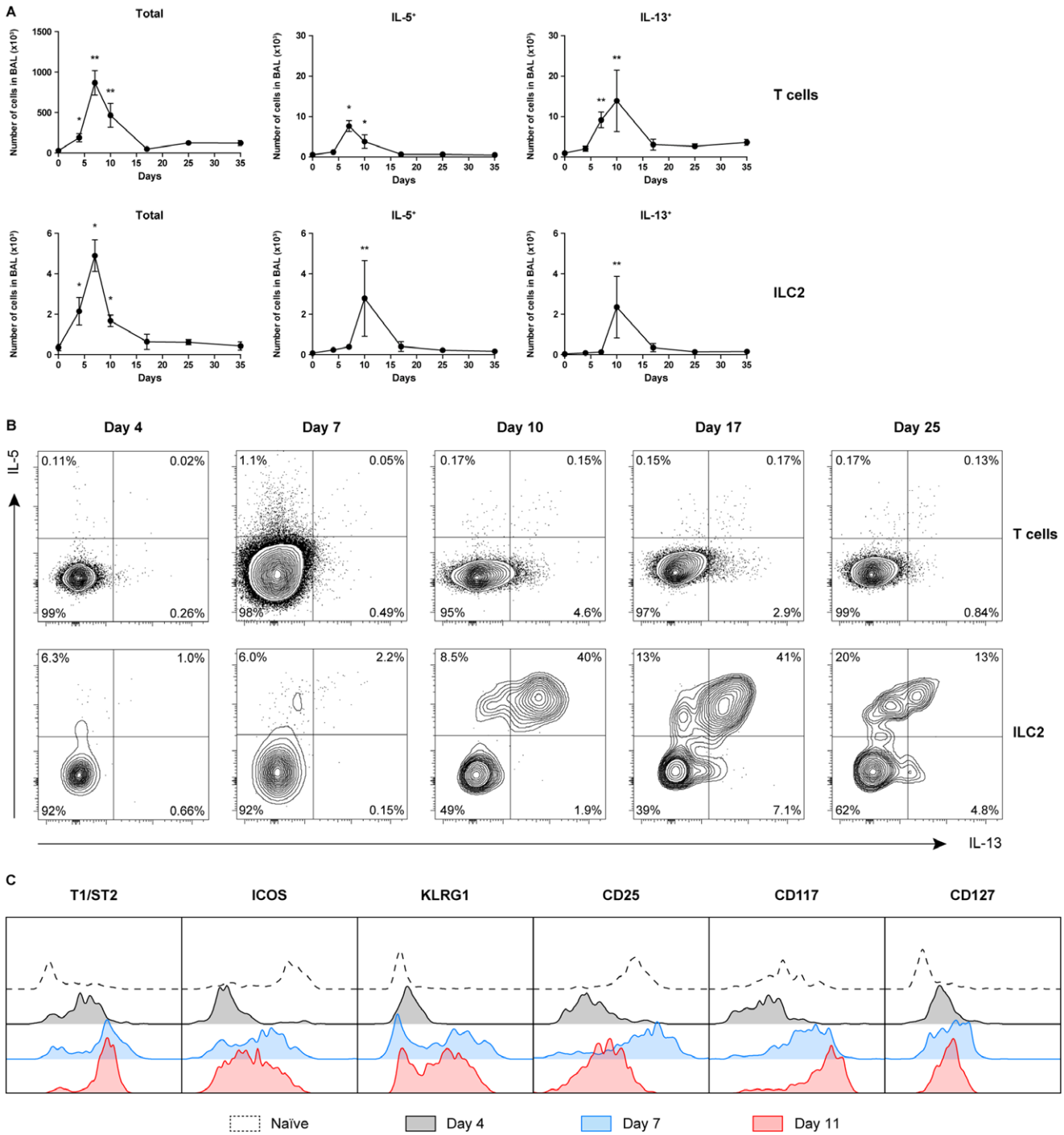

Figure 3. Delayed ILC2 cytokine production in influenza virus infection. (A) Quantification of total IL-5 and IL-13 producing T cells and ILC2s in BALF over time after X31 virus infection. Data are shown as mean ± SEM ($n = 5$) of a single experiment from two independent experiments. $^{*}p \leq 0.05$, $^{**}p \leq 0.01$. (B) Flow cytometric analysis of IL-5 and IL-13 expression in BALF T cells and ILC2s at day 4, 7, 10, 17, and 25 after X31 influenza virus infection. (C) Expression of ILC2 surface markers at day 4, 7, and 11. (B, C) Plots represent combined data using the concatenate option in FlowJo ($n = 5$–7) of a single experiment, representative of two independent experiments.

Influenza virus infection does not increase ILC2s in the BALF in HDM-mediated inflammation

We previously found in chronic HDM-driven allergic airway inflammation that the numbers of both T cells and ILC2s were significantly increased in the BALF (~50.000–100.000 and ~1.000–3.000, respectively). Hereby, ILC2s constituted ~40% of the IL-5^{+} lymphocytes and ~30% of IL-13^{+} lymphocytes and were mainly CD25^{low} [33, 38]. To explore the contribution of ILC2s in influenza-induced exacerbations of allergic airway

© 2018 The Authors. *European Journal of Immunology* published by WILEY-VCH Verlag GmbH & Co. KGaA, Weinheim.

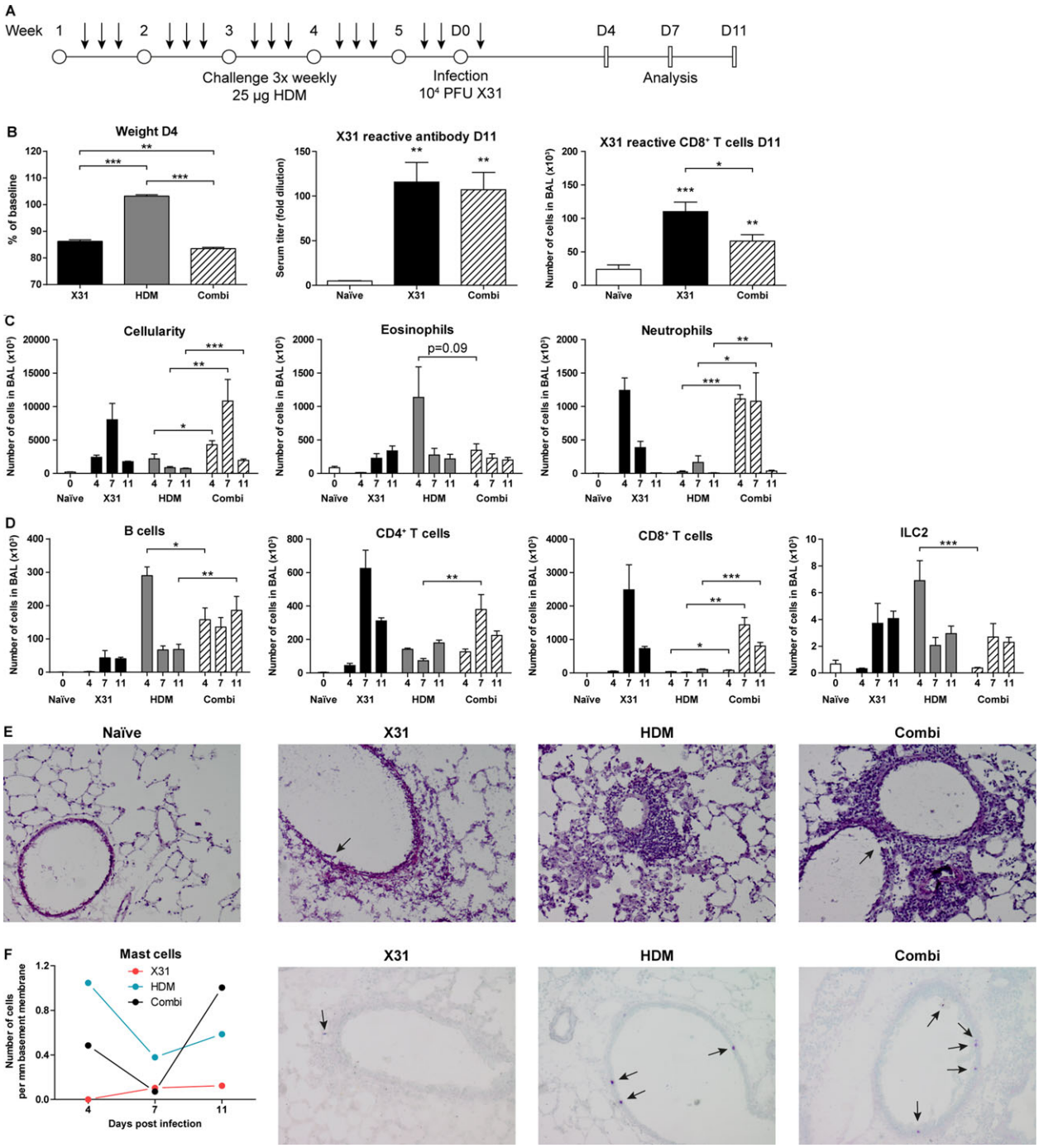

Figure 4. Influenza-induced exacerbations of allergic airway inflammation are primarily neutrophilic and lead to increased T cell numbers in BALF. (A) Scheme for administration of HDM (arrows) for induction of chronic asthma followed by X31 influenza virus inoculation and analyses at days 4, 7, and 11 post infection. (B) Weight loss of mice inoculated with X31 virus, compared to HDM-treated mice with or without X31 virus (*left*). X31 influenza specific antibody titer in serum (*middle*) and influenza virus-specific $CD8^+$ T cells in BALF (*right*). (C) Enumeration of total cells, eosinophils, and neutrophils infiltrating in the BALF at day 4, 7, and 11 post infection. (D) Quantification of B cells, $CD4^+$ T cells, $CD8^+$ T cells, and ILC2s in the BALF. (E) H&E stained cryosections of lungs from naïve, HDM only, X31 virus only, and combination-treated mice at day 4 post infection; original magnification ×100; arrows indicate epithelial damage. Representative images of $n = 2$ mice per group of a single experiment from two independent experiments. (F) Quantification of intraepithelial mast cells in toluidine blue stained cryosections of lungs from HDM only, X31 influenza virus only, and combination-treated mice. Data are shown as mean values ($n = 2$) from a single experiment. Representative images of day 11 post infection are displayed from a single experiment ($n = 2$); original magnification ×100; arrows indicate mast cells. (B–D) Data are shown as mean values ± SEM ($n = 7$) of a single experiment from two independent experiments. $^*p \leq 0.05$, $^{**}p \leq 0.01$, $^{***}p \leq 0.001$.

© 2018 The Authors. *European Journal of Immunology* published by WILEY-VCH Verlag GmbH & Co. KGaA, Weinheim.

inflammation, we developed a mouse model in which we first chronically exposed mice to HDM and subsequently infected them with X31 influenza virus. In these experiments, we included groups of control mice that were either chronically exposed to HDM only or that were infected with influenza virus without prior exposure to HDM. The immune response was analyzed by flow cytometry at day 4, day 7, and day 11 post infection (Fig. 4A).

