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IL-33 drives influenza-induced asthma exacerbations by halting innate and adaptive anti-viral immunity

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ABSTRACT

Background. Influenza virus triggers severe exacerbations of asthma for which no adequate treatment is available. It is known that IL-33 levels correlate with exacerbation severity, but its role in the immune-pathogenesis of exacerbations has remained elusive.

Objective. We hypothesized that IL-33 is necessary to drive asthma exacerbations. We intervened with the IL-33 cascade and sought to dissect its role, also in synergy with TSLP, in airway inflammation, anti-viral activity and lung function. We aimed to unveil the major source of IL-33 in the airways and IL-33-dependent mechanisms that underlie severe asthma exacerbation.

Methods: Mild asthmatic patients were experimentally infected with rhinovirus. Mice were chronically exposed to house dust mite (HDM) extract and then infected with influenza to resemble key features of exacerbations in humans. Interventions included anti-IL-33-receptor ST2 and/or anti-TSLP.

Results. We identified bronchial ciliated cells and Type-II alveolar cells as a major local source of IL-33 during virus-driven exacerbation in humans and mice, respectively. By blocking ST2 we demonstrated that IL-33 and not TSLP was necessary to drive exacerbations. IL-33 enhanced AHR and airway inflammation by suppressing innate and adaptive anti-viral responses and by instructing epithelial cells and dendritic cells (DCs) of HDM-sensitized mice to dampen IFN- β expression and prevent the Th1-promoting DCs phenotype. IL-33 also boosted luminal NETosis and halted cytolytic anti-viral activities, but did not affect the Th2-response.

Conclusion: Interventions targeting the IL-33/ST2 axis could prove an effective acute, short-term therapy for virus-induced asthma exacerbation.

Key Messages

- IL-33 drives influenza-induced severe exacerbation of experimental asthma while TSLP is redundant
- IL-33 suppresses innate and adaptive immunity to influenza and regulates the NETosis
- IL-33 instructs the airways epithelial cells and DCs to dampen the innate response to influenza and prevents the Th1-promoting DC phenotype.

Capsule Summary

IL-33 is implicated in asthma exacerbation but its role is unclear. We demonstrate that IL-33 drives influenza-induced asthma exacerbation suppressing anti-viral innate and adaptive responses in immune and epithelial cells.

Key Words

Asthma, exacerbation, IL-33, influenza, immune response, airway hyper-responsiveness, airways epithelial cells, NETosis, DCs.

Abbreviation

AHR	airway hyperresponsiveness
ASM	airway smooth muscle cells
ATII	type II alveolar cells
BAL	bronchoalveolar lavage
CDA	cell dense areas
DCs	dendritic cells
EC	epithelial cell
GrzA	granzyme A
HDM	house dust mite
IL	interleukin
ILC	innate lymphoid cell
IFN	interferon
iNKT	invariant natural killer T cell
LTi	lymphoid tissue inducer cells
mLN	mediastinal lymph nodes
MPO	myeloperoxidase
NET	neutrophil extracellular trap
NK	natural killer cell

82 PBEC primary bronchial epithelial cells
83 RV Rhinovirus
84 RSV Respiratory syncytial virus
85 Th1 T-helper 1
86 Th2 T-helper 2
87 TSLP thymic stromal lymphopoietin.
88

INTRODUCTION

Acute worsening of asthma symptoms (exacerbations), paralleled by exaggerated airway inflammation, represents a major burden to patients, health-care and the economy. Typically exacerbations are treated by enhanced dosing of inhaled corticosteroids¹, but severe exacerbations can be insensitive to inhaled, and even to systemic corticosteroids and thus constitute a major unmet medical need^{2,3}. Rhinovirus (RV), respiratory syncytial virus (RSV) and influenza A cause up to 80% of asthma exacerbations in children and 50% of exacerbations in adults⁴⁻⁶. Both defective type I and type III interferon (IFNs) anti-viral responses⁷⁻⁹ and exaggerated T-helper 2 (Th2) responses¹⁰ have been considered main drivers of asthma exacerbations. Recent studies have indicated that the alarmin IL-33, an IL-1 family member cytokine, is enhanced in asthma¹¹ and released in nasal lining fluid during virus-induced exacerbations, correlating with the inflammatory response and clinical symptoms¹⁰. A similar enhanced IL-33 production in lung tissue was shown in house dust mite (HDM)-sensitized mice infected with influenza-X31, a mouse model of severe influenza-induced asthma exacerbation that recapitulates all key features of the human disease, including the corticosteroid insensitivity of the inflammatory process^{2,12}.