Mice that received both HDM and X31 virus treatment lost marginally more weight than those only infected with X31 virus (Fig. 4B). Compared to influenza-only mice, the combination treated animals had similar virus-specific antibody titers in serum but showed a decrease in the numbers of $CD8^+$ T cells that carried a T cell receptor specific for the X31 epitope $NP_{366\text{-}374}$ (ASNENMETM) in the BALF (Fig. 4B). Importantly, the combination treatment resulted in a significantly higher influx of immune cells into the BALF than HDM alone at all time points investigated (flow cytometric gating strategy illustrated in Supporting Information Fig. S3). The infiltrating granulocytes in the combination group consisted predominantly of neutrophils, but a significant eosinophil presence was also observed. The total influx of cells in the BALF peaked at day 7, although both eosinophil and neutrophil numbers remained comparable to the levels seen at day 4. By day 11, neutrophils returned to baseline levels and the inflammation reached its resolution phase (Fig. 4C). The combination of HDM exposure and X31 virus infection led to increased numbers of B cells, $CD4^+$, and $CD8^+$ T cells compared to HDM only, particularly at day 7 or 11 (Fig. 4D).

Notably, X31 influenza virus infection significantly reduced ILC2 numbers in HDM-treated mice at day 4 to <1000 cells, despite a strong $CD4^+$ T cell presence, and recovered to a similar range as HDM-treated group by day 7 (Fig. 4D). Examination of lung cryosections stained with H&E confirmed a more severe inflammatory response in the combination treatment around the airways, compared to HDM alone and showed signs of epithelial damage similar to X31 virus infection alone (shown for day 4 in Fig. 4E). Mast cells were detected within the epithelial layer in mice chronically exposed to HDM but not after X31 virus infection alone, where only very low numbers of mast cells resided underneath the basement membrane. Interestingly, upon infection of HDM-treated mice with X31 influenza virus, the numbers of intraepithelial mast cells decreased and were almost undetectable by day 7. Mast cells reemerged in the epithelium after virus clearance by day 11 (Fig. 4F).

In summary, influenza virus infection following HDM-mediated allergic airway inflammation differentially affects $CD4^+$ T cells and ILC2s and induces a transient reduction of ILC2 numbers in the BALF at day 4, compared to HDM-mediated inflammation only.

Different kinetics of cytokine response by T cells and ILC2s in exacerbation of airway inflammation

Next, we used intracellular flow cytometry to evaluate the capacity of Th2 cells and ILC2 to produce cytokines. These analyses revealed that the numbers of IL-4^+ and IL-5^+ $CD4^+$ T cells were significantly enhanced at day 4 and 7 of the X31-induced exacerbation of HDM-mediated allergic airway inflammation, when compared to influenza virus infection or HDM-challenge alone (Fig. 5A). Interestingly, the numbers of IL-5^+, IL-13^+, and amphiregulin-expressing ILC2s were increased at day 7 and day 11 but not at day 4, as compared to HDM alone (Fig. 5A). The additive effect of X31 virus on IL-4^+ $CD4^+$ T cells and IL-5^+ ILC2s was also observed in the lungs (Supporting Information Fig. S4).

Overall, when we compared the relative contribution of $CD4^+$ T cells and ILC2s to the population of type 2 cytokine expressing cells, it was apparent that $CD4^+$ T cells were the major source of IL-5 or IL-13 in the early phase of influenza-induced exacerbation (Fig. 5B). At later time points following influenza virus infection, however, ILC2s contributed more to the total number of IL-5 and IL-13 producing cells, although $CD4^+$ T cells remained dominant (Fig. 5B). Moreover, the MFI values for IL-5 and IL-13 content in ILC2s at day 11 was significantly higher in combination-treated mice, indicating a higher cytokine production capacity per cell (Fig. 5C).

Taken together, these data imply that both T cells and ILC2s are major effector cells that contribute to the type 2 cytokine milieu in influenza-induced exacerbation of allergic airway inflammation, albeit with different kinetics.

ILC2s display an activated phenotype only after virus clearance

Finally, we compared the surface phenotype of ILC2s as an indicator of the activation status of ILC2s in chronic HDM-driven allergic airway inflammation, with and without X31 influenza virus-induced exacerbation, as described in Fig. 4A.

We noticed upregulation of T1/ST2 and KLRG1 paired with a downregulation of ICOS and CD25 in HDM-treated mice compared to naïve mice at day 4 (Fig. 6; quantified in Supporting Information Fig. S5). As expected, the activated phenotype appeared to diminish slowly over time, as T1/ST2 and KLRG1 surface expression decreased, but did not yet return to baseline levels at day 11. In the combination group, however, surface T1/ST2 and KLRG1 expression remained close the levels found in naïve mice, suggesting that X31 influenza virus infection resulted in suppression of ILC2s that were activated by the HDM exposure (Fig. 6; quantified in Supporting Information Fig. S5).

However, at day 7–11 ILC2s in combination-treated mice regained expression of various surface markers. Most interestingly, this resulted in an activated $T1/ST2^{high}ICOS^{high}KLRG1^{high}CD25^{high}$ surface phenotype of BALF ILC2s, which was significantly different from the phenotype in mice that were not infected with influenza virus (Fig. 6; quantified in Supporting Information Fig. S5).

Taken together, these findings show that after clearance of the virus by day 11, ILC2s present in the BALF manifest—concomitant with a high capacity to produce type 2 cytokines—an activated surface phenotype.

© 2018 The Authors. *European Journal of Immunology* published by WILEY-VCH Verlag GmbH & Co. KGaA, Weinheim.

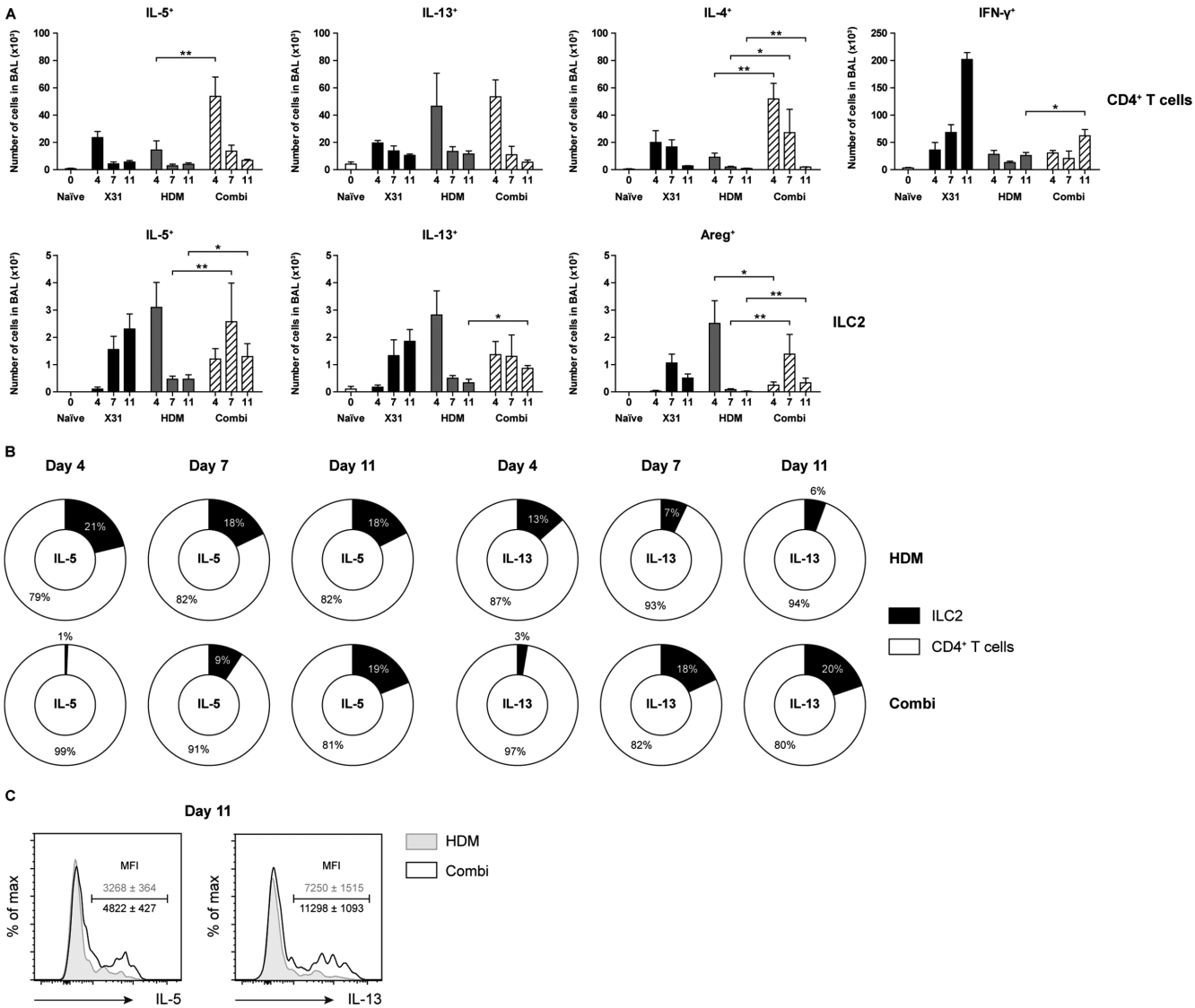

Figure 5. ILC2s are important contributors to type 2 cytokine producers in the late phase of influenza-induced exacerbations of allergic airway inflammation. (A) Quantification of the absolute number of cytokine producing CD4$^+$ T cells (top) and ILC2s (bottom). (B) Ratio of IL-5 and IL-13 producing ILC2s and CD4$^+$ T cells over time in BALF. (C) Flow cytometric comparison of IL-5 and IL-13 production by BALF ILC2s at day 11 post infection. Histogram overlay plots represent combined data using the concatenate option in FlowJo ($n = 7$) of a single experiment, representative of two independent experiments. (A–C) Data are shown as mean ± SEM ($n = 7$) of a single experiment representative of two independent experiments. $^*p \leq 0.05$, $^{**}p \leq 0.01$.

Discussion

Virus infection-induced exacerbations account for a major portion in healthcare costs associated with severe asthma [39]. However, the current knowledge of how a Th1 response is able to trigger a Th2-mediated disease is fragmented. IL-33 and the type 2 cytokines IL-4, IL-5, and IL-13 have been shown to be induced by rhinovirus infection with a correlation between disease severity and cytokine levels. Furthermore, in vitro data show that supernatant from rhinovirus-infected bronchial epithelial cells induces type 2 cytokine production in both T cells and ILC2s [40]. ILC2s have also been hypothesized to be the missing link in the underlying mechanisms between influenza virus infection and asthma exacerbation due to their accumulation in the lung after influenza virus infection and their ability to secrete type 2 cytokines to promote asthma symptoms [21].

Influenza-induced asthma exacerbation models have been reported, but the contribution of ILC2s is still unclear [25, 26]. In our study we present a model for influenza-induced exacerbation of allergic airway inflammation in which mice were chronically exposed to HDM [38] and subsequently infected with X31 influenza virus to induce exacerbation of airway inflammation. Using multicolor flow cytometry, we observed an increased cellularity of the BALF consisting of a mixed eosinophilic and neutrophilic infiltrate in influenza-infected HDM-exposed mice, compared to major eosinophilic inflammation present in mice that

© 2018 The Authors. *European Journal of Immunology* published by WILEY-VCH Verlag GmbH & Co. KGaA, Weinheim.

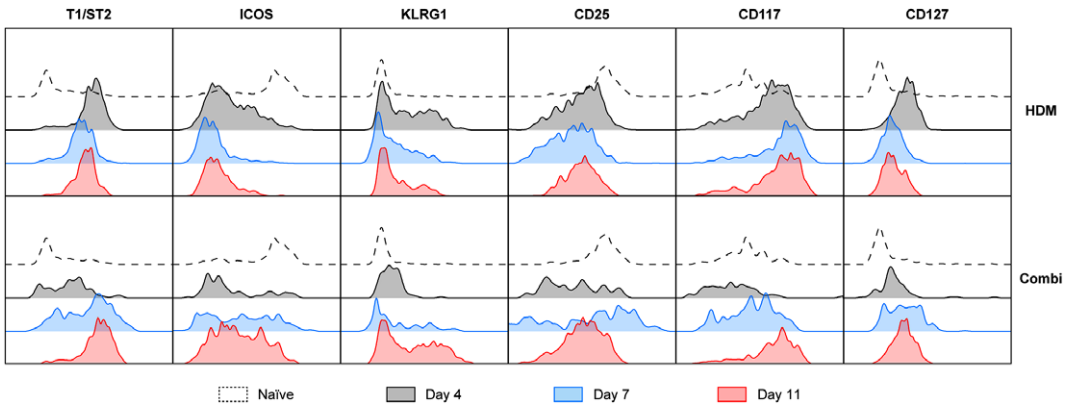

Figure 6. Influenza virus infection initially suppresses HDM-activated ILC2s but induces enhanced activation at day 11. Flow cytometric comparison of T1/ST2, ICOS, KLRG1, CD25, CD117, and CD127 on ILC2s derived from BALF at day 0, 4, 7, and 11. Histogram overlay plots represent combined data using the concatenate option in FlowJo ($n = 7$) of a single experiment, representative of two independent experiments. Quantification of these data is given in Supporting Information Fig. S5.

were only exposed to HDM. Concomitantly, at day 4 of the influenza virus infection, ILC2 numbers and their cytokine production capacity in BALF were suppressed. However, once the virus was cleared, BALF ILC2s resumed production of type 2 cytokines and gained an activated T1/ST2^{high}ICOS^{high}KLRG1^{high}CD25^{high} surface phenotype on day 11, which was significantly different from the phenotype in mice that were exposed to HDM only. In contrast, T cells in the BALF and mediastinal LNs of HDM-exposed mice showed particularly at day 4 of the influenza virus infection increased IL-4 and IL-5 production, compared to mice that were not infected with influenza virus. Therefore, CD4$^+$ T cells in this virus-induced HDM-driven inflammation exacerbation model were a more prominent contributor to the type 2 cytokine environment than ILC2s early after influenza virus infection. We conclude that both T cells and ILC2s contribute to influenza-induced exacerbation of allergic airway inflammation, but with remarkably different kinetics.

Irrespective of the presence of chronic HDM-driven inflammation, we found that during influenza virus infection ILC2s are induced in the BALF with remarkable kinetics: numbers of ILC2s peak at day 4–7 but substantial IL-5 and IL-13 cytokine production capacity was only detected later during infection at around day 10–11 in the resolution phase of influenza virus infection when virus particles had been eradicated and the type 1 immune response was reaching its conclusion. This would be in agreement with the proposed restorative functions of ILC2s [21], but also indicates that an ongoing influenza virus infection significantly suppresses ILC2 activation. This was also evident in our combination treatment in which the numbers of HDM-activated ILC2s were very low at day 4 after influenza virus infection. A likely explanation for this is that type 1 IFN, IFN-γ, and IL-27, which are abundantly present in influenza virus infections (Fig. 1), are able to inhibit ILC2s in a dose-dependent manner [28, 29, 31, 32]. In particular, it was very recently reported that virus-induced IFN-γ restricts protective ILC2 functions in the lung following challenge with the pandemic H1N1 CA04 influenza virus, whereby neutralizing IFN-γ protected mice from lethal infection [30]. Our finding that at day 4 of infection both IFN-β and IFN-γ are increased supports the hypothesis that these cytokines inhibit ILC2 induction. Other known cytokines that have an inhibitory effect on ILC2 function where either not significantly induced by the influenza virus infection (IFN-α) or not represented in the Affymetrix mouse microarray 430.2 (IL-27). It is very well possible that these cytokines act in concert to suppress ILC2 activity. It must be noted however, that different strains of influenza virus may result in alternative ILC2 kinetics. Comparison between the low-pathological H3N2 X31 strain and the high-pathological H1N1 PR8 strain show that a more robust CD8$^+$ T cell response is generated upon infection with the X31 influenza virus and is paired with higher levels of IL-12 in the microenvironment and more IFN-γ production by CD8$^+$ T cells [41]. Therefore in high-pathological influenza virus infections such as the PR8 strain, the reduction in IFN-γ levels as well as more severe epithelial damage may trigger earlier activation of ILC2s.

Nevertheless, upon virus clearance, ILC2s regained a T1/ST2^{high}ICOS^{high}KLRG1^{high}CD25^{high} phenotype paired with cytokine production and were major contributors to the type 2 cytokine milieu. Several lines of evidence support the notion that this surface phenotype signifies strong activation of ILC2s. First, high cell surface expression of T1/ST2 on ILC2s will increase their activation status because IL-33, produced by epithelial cells, e.g., upon allergen activation, is one of the most potent activators of ILC2s [42–44]. In this context, we previously showed that although in HDM-mediated airway inflammation the accumulation of ILC2s in BALF is IL-33 independent, IL-33 is critical for cytokine production by ILC2s [17]. In our combination treatment model, next to HDM-activated epithelial cells, NKT cells might be an additional source of IL-33, given that NKT cells were shown to secrete IL-33 on day 7–12 following infection with H1N1 PR8 influenza virus [22]. Alternatively, alveolar macrophages are also a source of IL-33 in H3N1 Mem/71 influenza virus-infected mice where ILC2 activation peaks at day 6–9 post infection [20]. Second, IL-2 and IL-7, which alone are not sufficient for ILC2 activation, are thought to enhance the

© 2018 The Authors. *European Journal of Immunology* published by WILEY-VCH Verlag GmbH & Co. KGaA, Weinheim.

in vivo effect of IL-33 [13, 45]. Therefore, high expression of IL-2R (CD25) and IL-7R (CD127)—together with high T1/ST2 expression—would optimally prime ILC2s for receiving activating signals from NKT cells, alveolar macrophages, or epithelial cells via IL-33. Rapid downregulation of CD25 on ILC2s by day 11 is evidence for tight regulation of this pathway and was followed by a diminishing IL-5/IL-13 profile. Third, ICOS:ICOS-ligand interaction promotes cytokine production, proliferation, and survival of ILC2s both under homeostatic and inflammatory conditions [46, 47] and moreover, ICOS on ILC2s may interact with B7RP-1-expressing dendritic cells in allergic inflammatory lung, which increases ILC2 activation [48]. Finally, KLRG1 has been reported as an ILC2 activation marker and is intermediate to highly expressed by mature ILC2s [49, 50]. Heterogeneous surface expression of CD117 on ILC2s during an influenza virus infection has been previously described. Because the authors did not observe functional differences between $CD117^{-}$ and $CD117^{+}$ ILC2s, they concluded that the marked accumulation of $CD117^{+}$ ILC2s during the recovery phase of influenza virus infection most likely reflects recruitment of these cells from the BM [22]. In this context, it is important to note that our $T1/ST2^{high}ICOS^{high}KLRG1^{high}CD25^{high}$ cells are different from the $KLRG1^{high}$ inflammatory ILC2 population that is responsive to IL-25 and is proposed to be transient progenitors mobilized by inflammation [49]. In contrast to these cells, which do not express T1/ST2, our activated ILC2s are $T1/ST2^{high}$.

Our comparison of chronic HDM-driven allergic airway inflammation, with and without X31 influenza virus-induced exacerbation (day 11), indicated a correlation between the presence of mast cells and ILC2 activation. Interestingly, mast cell-derived mediators, such as IL-2, IL-33, prostaglandin D_2, and leukotriene E_4, enhance ILC2 responses [51–53] and serine proteases secreted by activated mast cells cleave IL-33 into mature and more biologically active forms [54]. Conversely, IL-9 production by ILC2 can induce mast cell hyperplasia [55], leading to a positive feedback loop. Therefore, it is conceivable that mast-cell ILC2 interaction contributes to the increased activation of ILC2s in the recovery phase of influenza infection in our exacerbation model.

In summary, our mouse model of influenza-induced exacerbation of chronic HDM-driven allergic airway inflammation presents a mixed eosinophilic and neutrophilic infiltrate in the BALF and an initial suppression of ILC2 accumulation. After virus clearance, however, ILC2s are significant cytokine producers and show an activated $T1/ST2^{high}ICOS^{high}KLRG1^{high}CD25^{high}$ state, which would prime ILC2s for receiving activating signals from allergen-activated epithelial cells. It is attractive to speculate that these activated ILC2s share characteristics with the allergen-experienced ILC2s that persist after resolution of inflammation and were shown to respond more potently to unrelated allergens than naïve ILC2s did [56]. It was proposed that memory-like properties of allergen-experienced ILC2s may explain why asthma patients are often sensitized to multiple allergens. Likewise, it is conceivable that—next to T cells—memory-like properties of ILC2s that are induced upon viral infections may contribute to exacerbations of airway inflammation upon allergen exposure. Furthermore, type 2 cytokines such as those secreted by ILC2s possess pro-fibrotic functions through activation of fibroblasts and may result in additional remodeling of the airways [57, 58].

Materials and methods

Mice

Wildtype C57BL/6 mice were purchased from Envigo (United Kingdom). Homozygous *Gata3* reporter (GATIR) mice, which harbor an IRES-YFP sequence within the 3' untranslated region of the *Gata3* gene resulting in concomitant production of GATA3 and YFP protein, were bred on a C57BL/6 background [T.N.R. and H.J.F., manuscript in preparation; Stadhouders et al., manuscript in revision]. Mice were ~8–16 weeks old at the time of analysis. All animals were housed at the Erasmus MC Animal Facility under SPF conditions. All experiments were approved by the Erasmus MC Animal Ethics Committee.

Induction of chronic allergic airway inflammation

To induce chronic allergic airway inflammation, mice were anesthetized using isoflurane and treated intranasally three times per week for five consecutive weeks with 25 μg HDM extract (Greer, USA) or PBS (GIBCO Life Technologies, USA) as previously described [38].

X31 influenza virus infection and gene expression profiling

Inoculation and harvest of the X31 H3N2 strain of influenza virus (Medical Research Council, UK) has been described previously [59]. Mice were anesthetized using isoflurane and infected with 10^4 PFU X31 particles diluted in PBS via intranasal inoculation. Animals were sacrificed after infection at the indicated time points. Virus titers in lung tissue were determined as previously described [59, 60] X31-reactive antibody titers in serum was detected by hemagglutination inhibition assay as reported in the World Health Organization manual for laboratory diagnosis and virologic surveillance of influenza [61].

Gene expression profiling was performed in mice infected with 10^5 PFU X31 virus particles as described elsewhere [62]. In brief, the lungs were excised and stored in RLT buffer (Qiagen, Germany) at –80°C. Total RNA was then isolated using an RNeasy kit (Qiagen, Germany) and biotin labeled and hybridized to mouse DNA microarrays (Affymetrix, USA).