We hypothesized that IL-33 drives this influenza-induced asthma exacerbation. IL-33 acts through a receptor complex known as ST2 and IL-1 receptor accessory protein to initiate MyD88-dependent inflammatory pathways^{13,14}. As the IL-33 receptor complex is present on a range of innate and adaptive immune cells, IL-33 can exert pleiotropic effects¹⁵⁻²⁰. Therefore, we analysed in detail its role in inflammatory processes during influenza-induced exacerbation and on various cell types, among which innate lymphoid cells (ILCs). Furthermore, as IL-33 was found to drive the severity of Respiratory Syncytial Virus (RSV) infections in naïve mice²¹, we also investigated the effect of IL-33 in naïve *versus* HDM-sensitized mice upon influenza infection. And finally, we also determined the role of thymic stromal lymphopoietin (TSLP), a mediator produced by bronchial epithelial cells and that synergizes with IL-33²²⁻²⁴, during virus-induced exacerbation of experimental asthma.

We demonstrated that IL-33 drives the increased airway hyperresponsiveness (AHR), viral replication and both lymphoid and myeloid populations infiltrate in airway lumen and parenchyma, whereas TSLP was redundant in this model. IL-33 suppressed Th1-like, innate anti-viral responses without affecting Th2-like responses and promoted NETosis during asthma exacerbation. Importantly, IL-33 instructed airway epithelial cells and DCs to dampen IFN- β expression and prevented the development of Th-1-promoting DCs phenotype. Therefore, blocking IL-33 could prove a novel acute therapy against virus-induced asthma exacerbations.

MATERIAL AND METHODS

Detailed Methods are available in the online supplement.

Mice, allergen, virus and treatments. All experiments described were approved by the Animal Research Ethics Board of the Academic Medical Center (protocol numbers DSK102810 and DSK49-AA) in adherence to the NIH Guide for the Care and Use of Laboratory Animals, performed according to institutional guidelines, EU and Netherlands legislation. Male 8 weeks old C57BL/6J mice purchased from Charles River Laboratories (France) were housed under specific pathogen-free conditions and maintained on a 12-hour light-dark cycle with access to food and water *ad libitum*. Groups of C57BL/6J mice were randomly assigned (Simple Randomization) to treatment for five days a week for five consecutive weeks with either the aeroallergen HDM²⁵ (Greer Laboratories, Lenoir, NC, USA) or NaCl through intranasal (i.n.) administration. Lyophilized HDM extract was re-suspended in sterile NaCl at a concentration of 5 mg (protein)/ml and 25 µg (5 µl) protein per dose per mouse was administered i.n. in a final volume of 20 µl (in NaCl) in mice lightly anesthetized with isofluorane. The H3N2 influenza A virus HK X31 stocks and viral titers were obtained by infecting Madin-Darby-Canine-Kidney (MDCK) cells as described before¹². The viral stock suspension was diluted in NaCl and animals (lightly anesthetized with isofluorane) received i.n. 20TCID₅₀ (50% of the tissue culture infective dose) in 50 µl. Anti-mouse ST2 (αST2), anti-mouse TSLP (αTSLP) and the isotype control antibodies, kindly provided from Drs. Dirk Smith and Michael C. Comeau²⁶, were re-suspended in sterile NaCl and 150, 350 or 250 µg in 100 µl/mouse, respectively, and administered when indicated via the intraperitoneal (i.p) route. Prednisone (Pred) (Sigma-Aldrich) was diluted in sterile NaCl and 5 mg/kg in 100 µl and administered separately via the intraperitoneal (i.p) route. Prednisone, αST2 or αTSLP were given at separate time points because of their intrinsic pharmacodynamics and pharmacokinetic characteristics. These treatments are all therapeutic and not prophylactic. The different schedules for each treatment are based on extensive pilot studies on HDM-sensitized mice and X31-infected mice (Respiratory R&D, GSK, Stevenage, UK; unpublished data kindly provided by Robert Shaw) and were expected to be efficacious, with evident beneficial anti-inflammatory effects, within the shortest period after administration and without eliciting serious side effects (i.e. body weight loss, dysregulation of immune functions).