Flow cytometry

Single cell suspensions were prepared from lungs and lymph nodes by mechanical disruption without digestive enzymes

© 2018 The Authors. *European Journal of Immunology* published by WILEY-VCH Verlag GmbH & Co. KGaA, Weinheim.

using a 100 μm cell strainer (BD Biosciences, USA) in PBS supplemented with 0.5% BSA and 5 mM EDTA (Sigma–Aldrich, USA). For determination of intracellular cytokine content, cells were stimulated with PMA and ionomycin (Sigma–Aldrich, USA) at 37°C for 4 h prior to staining. Flow cytometry surface and intracellular staining was performed as described previously [44, 63]. In brief, intracellular transcription factors were stained by fixation and permeabilization using eBioscience™ FOXP3/transcription factor staining buffer set (Thermo Fisher Scientific, USA) followed by 1 h incubation with fluorescent antibodies at 4°C. Intracellular cytokines were stained by fixation with 2% PFA (Thermo Fisher Scientific, USA) and permeabilization with 0.5% saponin (Sigma–Aldrich, USA) followed by 1 h incubation with fluorescent antibodies at 4°C. Dead cells were excluded using LIVE/DEAD™ fixable aqua dead cell stain kit (Thermo Fisher Scientific, USA) and only single cells were gated for further analysis using FSC-W and FSC-A. Lineage markers included CD3, CD4, CD5, CD8α, CD11b, CD11c, CD19, B220, NK1.1, FcεRIα, TER-119, and Gr-1. X31-reactive $CD8^+$ T cells were detected using MHC dextramer against epitope $NP_{366\text{-}374}$ (ASNENMETM). A comprehensive list of antibodies used for flow cytometry is presented in Supporting Information Table S1. Samples were acquired using a LSR II flow cytometer and FACSDiva (BD Biosciences, USA) and analyzed by FlowJo X (Tree Star Inc., USA). Gating strategy is shown in in Fig. 2 and Supporting Information Fig. S3.

Histology

Lungs were inflated with OCT embedding medium (Sakura, the Netherlands) diluted in equal volume of PBS prior to being snap frozen in liquid nitrogen. Lungs from *Gata3* reporter mice were inflated with a 1:1 mixture of OCT embedding medium and PFA to preserve YFP fluorescence. Cryosections (7 μm) were cut at –20°C using a cryostat (Thermo Fisher Scientific, USA) and fixed in 4% PFA for 10 min. Then slides were stained with primary antibodies for 1 h and secondary antibodies for 30 min at room temperature. A comprehensive list of antibodies used for confocal microscopy is presented in Supporting Information Table S2. Slides were incubated with DAPI (Invitrogen, USA) for 5 min and sealed with Vectashield (Vector Laboratories, USA). An LSM510Meta confocal microscope equipped with 405, 488, 543, and 633 nm lasers (Zeiss, Germany) was used to examine the slides. Images were processed and analyzed in Fiji, an open source scientific image processing application based on ImageJ.

For H&E stain, cryosections (7 μm) were fixed by dipping in 4% PFA followed by 30 s in Gill's Haematoxylin and 1 min in 4% Eosin B acetified with glacial acetic acid (Sigma–Aldrich). Samples were dehydrated by sequential rinsing in ethanol up to 100% and cleared in xylene for 2 min (Sigma–Aldrich, USA). Sections were mounted in Entallan (Merck, USA) and examined under a light microscope (Zeiss, Germany).

The presence of intraepithelial mast cells was analyzed by staining cryosections (6 μm) with toluidine blue (Sigma–Aldrich) at pH 0.5 for 1–2 min, which were immediately examined under a light microscope. Quantification was performed by counting the number of mast cells per mm basement membrane in bronchioles of the lower airways.

Statistical analysis

Mann–Whitney *U* tests were used for statistical comparisons between groups and a *p*-value <0.05 was considered statistically significant. All analyses were performed using Prism 5 (GraphPad Software, USA).

Acknowledgments: These studies were supported by the Lung Foundation Netherlands (grant numbers 3.2.12.067 and 3.2.12.087 to R.W.H.). We would like to acknowledge Carolien van de Sandt and Henk-Jan van den Ham (Department of Viroscience, Erasmus MC), Karolina Leman, Heleen Vroman, Mirjam Kool, Dior Beerens, and Maarten Brem (Department of Pulmonary Medicine, Erasmus MC), Henk Dronk and the Erasmus MC Animal Facility staff for their assistance at several stages of the project. We also thank Hans Jörg Fehling (University Clinics, Ulm, Germany) for providing the GATIR mouse model. B.W.S.L. conceived the study, designed and performed experiments, analyzed data and wrote the manuscript. M.J.W.d.B., M.L., M.v.N., I.M.B., and C.H.G. performed experiments, and A.A. performed data analyses. A.K. performed experiments and analyzed the data. C.H.G. and G.F.R. designed and characterized the X31 influenza virus infection mouse model. R.W.H. conceived the study and designed experiments and wrote the manuscript.

Conflict of interest: The authors declare no financial or commercial conflict of interest.

References

1 **Johnston, S. L., Pattemore, P. K., Sanderson, G., Smith, S., Lampe, F., Josephs, L., Symington, P. et al.**, Community study of role of viral infections in exacerbations of asthma in 9–11 year old children. *BMJ* 1995. **310**: 1225–1229.

2 **Makela, M. J., Puhakka, T., Ruuskanen, O., Leinonen, M., Saikku, P., Kimpimaki, M., Blomqvist, S. et al.**, Viruses and bacteria in the etiology of the common cold. *J. Clin. Microbiol.* 1998. **36**: 539–542.

3 **Khetsuriani, N., Kazerouni, N. N., Erdman, D. D., Lu, X., Redd, S. C., Anderson, L. J. and Teague, W. G.**, Prevalence of viral respiratory tract infections in children with asthma. *J. Allergy Clin. Immunol.* 2007. **119**: 314–321.

4 **Mahut, B., Refabert, L., Marchac, V., Iniguez, J. L., Aubertin, G., Tamalet, A., Lebras, M. N. et al.**, Influenza-like illness responsible for severe exacerbations in asthmatic children during H1N1 pandemic: a survey before vaccination. *J. Asthma* 2011. **48**: 224–227.

© 2018 The Authors. *European Journal of Immunology* published by WILEY-VCH Verlag GmbH & Co. KGaA, Weinheim.

5 **Dao, C. N., Kamimoto, L., Nowell, M., Reingold, A., Gershman, K., Meek, J., Arnold, K. E. et al.**, Adult hospitalizations for laboratory-positive influenza during the 2005–2006 through 2007–2008 seasons in the United States. *J. Infect. Dis.* 2010. **202**: 881–888.

6 **Jacoby, D. B., Tamaoki, J., Borson, D. B. and Nadel, J. A.**, Influenza infection causes airway hyperresponsiveness by decreasing enkephalinase. *J. Appl. Physiol. (1985)* 1988. **64**: 2653–2658.

7 **Yamaya, M., Shinya, K., Hatachi, Y., Kubo, H., Asada, M., Yasuda, H., Nishimura, H. et al.**, Clarithromycin inhibits type a seasonal influenza virus infection in human airway epithelial cells. *J. Pharmacol. Exp. Ther.* 2010. **333**: 81–90.

8 **Yamaya, M., Nishimura, H., Shinya, K., Hatachi, Y., Sasaki, T., Yasuda, H., Yoshida, M., A. et al.**, Inhibitory effects of carbocisteine on type A seasonal influenza virus infection in human airway epithelial cells. *Am. J. Physiol. Lung Cell Mol. Physiol.* 2010. **299**: L160–168.

9 **Wang, S., Le, T. Q., Kurihara, N., Chida, J., Cisse, Y., Yano, M. and Kido, H.**, Influenza virus-cytokine-protease cycle in the pathogenesis of vascular hyperpermeability in severe influenza. *J. Infect. Dis.* 2010. **202**: 991–1001.

10 **Fahy, J. V.**, Type 2 inflammation in asthma–present in most, absent in many. *Nat. Rev. Immunol.* 2015. **15**: 57–65.

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

12 **Klose, C. S. and Artis, D.**, Innate lymphoid cells as regulators of immunity, inflammation and tissue homeostasis. *Nat. Immunol.* 2016. **17**: 765–774.

13 **Halim, T. Y., Krauss, R. H., Sun, A. C. and Takei, F.**, Lung natural helper cells are a critical source of Th2 cell-type cytokines in protease allergen-induced airway inflammation. *Immunity* 2012. **36**: 451–463.

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

15 **Doherty, T. A., Khorram, N., Chang, J. E., Kim, H. K., Rosenthal, P., Croft, M. and Broide, D. H.**, STAT6 regulates natural helper cell proliferation during lung inflammation initiated by Alternaria. *Am. J. Physiol. Lung Cell Mol. Physiol.* 2012. **303**: L577–588.

16 **Doherty, T. A., Khorram, N., Sugimoto, K., Sheppard, D., Rosenthal, P., Cho, J. Y., Pham, A. et al.**, Alternaria induces STAT6-dependent acute airway eosinophilia and epithelial FIZZ1 expression that promotes airway fibrosis and epithelial thickness. *J. Immunol.* 2012. **188**: 2622–2629.

17 **Li, B. W., de Bruijn, M. J., Tindemans, I., Lukkes, M., KleinJan, A., Hoogsteden, H. C. and Hendriks, R. W.**, T cells are necessary for ILC2 activation in house dust mite-induced allergic airway inflammation in mice. *Eur. J. Immunol.* 2016. **46**: 1392–1403.

18 **Corry, D. B., Grunig, G., Hadeiba, H., Kurup, V. P., Warnock, M. L., Sheppard, D., Rennick, D. M. and Locksley, R. M.**, Requirements for allergen-induced airway hyperreactivity in T and B cell-deficient mice. *Mol. Med.* 1998. **4**: 344–355.

19 **Doherty, T. A., Soroosh, P., Broide, D. H. and Croft, M.**, CD4+ cells are required for chronic eosinophilic lung inflammation but not airway remodeling. *Am. J. Physiol. Lung Cell Mol. Physiol.* 2009. **296**: L229–235.

20 **Chang, Y. J., Kim, H. Y., Albacker, L. A., Baumgarth, N., McKenzie, A. N., Smith, D. E., Dekruyff, R. H. et al.**, Innate lymphoid cells mediate influenza-induced airway hyper-reactivity independently of adaptive immunity. *Nat. Immunol.* 2011. **12**: 631–638.

21 **Monticelli, L. A., Sonnenberg, G. F., Abt, M. C., Alenghat, T., Ziegler, C. G., Doering, T. A., Angelosanto, J. M. et al.**, Innate lymphoid cells promote lung-tissue homeostasis after infection with influenza virus. *Nat. Immunol.* 2011. **12**: 1045–1054.

22 **Gorski, S. A., Hahn, Y. S. and Braciale, T. J.**, Group 2 innate lymphoid cell production of IL-5 is regulated by NKT cells during influenza virus infection. *PLoS Pathog.* 2013. **9**: e1003615.

23 **Chung, K. F., Wenzel, S. E., Brozek, J. L., Bush, A., Castro, M., Sterk, P. J., Adcock, I. M. et al.**, International ERS/ATS guidelines on definition, evaluation and treatment of severe asthma. *Eur. Respir. J.* 2014. **43**: 343–373.

24 **Singh, A. M. and Busse, W. W.**, Asthma exacerbations. 2: aetiology. *Thorax* 2006. **61**: 809–816.

25 **Kim, H. S., Lee, H., Kim, H. S., Won, S., Lee, E. K., Bang, K., Chun, Y. H. et al.**, Effect of influenza virus infection in a murine model of asthma. *Iran J. Allergy Asthma Immunol.* 2015. **14**: 392–401.

26 **Ravanetti, L., Dijkhuis, A., Sabogal Pineros, Y. S., Bal, S. M., Dierdorp, B. S., Dekker, T. et al.**, An early innate response underlies severe influenza-induced exacerbations of asthma in a novel steroid-insensitive and anti-IL-5-responsive mouse model. *Allergy* 2016.