Airway hyperresponsiveness measurements. AHR responses were assessed at the indicated time points by methacholine-induced airflow obstruction in conscious mice placed in a whole-body plethysmograph (Buxco Electronics, Troy, NY) as described previously¹².

Mouse trachea epithelial cell analysis. Tracheae from euthanized mice were longitudinally dissected and epithelial cells were collected by gentle brushing using a sterile, RNase-free,

cotton swap and vigorously re-suspended in sterile saline. Cells were pelleted at 240 g for 10 min at 4°C and used for RNA extraction with TRIzol (ThermoFisher scientific), following manufacturer's instructions.

Samples were analyzed for IFN- β expression by qPCR (see corresponding paragraph) and for IL-33 and TSLP expression by the Affymetrix GeneChip® Mouse Gene 2.1 ST array (Affymetrix, CA, USA) and analyzed as detailed in the online supplement. Details on collection and processing of other murine samples are in the online supplement.

***In vivo* rhinovirus-16 (RV16) challenge in healthy controls and asthma patients.** PBECs were obtained from 17 atopic individuals with mild asthma, steroid-naïve, and 4 non-atopic, non-asthmatic healthy individuals without a recent infection and no serum-neutralizing antibodies to RV16 at screening (≤ 1 in 4), before and 6 days after RV16 exposure (Y.S. Sabogal Pineros²⁷; A. Ravi accepted for publication; Sept 2018, AJRCCM). Clinical characteristics of asthmatic and healthy subjects recruited by strict inclusion and exclusion criteria are reported in **Supplementary Table 1**. The study protocol has been approved by the Medical Ethics committee of the Academic Medical Center, according to institutional guidelines, EU and Netherlands legislation. All subjects provided written informed consent and were successfully infected intranasally with 10TCID₅₀ RV16 in 750 μ l as based on WURSS scores and/or nasal swab viral load and/or seroconversion. Respiratory-symptom scores were recorded and bronchoscopy was performed at baseline and 6 days after RV16 inoculation.

Human primary bronchial epithelial cells (PBEC) analysis. Human PBEC were collected by bronchial brush during bronchoscopy and directly dissolved in 1ml of TRIzol™ (Invitrogen, ThermoFischer scientific). RNA was isolated, processed and sequenced as detailed in the online supplement.

Immuno-staining. Mouse lungs were collected at indicated time points post-infection (p.i.) without performing BAL and fixed for one day with 4% paraformaldehyde in PBS. Lung tissues were paraffin-embedded and stained with anti-IL-33 as detailed in the online supplement. For the NET immune-reactivity, lung sections were stained in blocking buffer with rabbit antibodies to histone H3 citrullinated (H3Cit; Abcam; ab5103) and with goat antibodies to MPO (R&D system), 2 hours at 37°C. After washing samples with PBS, secondary donkey anti-rabbit and anti-goat IgG antibodies conjugated with AlexaFluor-568 (ThermoFisher) and AlexaFluor 488 (ThermoFisher) were added in blocking buffer containing DAPI (R&D system) and incubated for 2 hours in the dark. Finally, samples were mounted with 10 μ l of ProLong Antifade reagent (ThermoFisher) on glass slides and stored at room temperature in the dark overnight.

For staining with anti-IL-33 of human primary bronchial epithelial cells, brush biopsies were collected during bronchoscopy in 1ml of sterile NaCl, cytopspins were prepared (2 minutes at 550 rpm), fixed with 4% paraformaldehyde for 10 minutes, permeabilized with 0.1% Tween 20 for 10 minutes and incubated for 1 hour with 10% goat serum to block aspecific binding sites. The slides were incubated with 1:250 dilution of anti-IL-33 monoclonal mouse IgG1 (Nessy -1; #ALX-804-840/1) or mouse isotype control (Dako, #X0931) for 1hour and subsequently with AF568 goat anti- mouse (#A-11004) for 1 hour and treated with DAPI prolong gold anti-fade reagent (ThermoFisher).