27 **Monticelli, L. A., Buck, M. D., Flamar, A. L., Saenz, S. A., Tait Wojno, E. D., Yudanin, N. A., Osborne, L. C. et al.**, Arginase 1 is an innate lymphoid-cell-intrinsic metabolic checkpoint controlling type 2 inflammation. *Nat. Immunol.* 2016. **17**: 656–665.

28 **Molofsky, A. B., Van Gool, F., Liang, H. E., Van Dyken, S. J., Nussbaum, J. C., Lee, J., Bluestone, J. A. et al.**, Interleukin-33 and interferon-gamma counter-regulate group 2 innate lymphoid cell activation during immune perturbation. *Immunity* 2015. **43**: 161–174.

29 **Han, M., Hong, J. Y., Jaipalli, S., Rajput, C., Lei, J., Hinde, J. L., Chen, Q. et al.**, IFN-gamma blocks development of an asthma phenotype in rhinovirus-infected baby mice by inhibiting type 2 innate lymphoid cells. *Am. J. Respir. Cell Mol. Biol.* 2017. **56**: 242–251.

30 **Califano, D., Furuya, Y., Roberts, S., Avram, D., McKenzie, A. N. J. and Metzger, D. W.**, IFN-gamma increases susceptibility to influenza A infection through suppression of group II innate lymphoid cells. *Mucosal. Immunol.* 2017.

31 **Moro, K., Kabata, H., Tanabe, M., Koga, S., Takeno, N., Mochizuki, M., Fukunaga, K. et al.**, Interferon and IL-27 antagonize the function of group 2 innate lymphoid cells and type 2 innate immune responses. *Nat. Immunol.* 2016. **17**: 76–86.

32 **Duerr, C. U., McCarthy, C. D., Mindt, B. C., Rubio, M., Meli, A. P., Pothlichet, J., Eva, M. M. et al.**, Type I interferon restricts type 2 immunopathology through the regulation of group 2 innate lymphoid cells. *Nat. Immunol.* 2016. **17**: 65–75.

33 **Li, B. W. S., Stadhouders, R., de Bruijn, M. J. W., Lukkes, M., Beerens, D., Brem, M. D., KleinJan, A. et al.**, Group 2 innate lymphoid cells exhibit a dynamic phenotype in allergic airway inflammation. *Front Immunol.* 2017. **8**: 1684.

34 **Lim, A. I., Menegatti, S., Bustamante, J., Le Bourhis, L., Allez, M., Rogge, L., Casanova, J. L. et al.**, IL-12 drives functional plasticity of human group 2 innate lymphoid cells. *J. Exp. Med.* 2016. **213**: 569–583.

35 **Bal, S. M., Bernink, J. H., Nagasawa, M., Groot, J., Shikhagaie, M. M., Golebski, K., van Drunen, C. M. et al.**, IL-1beta, IL-4 and IL-12 control the fate of group 2 innate lymphoid cells in human airway inflammation in the lungs. *Nat. Immunol.* 2016. **17**: 636–645.

36 **Silver, J. S., Kearley, J., Copenhaver, A. M., Sanden, C., Mori, M., Yu, L., Pritchard, G. H. et al.**, Inflammatory triggers associated with exacerbations of COPD orchestrate plasticity of group 2 innate lymphoid cells in the lungs. *Nat. Immunol.* 2016. **17**: 626–635.

37 **Ohne, Y., Silver, J. S., Thompson-Snipes, L., Collet, M. A., Blanck, J. P., Cantarel, B. L., Copenhaver, A. M. et al.**, IL-1 is a critical regulator of group 2 innate lymphoid cell function and plasticity. *Nat. Immunol.* 2016. **17**: 646–655.

© 2018 The Authors. *European Journal of Immunology* published by WILEY-VCH Verlag GmbH & Co. KGaA, Weinheim.

38 **Vroman, H., Bergen, I. M., Li, B. W., van Hulst, J. A., Lukkes, M., van Uden, D., Hendriks, R. W. et al.**, Development of eosinophilic inflammation is independent of B-T cell interaction in a chronic house dust mite-driven asthma model. *Clin. Exp. Allergy* 2017. **47**: 551–564.

39 **Bahadori, K., Doyle-Waters, M. M., Marra, C., Lynd, L., Alasaly, K., Swiston, J. and FitzGerald, J. M.**, Economic burden of asthma: a systematic review. *BMC Pulm. Med.* 2009. **9**: 24.

40 **Jackson, D. J., Makrinioti, H., Rana, B. M., Shamji, B. W., Trujillo-Torralbo, M. B., Footitt, J., Jerico, D.-R. et al.**, IL-33-dependent type 2 inflammation during rhinovirus-induced asthma exacerbations in vivo. *Am. J. Respir. Crit Care Med.* 2014. **190**: 1373–1382.

41 **Rutigliano, J. A., Sharma, S., Morris, M. Y., Oguin, T. H., McClaren, 3rd, J. L., Doherty, P. C. and Thomas, P. G.**, Highly pathological influenza A virus infection is associated with augmented expression of PD-1 by functionally compromised virus-specific CD8+ T cells. *J. Virol.* 2014. **88**: 1636–1651.

42 **Bartemes, K. R., Iijima, K., Kobayashi, T., Kephart, G. M., McKenzie, A. N. and Kita, H.**, IL-33-responsive lineage- CD25+ CD44(hi) lymphoid cells mediate innate type 2 immunity and allergic inflammation in the lungs. *J. Immunol.* 2012. **188**: 1503–1513.

43 **Kim, H. Y., Chang, Y. J., Subramanian, S., Lee, H. H., Albacker, L. A., Matangkasombut, P., Savage, P. B. et al.**, Innate lymphoid cells responding to IL-33 mediate airway hyperreactivity independently of adaptive immunity. *J. Allergy Clin. Immunol.* 2012. **129**: 216–227.

44 **Klein Wolterink, R. G., Kleinjan, A., van Nimwegen, M., Bergen, I., de Bruijn, M., Levani, Y. and Hendriks, R. W.**, Pulmonary innate lymphoid cells are major producers of IL-5 and IL-13 in murine models of allergic asthma. *Eur. J. Immunol.* 2012. **42**: 1106–1116.

45 **Roediger, B., Kyle, R., Tay, S. S., Mitchell, A. J., Bolton, H. A., Guy, T. V., Tan, S. Y. et al.**, IL-2 is a critical regulator of group 2 innate lymphoid cell function during pulmonary inflammation. *J. Allergy Clin. Immunol.* 2015. **136**: 1653–1663.

46 **Maazi, H., Patel, N., Sankaranarayanan, I., Suzuki, Y., Rigas, D., Soroosh, P., Freeman, G. J. et al.**, ICOS:ICOS-ligand interaction is required for type 2 innate lymphoid cell function, homeostasis, and induction of airway hyperreactivity. *Immunity* 2015. **42**: 538–551.

47 **Paclik, D., Stehle, C., Lahmann, A., Hutloff, A. and Romagnani, C.**, ICOS regulates the pool of group 2 innate lymphoid cells under homeostatic and inflammatory conditions in mice. *Eur. J. Immunol.* 2015. **45**: 2766–2772.

48 **Kamachi, F., Isshiki, T., Harada, N., Akiba, H. and Miyake, S.**, ICOS promotes group 2 innate lymphoid cell activation in lungs. *Biochem. Biophys. Res. Commun.* 2015. **463**: 739–745.

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

50 **Koyasu, S.**, Inflammatory ILC2 cells: disguising themselves as progenitors? *Nat. Immunol.* 2015. **16**: 133–134.

51 **Moretti, S., Renga, G., Oikonomou, V., Galosi, C., Pariano, M., Iannitti, R. G., Borghi, M. et al.**, A mast cell-ILC2-Th9 pathway promotes lung inflammation in cystic fibrosis. *Nat. Commun.* 2017. **8**: 14017.

52 **Shimokawa, C., Kanaya, T., Hachisuka, M., Ishiwata, K., Hisaeda, H., Kurashima, Y., Kiyono, H. et al.**, Mast cells are crucial for induction of group 2 innate lymphoid cells and clearance of helminth infections. *Immunity* 2017. **46**: 863–874.

53 **Salimi, M., Stoger, L., Liu, W., Go, S., Pavord, I., Klenerman, P., Ogg, G. and Xue, L.**, Cysteinyl leukotriene E4 activates human group 2 innate lymphoid cells and enhances the effect of prostaglandin D2 and epithelial cytokines. *J. Allergy Clin. Immunol.* 2017. **140**: 1090–1100

54 **Lefrancais, E., Duval, A., Mirey, E., Roga, S., Espinosa, E., Cayrol, C. and Girard, J. P.**, Central domain of IL-33 is cleaved by mast cell proteases for potent activation of group-2 innate lymphoid cells. *Proc. Natl. Acad. Sci. USA* 2014. **111**: 15502–15507.

55 **Verma, M., Liu, S., Michalec, L., Sripada, A., Gorska, M. M. and Alam, R.**, Experimental asthma persists in IL-33 receptor knockout mice because of the emergence of thymic stromal lymphopoietin-driven IL-9(+) and IL-13(+) type 2 innate lymphoid cell subpopulations. *J. Allergy. Clin. Immunol.* 2017: S0091-6749(17)31747-5.

56 **Martinez-Gonzalez, I., Matha, L., Steer, C. A., Ghaedi, M., Poon, G. F. and Takei, F.**, Allergen-experienced group 2 innate lymphoid cells acquire memory-like properties and enhance allergic lung inflammation. *Immunity* 2016. **45**: 198–208.

57 **Fichtner-Feigl, S., Strober, W., Kawakami, K., Puri, R. K. and Kitani, A.**, IL-13 signaling through the IL-13alpha2 receptor is involved in induction of TGF-beta1 production and fibrosis. *Nat. Med.* 2006. **12**: 99–106.

58 **He, C. H., Lee, C. G., Dela Cruz, C. S., Lee, C. M., Zhou, Y., Ahangari, F., Ma, B. et al.**, Chitinase 3-like 1 regulates cellular and tissue responses via IL-13 receptor alpha2. *Cell Rep.* 2013. **4**: 830–841.

59 **GeurtsvanKessel, C. H., Willart, M. A., van Rijt, L. S., Muskens, F., Kool, M., Baas, C., Thielemans, K. et al.**, Clearance of influenza virus from the lung depends on migratory langerin+CD11b- but not plasmacytoid dendritic cells. *J. Exp. Med.* 2008. **205**: 1621–1634.

60 **Masurel, N., Ophof, P. and de Jong, P.**, Antibody response to immunization with influenza A/USSR/77 (H1N1) virus in young individuals primed or unprimed for A/New Jersey/76 (H1N1) virus. *J. Hyg. (Lond)* 1981. **87**: 201–209.

61 WHO, *Manual for the laboratory diagnosis and virological surveillance of influenza*. WHO, Geneva 2011.

62 **GeurtsvanKessel, C. H., Willart, M. A., Bergen, I. M., van Rijt, L. S., Muskens, F., Elewaut, D., Osterhaus, A. D. et al.**, Dendritic cells are crucial for maintenance of tertiary lymphoid structures in the lung of influenza virus-infected mice. *J. Exp. Med.* 2009. **206**: 2339–2349.

63 **KleinJan, A., Klein Wolterink, R. G., Levani, Y., de Bruijn, M. J., Hoogsteden, H. C., van Nimwegen, M. and Hendriks, R. W.**, Enforced expression of Gata3 in T cells and group 2 innate lymphoid cells increases susceptibility to allergic airway inflammation in mice. *J. Immunol.* 2014. **192**: 1385–1394.