All samples were analyzed by fluorescence microscopy using standard filter sets. Controls were stained with secondary antibody only and non-specific fluorescence staining was not detected when secondary antibodies were tested alone. Images were acquired on a LEICA TCS SP8-SMD confocal microscope.

Flow cytometry analysis and sorting. BAL, lungs, blood, mLNs or spleen single cell suspensions were stained with different mixtures (as indicated in the online supplement) of α GalCer-loaded CD1d tetramer (proImmune) and fluorochrome-conjugated anti-mouse antibodies (clone id in brackets) against CD3 (145-2C11), CD4 (GK1.5), CD8 (53-6.7), CD19 (1D3), SiglecF (E50-2440), CD44 (IM7), CD117 (2B8), LY-6A/E (D7), CD127 (SB/199), NK1.1 (PK136), CD103 (M290), CD86 (GL-1), OX40L (RM104L) (all from BD Biosciences); CCR3 (83101), IL-17BR (752101), CD101 (307707) (all from R&D systems); MHCII (M5/144.15.2), NKp46 (29A1.4), ST2 (RMST2-2), CD80 (16-10A1), CD11c (N418), CD11b (M1/70) (all from eBioscience); CD45 (30-F11), CD200R3 (Ba13), CD86 (GL-1), Ly-6G (1A8), CD169 (3D6.112), CD64 (X54-5/7.1), CD103 (2E7) (all from BioLegend). Additional information is given in the online supplement.

Ex vivo cells stimulation for intracellular cytokines staining. Freshly isolated lung cells were stimulated for 6 hours with UV-inactivated influenza X31 (20TCID₅₀/ml), Der-p1 (0.5 μ g/ml) and LPS (0.5 μ g/ml) or with PMA (10 ng/ml; Sigma) and ionomycin (500 nM; Merck) in the presence of GolgiPlug (BD) for the final 4 hours of culture. A Cytofix/Cytoperm kit (BD) was used for cell permeabilization, staining and subsequent washing. The following antibodies (clone id in brackets) were used at the concentrations recommended by the manufacturers: IL-21 (FFA21), IL-13 (eBio13A), IFN- γ (XMG1.2) (eBioscience); IL-17 (TC11-18H10.1) (BioLegend); IL-6 (MP5-20F3) (BD Biosciences). Data were acquired on an LSRFortessa or FACSCanto II instrument and were analyzed with FlowJo software.

Gene expression assessment by qPCR. Total RNA was extracted from PBECS, tracheal epithelial cells (EC), CD11b+DCs, moDCs or lung cell suspension with TRIzol, according to

manufacturer instructions (ThermoFisher scientific), and 200 ng was reverse-transcribed for cDNA (complementary DNA) synthesis (SuperScript™, First-Strand Synthesis System for RT-PCR, Invitrogen, ThermoFisher scientific). Quantitative PCR was performed with both primers and probe map within a single exon for IL-12p35 (il12a, assay ID Mm00434169) and IFN- β (ifnb1, assay ID Mm00439552) according to manufacturer instructions (ThermoFisher scientific). Each gene was normalized to HPRT (hpert, assay ID Mm03024075_m1, ThermoFisher scientific) and expressed as copies per μ L of cDNA reaction.

Determination of viral outgrowth by qPCR. Viral load was determined by real-time quantitative PCR in lung cell suspension as described previously¹². The viral load present in a sample was calculated using a standard curve of particle-coated influenza virus included in every assay run. The following primers were used: 5'- GACAAGACCAATCCTGTCACYTCTG -3' (forward); 5'- AAGCGTCTACGCTGCAGTCC -3' (reverse), 5'- TTCACGCTCACCGTGCCCAGTGAGC -3' (50-FAM labeled probe).

Histopathological analysis. The left lungs from four additional animals per group, not having undergone BAL and not used for FACS analysis, were harvested at the indicated time points, fixed overnight in 10% formalin and embedded in paraffin. Sections (4 μ m) were stained with hematoxylin and eosin (H&E) and Periodic Acid-Schiff (PAS). Multiple images of the primary airway were taken with OpenLab software (version 3.0.3; Improvision) via a camera and microscope (Leica Microsystems).