Abbreviations: **HDM**: house dust mite · **ILC2**: group 2 innate lymphoid cell · **YFP**: yellow fluorescent protein

Full correspondence: Dr. Rudi W. Hendriks, Department of Pulmonary Medicine, Erasmus MC Rotterdam, PO box 2040, 3000 CA Rotterdam, The Netherlands
Fax: +31(0)107044728
e-mail: r.hendriks@erasmusmc.nl

Received: 17/11/2017
Revised: 14/3/2018
Accepted: 9/5/2018
Accepted article online: 15/5/2018

© 2018 The Authors. *European Journal of Immunology* published by WILEY-VCH Verlag GmbH & Co. KGaA, Weinheim.

European Journal of Immunology

T cells and ILC2s are major effector cells in influenza-induced exacerbation of allergic airway inflammation in mouse

Bobby W.S. Li, Marjolein J.W. de Bruijn, Melanie Lukkes, Menno van Nimwegen, Ingrid M. Bergen, Alex KleinJan, Corine H. GeurtsvanKessel, Arno Andeweg, Guus F. Rimmelzwaan and Rudi W. Hendriks

Correspondence: Dr. Rudi W. Hendriks, Department of Pulmonary Medicine Erasmus MC Rotterdam , 3000 CA Rotterdam, The Netherlands

---

Review Timeline:	Submission date:	17-Nov-2017
	First Editorial decision:	21-Dec-2017
	Revision received:	14-Mar-2018
	Accepted:	09-May-2018

Handling Executive Committee member: Prof. Annette Oxenius

Please note that the correspondence below does not include the standard editorial instructions regarding preparation and submission of revised manuscripts, only the scientific revisions requested and addressed.

First Editorial Decision
21-Dec-2017

Dear Mr. Li,

Manuscript ID eji.201747421 entitled "T cells and ILC2s are major effector cells in influenza-induced asthma exacerbation" which you submitted to the European Journal of Immunology has been reviewed. The comments of the referees are included at the bottom of this letter.

A revised version of your manuscript that takes into account the comments of the referees will be

reconsidered for publication. Should you disagree with any of the referees ' concerns, you should address this in your point-by-point response and provide solid scientific reasons for why you will not make the requested changes.

You should also pay close attention to the editorial comments included below. **In particular, please edit your figure legends to follow Journal standards as outlined in the editorial comments. Please state the number of independent experiments and the number of samples per experiment. For flow cytometry results, please also show the full gaing strategy. Failure to do this will result in delays in the re-review process.**

Please note that submitting a revision of your manuscript does not guarantee eventual acceptance, and that your revision will be re-reviewed by the referees before a decision is rendered.

If the revision of the paper is expected to take more than three months, please inform the editorial office. Revisions taking longer than six months may be assessed by new referee(s) to ensure the relevance and timeliness of the data.

Once again, thank you for submitting your manuscript to European Journal of Immunology and we look forward to receiving your revision.

Yours sincerely,
Marta Vuerich

On behalf of
Prof. Annette Oxenius

Dr. Marta Vuerich
Editorial Office
European Journal of Immunology
e-mail: ejied@wiley.com
www.eji-journal.eu

****

Reviewer: 1

Comments to the Author

European Journal of Immunology

Influenza virus infection is an important cause of severe asthma exacerbations, but it remains unclear how a Th1-mediated antiviral response triggers a prototypical Th2 disease. In this manuscript Dr. Hendriks and colleagues investigated CD4+ T cells and group 2 innate lymphoid cells (ILC2s) in influenza virus-infected mice and found that ILC2s accumulated in the lung rapidly after influenza virus infection, but the induction of IL-5 and IL-13 secretion was delayed and concomitant with T cell activation. In an influenza-induced asthma exacerbation model the authors further noticed an initial reduction of ILC2 numbers and cytokine production in the broncho-alveolar lavage compared to chronic house dust mite (HDM)-mediated airway inflammation alone. ILC2s had a phenotype characterized by low T1/ST2, ICOS, KLRG1 and CD25 expression, resembling naive ILC2s. Consequently, the contribution of ILC2s to type 2 cytokine production in the early stage of the influenza-induced asthma exacerbation was limited. In contrast, T cells showed increased IL-4 and IL-5 production when exposed to both HDM and influenza virus. Nevertheless, upon clearance of the virus, ILC2s regained an activated T1/ST2highICOShighKLRG1highCD25high phenotype paired with cytokine production and were major contributors to the type 2 cytokine milieu. Collectively, the observations by the authors nicely demonstrate that both T cells and ILC2s contribute to influenza-induced asthma exacerbation, but with different kinetics.
This is beautiful manuscript. Rationale and hypothesis clearly displayed, experiments well chosen and performed, claims are substantiated by the data. The work details important findings on how pulmonary viral infections contribute to pulmonary allergic reactions and clarifies some uncertainties and controversies regarding the involvement of ILC2. I only have minor comments that should be easy to address to strengthen the clarity and message of the paper.

ïƒ Figure 2B: Regarding the identification of ILC2 it would be important to extend the FACS gating and add/show expression for ST2, ICOS, Thy1, KLRG1.

ïƒ Figure 2E & S1: Are Gata3+ cells really ILC2? Gata3 is not a determining factor of ILC2 only as many cells in the lungs (including other ILCs, Th2, Treg cells). As such the data should be interpreted with more caution.

ïƒ Intracellular cytokine staining need to be shown with controls throughout the manuscript e.g. Figure 3B, Figure 5; isotype and FMO controls need to be shown for IL-5 and IL-13.

ïƒ The choice of the Influenza strain should be justified and discussed in light of other data published on this topic (Artis, Umetsu, Fritz, Metzger) â€“ how strain specific are the observations?

ïƒ Gating strategies for eosinophils, neutrophils, B and T cells together with ILC2 should be shown clearly (FACS plots gating and explained in the methods section with antibody clones).

ïƒ Were other cells implicated in type 2 immune responses such as type 2 DCs, basophils, mast cells, M2 macrophages analyzed? If the authors have data on it I would like to invite them to add it.

ïƒ Figure 4E: scale bars should be added to the pictures.

ïƒ All flow cytometry antibody clones and sources should be given in the methods sections. ILC2 staining and intracellular staining procedure for cytokine analysis should be detailed clearly. Also the tissue digestion protocol and live-dead/duplex exclusion should be written in more details.

Reviewer: 2

Comments to the Author
The authors characterize the contribution of ILC2 and CD4 T cells to type 2 immunity following infection with influenza and in a model of influenza-induced (HDM) asthma exacerbation.

The paper is largely descriptive and does not provide much mechanistic insight. On the other hand, the experiments are performed to a high standard and provide a good and thorough overview of type 2 immune cells in the two models.

The paper seems publishable in the Journal with one caveat. It is entirely unclear from the data if the infection of mice HDM pre-treated with influenza leads to asthma exacerbation (Figure 4). Airway resistance should be evaluated in the various treatment groups and correlated with type 2 immunity. Without these data the major claim of the paper is not supported by the data.

First Revision – authors' response

14_Mar-2018

Point-by-point reply to:

T cells and ILC2s are major effector cells in influenza-induced exacerbation of allergic airway inflammation

Bobby W.S. Li[1], Marjolein J.W. de Bruijn[1], Melanie Lukkes[1], Menno van Nimwegen[1], Ingrid M. Bergen[1], Corine H. GeurtsvanKessel[2], A. KleinJan[1], Arno Andeweg[2], Guus F. Rimmelzwaan[2] and Rudi W. Hendriks[1]
[1]Department of Pulmonary Medicine, Erasmus MC Rotterdam, Rotterdam, the Netherlands
[2]Department of Viroscience, Erasmus MC Rotterdam, Rotterdam, the Netherlands

European Journal of Immunology

Reviewer 1

Influenza virus infection is an important cause of severe asthma exacerbations, but it remains unclear how a Th1-mediated antiviral response triggers a prototypical Th2 disease. In this manuscript Dr. Hendriks and colleagues investigated $CD4^+$ T cells and group 2 innate lymphoid cells (ILC2s) in influenza virus-infected mice and found that ILC2s accumulated in the lung rapidly after influenza virus infection, but the induction of IL-5 and IL-13 secretion was delayed and concomitant with T cell activation. In an influenza-induced asthma exacerbation model the authors further noticed an initial reduction of ILC2 numbers and cytokine production in the broncho-alveolar lavage compared to chronic house dust mite (HDM)-mediated airway inflammation alone. ILC2s had a phenotype characterized by low T1/ST2, ICOS, KLRG1 and CD25 expression, resembling naïve ILC2s. Consequently, the contribution of ILC2s to type 2 cytokine production in the early stage of the influenza-induced asthma exacerbation was limited. In contrast, T cells showed increased IL-4 and IL-5 production when exposed to both HDM and influenza virus. Nevertheless, upon clearance of the virus, ILC2s regained an activated $T1/ST2^{high}ICOS^{high}KLRG1^{high}CD25^{high}$ phenotype paired with cytokine production and were major contributors to the type 2 cytokine milieu. Collectively, the observations by the authors nicely demonstrate that both T cells and ILC2s contribute to influenza-induced asthma exacerbation, but with different kinetics.
This is beautiful manuscript. Rationale and hypothesis clearly displayed, experiments well-chosen and performed, claims are substantiated by the data. The work details important findings on how pulmonary viral infections contribute to pulmonary allergic reactions and clarifies some uncertainties and controversies regarding the involvement of ILC2. I only have minor comments that should be easy to address to strengthen the clarity and message of the paper.
- Reply: We thank the referee for these positive remarks.

Comments

1. Figure 2B: Regarding the identification of ILC2 it would be important to extend the FACS gating and add/show expression for ST2, ICOS, Thy1, KLRG1.
- Reply: We have extended the FACS gating and now show surface expression of ICOS, KLRG1 and T1/ST2 on the gated ILC2 in Figure 2B. Figure legends are adapted accordingly. We have previously shown that ILC2 gated in this way consistently express high levels of Thy1 following IL-33 or HDM exposure (Li et al. Front Immunol. 2017).

2. Figure 2E & S1: Are Gata3+ cells really ILC2? Gata3 is not a determining factor of ILC2 only as many cells in the lungs (including other ILCs, Th2, Treg cells). As such the data should be interpreted with more caution.
- Reply: We agree that other cell types are known to express GATA3, including ILC1 and IL3, CD4 and CD8 cells. However, in our hands, confocal microscopy analysis only detects cells that express high levels of GATA3. We detect YFP only in a small fraction of T cells (Th2 cells) and not in all CD4+ and CD8+ T cells (which do express GATA3, but at low levels). We have now added a sentence to the legends of Figure 2 and Figure S1 to provide additional clarification.

3. Intracellular cytokine staining need to be shown with controls throughout the manuscript e.g. Figure 3B, Figure 5; isotype and FMO controls need to be shown for IL-5 and IL-13.

- Reply: As a standard procedure, we use gated cells of which we know that they are negative for the cytokine as a control, rather than isotypes or FMO. The point is here that controls such as isotypes or FMOs would not detect non-specific binding of the antibody. In the case of Figure 3B, we have placed the quadrant on the basis of cells of which we know are IL-5 and IL-13 negative (Lineage^{+}CD4^{-}).