Cytokine and chemokines analysis. Levels of cytokines, chemokines and soluble mediators in BAL and lung homogenates were measured using a Luminex 100 Total System based on xMAP multiplexing technology with Bio-Plex Pro™ Mouse Cytokine Th17 Panel B and 8-Plex Group III kits (Bio-Rad Laboratories Ltd. UK) or by ELISA (Granzyme B Mouse Uncoated ELISA Kit, Invitrogen, Thermofisher; Mouse Myeloperoxidase DuoSet ELISA, R&D system) according to the manufacture's specifications.

Data analysis. Data are expressed as indicated. Results were interpreted using Prism 5 (GraphPad). Differences were considered statistically significant when $p < 0.05$. Experiments were conducted with four to six mice per group per time point and data are representative for four independent replicates unless differently indicated. The numbers of mice used are indicated in the figure legends.

RESULTS

IL-33 drives airway hyperresponsiveness, inflammation and Th2 immunity in experimental chronic allergic asthma

To test the hypothesis that IL-33 drives allergic airways inflammation and asthma exacerbations, we first addressed the role of IL-33 in sustaining chronic airway inflammation in HDM-sensitized mice²⁵ (**Supplementary Fig. 1a**). Treatment with an anti-ST2 antibody (α ST2) abrogated airway hyperresponsiveness (AHR) (**Fig. 1a**), to a similar extent as systemic corticosteroids (prednisone; Pred). Similarly, α ST2 and prednisone significantly reduced numbers of total bronchoalveolar lavage (BAL) cells, as well as total and inflammatory (CD101+)²⁸ eosinophils, neutrophils, alveolar macrophages and CD11b+c DCs in BAL (**Fig. 1b**). In lung parenchyma, α ST2 and prednisone reduced numbers of CD4+ lymphocytes, innate natural killer T cells (iNKT) and efficiently abrogated intracellular IL-13 in both innate and adaptive lymphocytes. Notably, only α ST2 and not prednisone reduced the numbers of natural killer (NK) cells and efficiently abrogated intracellular IL-17, but not that of IFN- γ (**Supplementary Fig. 2**). Importantly, type 2 innate lymphoid cells (ILC2) and natural cytotoxicity receptor (NCR)+ILC3 were corticosteroid-resistant. Numbers of lung ILC2 and NCR+ILC3 were neither affected by α ST2, but IL-33 signalling drove IL-13 expression in ILC2 and suppressed intracellular IFN- γ in NCR+ILC3 (**Fig. 1b**).

Lung epithelial cells are a major source of IL-33 in virus-induced asthma exacerbation in mice and humans

Next, we analyzed the presence of IL-33 and the innate lymphocyte profile during virus-induced asthma exacerbations. Bronchial epithelial cells, predominantly ciliated cells, from asthma patients 6 days after being inoculated *in vivo* with RV16, showed enhanced expression of IL-33 protein (**Fig. 2a**). Even 6 days post-infection IL-33 gene expression was still significantly increased in primary bronchial epithelial cells (PBEC) from mild asthma patients as compared to healthy controls (**Fig. 2b**) supporting findings in the mouse model of severe asthma exacerbation¹² (**Supplementary Fig. 1b**), where influenza enhanced the transcription of IL-33 in tracheal epithelial cells (EC) compared to controls (HDM or NaCl/X31 at day 4 post-influenza) (**Supplementary Fig. 3a**). Notably, IL-33 gene expression levels in PBEC from mild asthma patients were not different before and 6 days after RV16 infection (data not shown). In lungs of HDM/X31-treated mice the major source of IL-33 are type II alveolar cells (ATII) (**Fig. 2c**), where IL-33 loss, likely associated with its release²⁹, is restricted to patchy areas of aggravated inflammation (cells dense areas, CDA). Gradual but not complete loss of IL-33 in CDA occurred upon influenza infection and was enhanced by HDM chronic exposure resulting in cell dense areas with complete lack of IL-33 (**Fig. 2c, d**).