4. The choice of the Influenza strain should be justified and discussed in light of other data published on this topic (Artis, Umetsu, Fritz, Metzger) – how strain specific are the observations?
- Reply: We thank the reviewer for this insight and have included an extra paragraph in the discussion regarding low- and high-pathological influenza virus strains (page 14, lines 21 – 25 and page 15, lines 1 – 2). Importantly, a marked innate response followed by an accumulation of eosinophils, neutrophils, dendritic cells and T cells was previously reported in an influenza virus infection model preceded by chronic exposure to HDM (Ravanetti et al. Allergy, 2016). In these experiments X31 was used, but ILC2s were not studied. We have now mention this in the introduction (page 5, line 14).

5. Gating strategies for eosinophils, neutrophils, B and T cells together with ILC2 should be shown clearly (FACS plots gating and explained in the methods section with antibody clones).
- Reply: We have added the FACS gating strategy of eosinophils, neutrophils, B cells, CD4+ T cells and CD8+ T cells as Supplementary Figure S3.

6. Were other cells implicated in type 2 immune responses such as type 2 DCs, basophils, mast cells, M2 macrophages analyzed? If the authors have data on it I would like to invite them to add it.
- Reply: We thank the reviewer for this suggestion. We have added histology data on mast cells in Figure 4F and have adapted the methods and results section accordingly. In addition, we have elaborated on the findings in the context of mast cell – ILC2 interaction in the discussion (page 16 lines 7 – 14).

7. Figure 4E: scale bars should be added to the pictures.
- Reply: Image scales were not recorded. However we have added the magnification factor in the figure legends.

8. All flow cytometry antibody clones and sources should be given in the methods sections. ILC2 staining and intracellular staining procedure for cytokine analysis should be detailed clearly. Also the tissue digestion protocol and live-dead/duplex exclusion should be written in more details.
- Reply: We now provide a list of antibodies (including clones and suppliers) used for flow cytometry and confocal microscopy in the supplementary data (Supplementary Tables S1 and S2). We have further clarified the tissue preparation and staining protocols as well as the gating strategy in the methods section (page 17, lines 10 – 20).

European Journal of Immunology

Reviewer 2

The authors characterize the contribution of ILC2 and CD4 T cells to type 2 immunity following infection with influenza and in a model of influenza-induced (HDM) asthma exacerbation.

The paper is largely descriptive and does not provide much mechanistic insight. On the other hand, the experiments are performed to a high standard and provide a good and thorough overview of type 2 immune cells in the two models.

The paper seems publishable in the Journal with one caveat. It is entirely unclear from the data if the infection of mice HDM pre-treated with influenza leads to asthma exacerbation (Figure 4). Airway resistance should be evaluated in the various treatment groups and correlated with type 2 immunity. Without these data the major claim of the paper is not supported by the data.

- Reply: We agree with reviewer 2 that airway resistance is an important parameter in the quantitative evaluation of asthma exacerbations. However, in our experience in the house dust mite models, lung function measurements are not highly sensitive. Therefore, we expect that detection of differences between chronic HDM alone and chronic HDM + influenza virus infection will require large numbers of mice. Moreover, given the complex kinetics in the contribution of T cells and IIC2, such an analysis should be done at several time points. A meaningful analysis of airway resistance would therefore require so many mice, that our ethical committee likely would not allow these experiments. Most importantly, these experiments would not address our key question which cells are responsible for the exacerbation of the type II response, T cells or ILC2? Nevertheless, we do agree with the referee that an increase in inflammation would not necessarily result in increased airway resistance. We have therefore changed the wording throughout the manuscript and replaced "asthma exacerbation" with "exacerbation of allergic airway inflammation".

Second Editorial Decision

09-Apr-2018

Dear Dr. Hendriks,

It is a pleasure to provisionally accept your manuscript entitled "T cells and ILC2s are major effector cells in influenza-induced exacerbation of allergic airway inflammation" for publication in the European Journal of Immunology. For final acceptance, please follow the instructions below and return the requested items as soon as possible as we cannot process your manuscript further until all items listed below are dealt with.

Please note that EJI articles are now published online a few days after final acceptance (see Accepted Articles: https://onlinelibrary.wiley.com/toc/15214141/0/ja). The files used for the Accepted Articles are the final files and information supplied by you in Manuscript Central. You should therefore check that all the information (including author names) is correct as changes will NOT be permitted until the proofs stage.

We look forward to hearing from you and thank you for submitting your manuscript to the European Journal of Immunology.

Yours sincerely,
Marta Vuerich

on behalf of
Prof. Annette Oxenius

Dr. Marta Vuerich
Editorial Office
European Journal of Immunology
e-mail: ejied@wiley.com
www.eji-journal.eu

Supplementary information to:

T cells and ILC2s are major effector cells in influenza-induced exacerbation of allergic airway inflammation

Bobby W.S. Li[1], Marjolein J.W. de Bruijn[1], Melanie Lukkes[1], Menno van Nimwegen[1], Ingrid M. Bergen[1], Corine H. GeurtsvanKessel[2], Alex KleinJan[1], Arno Andeweg[2], Guus F. Rimmelzwaan[2] and Rudi W. Hendriks[1]

[1]Department of Pulmonary Medicine, Erasmus MC Rotterdam, Rotterdam, the Netherlands

[2]Department of Viroscience, Erasmus MC Rotterdam, Rotterdam, the Netherlands

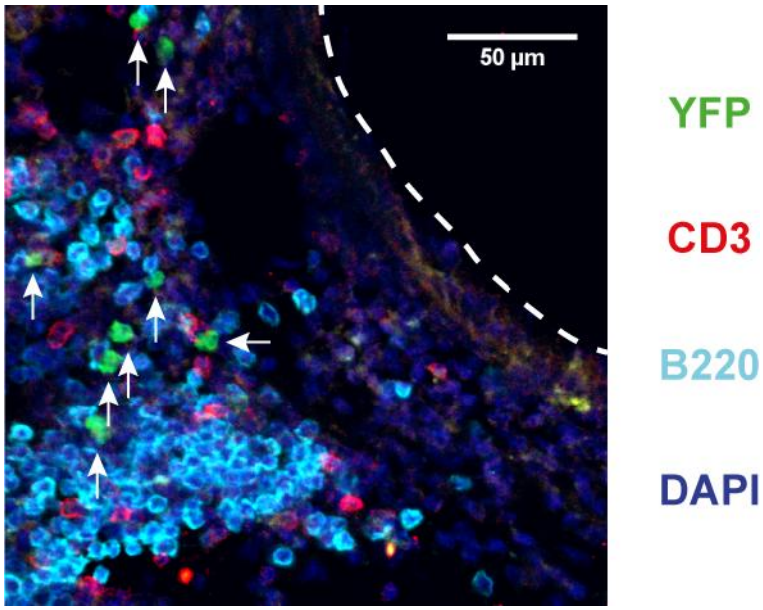

Figure S1. Severe inflammatory response to influenza recruits T cells, B220^{+} cells and ILC2s at day 4 post infection.

Lung cryosection from X31 influenza virus infected *Gata3* reporter mice counterstained with CD3 (T cells) and B220 (B cells and plasmacytoid dendritic cells). Airway epithelium is outlined by the dashed line. ILC2s, indicated with arrows, are identified as CD3^{-}YFP^{+} cells. In the confocal microscopy conditions, GATA3^{low} cells (such as non-Th2 CD4^{+} T cells, CD8^{+} T cells and other ILC lineages) are not detectable as YFP^{+} cells. Representative image of n = 3 mice per group from three independent experiments.

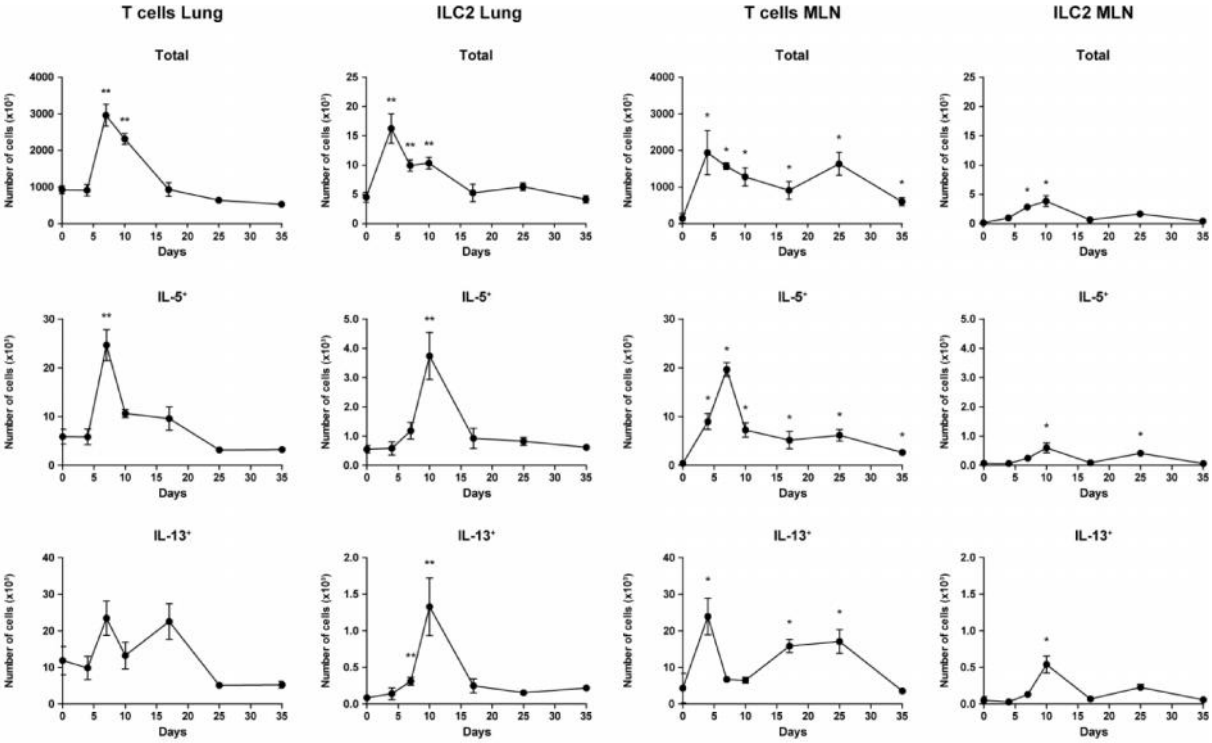

Figure S2. Kinetics of type 2 cytokine production by ILC2s and T cells after influenza virus infection. Quantification of the number of IL-5 and IL-13-producing T cells and ILC2s in the lungs and MLN of mice infected with X31 influenza virus. Data are shown as mean values ± SEM (n = 5) of a single experiment from two independent experiments; * $p \leq 0.05$, ** $p \leq 0.01$.

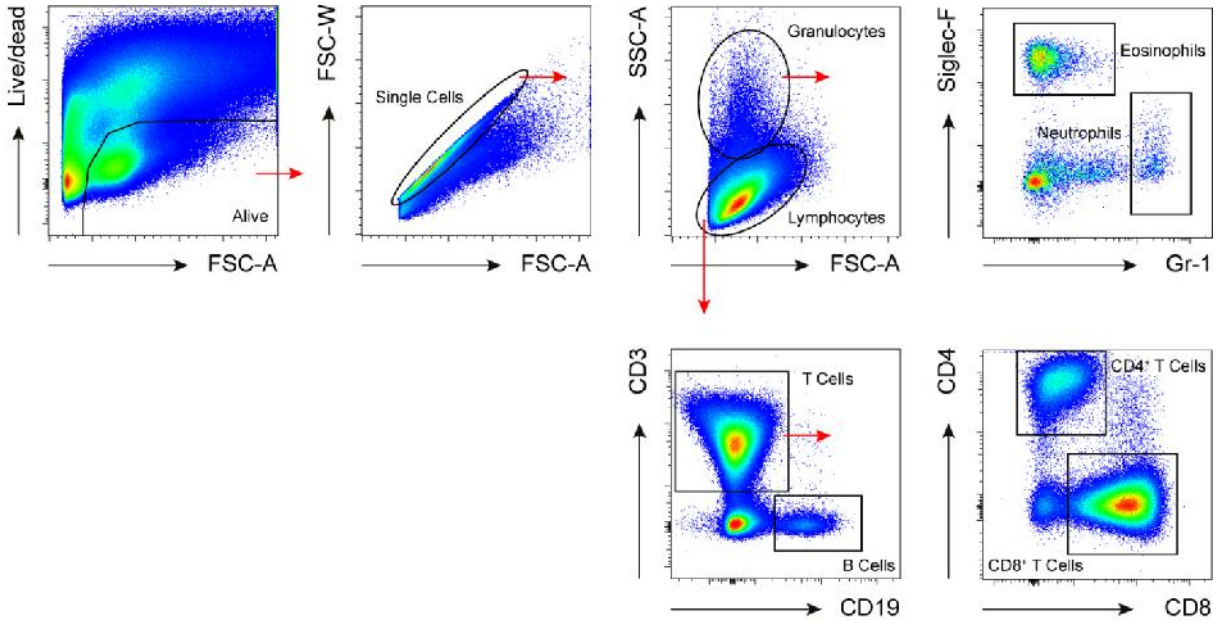

Figure S3. Flow cytometric analysis of eosinophils, neutrophils, B cells, CD4$^+$ T cells and CD8$^+$ T cells. Plots represent combined data from BAL fluid of HDM-treated and X31 virus-infected mice using the concatenate option in FlowJo (n = 7) of a single experiment, representative of multiple independent experiments.

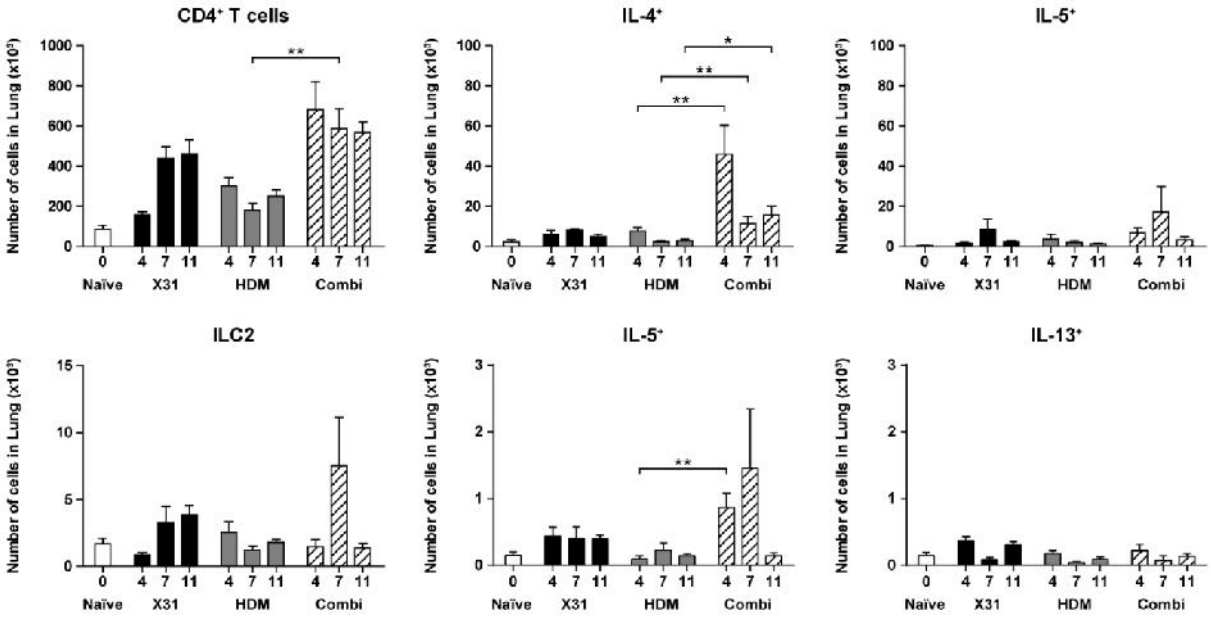

Figure S4. Synergistic effect of influenza virus infection in lungs of HDM-treated mice.

Quantification of the number of type 2 cytokine producing $CD4^+$ T cells (*top*) and ILC2s (*bottom*) in lungs. Not that there are significant synergistic effects of influenza virus infection on the induction of $IL\text{-}4^+$ T cells and $IL\text{-}5^+$ ILC2s in the lung. Data are shown as mean values ± SEM (n = 7) of a single experiment from two independent experiments. * $p \leq 0.05$, ** $p \leq 0.01$.

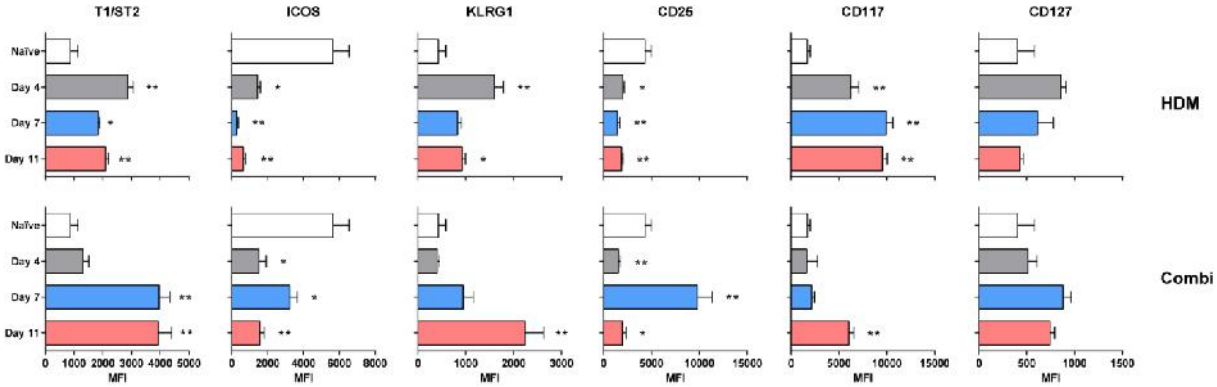

Figure S5. Quantification of surface expression of T1/ST2, ICOS, KLRG1, CD25, CD117 and CD127 on ILC2s in BAL fluid at day 0, day 4, day 7 and day 11 post influenza infection.

Mean fluorescence intensity (MFI) values corresponding to histograms shown in Figure 6. Data are shown as mean values ± SEM (n = 7) of a single experiment from two independent experiments. * $p \leq 0.05$, ** $p \leq 0.01$.

Table S1 Antibodies used for flow cytometry.

Antibody	Conjugate	Clone	Company
Amphiregulin	Biotin	Polyclonal	R&D Systems
B220	PE	RA3-6B2	eBioscience
CD117	Brilliant Violet 650	2B8	BD Biosciences
CD11b	PE	M1/70	eBioscience
CD11b	PerCP-Cy5.5	M1/70	BD Biosciences
CD11b	Alexa Fluor 700	M1/70	eBioscience
CD11c	PE	N418	eBioscience
CD11c	PE-Texas Red	N418	Invitrogen
CD11c	eFluor 450	N418	eBioscience
CD127	PE-Cy7	A7R34	eBioscience
CD127	eFluor 450	A7R34	eBioscience
CD19	PE	1D3	BD Biosciences
CD19	PerCP-Cy5.5	eBio1D3	eBioscience
CD19	Biotin	1D3	BD Biosciences
CD19	Alexa Fluor 700	eBio1D3	eBioscience
CD25	PerCP-Cy5.5	PC61.5	eBioscience
CD25	Brilliant Violet 605	PC61	BioLegend
CD3	PE	145-2c11	eBioscience
CD3	PE-CF594	145-2c11	BD Biosciences
CD4	Alexa Fluor 700	GK1.5	eBioscience
CD4	PerCP-Cy5.5	RM4-5	eBioscience
CD4	Brilliant Violet 605	RM4-5	BD Biosciences
CD4	Brilliant Violet 711	RM4-5	BD Biosciences
CD45	PE-CF594	I3/2.3	Abcam
CD45	Pe-Cy7	30-F11	eBioscience
CD5	PE	53-7.3	eBioscience
CD8	APC	53-7.3	eBioscience
CD8	APC-EF780	53-7.3	eBioscience
CD8	PE	53-6.7	eBioscience
CD86	PE-Cy7	GL1	BD Biosciences
CD90.2	FITC	53-2.1	BD Biosciences
FcεRIα	PE	MAR-1	eBioscience
FoxP3	Alexa Fluor 700	FJK-16s	eBioscience
FoxP3	PE-Cy7	FJK-16s	eBioscience
Gata3	eFluor 660	TWAJ-14	eBioscience
Gr-1	PE	RB6-8C5	BD Biosciences
Gr-1	APC-eFluor 780	RB6-8C5	eBioscience
ICOS	APC	C398.4A	eBioscience
ICOS	PE-Cy7	7E.17G9	eBioscience
IFN-γ	APC	XMG1.2	BD Biosciences
IFN-γ	Brilliant Violet 650	XMG1.2	BD Biosciences
IL-10	PerCP-Cy5.5	JES5-16E3	eBioscience
IL-13	eFluor 450	eBio13A	eBioscience
IL-13	eFluor 660	eBio13A	eBioscience
IL-17	Alexa Fluor 700	TC11-18H10.1	BD Biosciences
IL-4	Brilliant Violet 711	11B11	BD Biosciences
IL-4	Biotin	BVD4-1D11	eBioscience

IL-4	PE-Cy7	11B11	BD Biosciences
IL-5	APC	TRFK-5	BD Biosciences
IL-5	Biotin	TRFK4	BD Biosciences
IL-9	PerCP-Cy5.5	D9302C12	BD Biosciences
IL-9	PE	D9302C12	BD Biosciences
Ki-67	FITC	SolA15	eBioscience
Ki-67	Alexa Fluor 700	SolA15	eBioscience
KLRG1	APC	2F1	BD Biosciences
KLRG1	PE-CF594	2F1	BD Biosciences
MHCII	Alexa Fluor 700	M5/114.15.3	eBioscience
MHCII	Brilliant Violet 650	M5/114.15.2	BD Biosciences
NK1.1	PE	PK136	eBiosciences
NK1.1	APC	PK136	BD Biosciences
RORγt	PE	Q31-378	BD Biosciences
Sca-1	Pacific Blue	D7	BioLegend
Sca-1	Brilliant Violet 786	D7	BD Biosciences
Siglec-F	PE	E50-2440	BD Biosciences
Siglec-F	PE-CF594	E50-2440	BD Biosciences
Streptavidin	APC-eFluor 780		eBioscience
Streptavidin	Brilliant Violet 786		BD Biosciences
T1/ST2	Biotin	DJ8	MD Bioproducts
T1/ST2	FITC	DJ8	MD Bioproducts
T-bet	Brilliant Violet 421	O4-46	BD Biosciences
TER-119	PE	TER-119	eBioscience

Table S2 Antibodies used for confocal microscopy.

Antibody	**Conjugate**	**Clone**	**Company**
B220	Biotin	TY25	eBioscience
CD3	Biotin	145-2C11	BD Biosciences
αrat	Cy5	Polyclonal	Jackson ImmunoResearch
αhamster	Cy3	Polyclonal	Jackson ImmunoResearch