ILC2 numbers are not enhanced and do not correlate with disease severity in a mouse model of influenza-induced asthma exacerbation

Previously we have shown that myeloid innate cells (eosinophils, neutrophils) and lymphocytes were increased during influenza-induced asthma exacerbations¹². Given the crucial role of IL-33 in driving innate immune responses³⁰, we extended our earlier¹² analyses to other innate immune cells. Compared to HDM-sensitized mice, ILC2 were not increased after influenza infection at any time, in BAL or lung parenchyma (**Fig. 2e**), nor in spleen and mediastinal lymph nodes (mLN) (data not shown). ILC2 numbers did not correlate with any key feature of asthma exacerbations such as AHR, BAL inflammatory cell numbers, nor with lung IL-33 (data not shown). In the early phase of the response to influenza in HDM-sensitized mice, BAL NK cells were enhanced and so were iNKT cells and basophils in lung parenchyma. Two subsets of ILC3, NCR+ILC3 and CD4+Lymphoid tissue inducer cells (LTi)³¹⁻³³, respectively, were significantly reduced in lung tissue at day 1 and at day 6, and increased at day 6 p.i. (**Supplementary Fig. 3b**). Together these results show that experimental severe asthma exacerbations do not correlate with robust changes of ILC2 numbers within the airways, but instead do correlate with early decreased NCR+ILC3 numbers and enhanced basophil, NK, iNKT and LTi numbers.

IL-33 is necessary to drive influenza-induced exacerbation of experimental asthma while TSLP is redundant

The cytokine TSLP, proposed to promote Th2 airway inflammation and induce corticosteroid resistance in a subset of innate lymphocytes, is known to synergize with IL-33^{23, 34, 35}. TSLP protein was increased in lung parenchyma of HDM-sensitized mice (independent of influenza infection) compared to naïve mice between days 1 and 4 post-influenza infection (**Supplementary Fig. 4a**). In contrast, TSLP protein significantly increased 8 days p.i. in naïve mice but not in HDM-sensitized mice. Interestingly, transcriptome analysis of EC revealed no differences between groups with respect to TSLP mRNA expression (**Supplementary Fig. 4b**). TSLP mRNA was not determined in lung homogenate and specific immune cells. Next, to dissect the individual or combinatorial contributions of IL-33 and TSLP in driving the exacerbation, HDM/X31 mice were treated with either α ST2 or α TSLP 3 days before influenza infection and every 3 days thereafter. In addition, one group received isotype control antibodies and another group both α ST2 and α TSLP antibodies to address the potential synergism between IL-33 and TSLP (**Supplementary Fig. 1c**). Blocking both pathways attenuated AHR changes at day 7 p.i. Interestingly, α TSLP worsened AHR compared to the other groups (**Fig. 3a**). Body weight loss was markedly reduced upon treatment with α ST2 or α ST2 in combination with α TSLP, as compared to α TSLP only and isotype control (**Fig. 3b**). The viral load was partially reduced in

mice treated with α TSLP at day 6 but more markedly reduced upon α ST2 treatment at day 8 p.i. compared to both the isotype controls and those treated with both antibodies (**Fig. 3c**). We extensively evaluated the inflammatory infiltrate in BAL, lung parenchyma and mediastinal lymph nodes (mLNs). Either α ST2 or α ST2 in combination with α TSLP significantly reduced numbers of total BAL cells, eosinophils, neutrophils, alveolar macrophages, CD4+, CD8+, B lymphocytes, basophils, NK cells on days 6 and 8. CD11b+cDCs, iNKT cells, ILC2, NCR+ILC3, LTi were inhibited on either one of these days (**Fig. 3d**). Interestingly, blocking the IL-33 pathway did not affect the inflammatory eosinophil population (CD101+)²⁸, but reduced the potentially antigen-presenting eosinophils (CD86+MHCII+) in BAL (**Supplementary Fig. 5a**). Furthermore, α ST2 attenuated inflammatory cells in lung parenchyma (**Supplementary Fig. 5b**), whereas CD101+ or CD86+MHCII+ eosinophils were not affected (**Supplementary Fig. 5c**). α ST2 also attenuated inflammatory cells at day 6 in mLN (**Supplementary Fig. 6a**), which was also clearly reflected macroscopically (**Fig. 3e**). α TSLP did not affect cell numbers in BAL, lung and mLNs, although there appeared to be impaired immune trafficking and a partial retention of myeloid and lymphoid cell subsets in mLNs (**Supplementary Fig. 6a**), which was clearer in spleen (**Supplementary Fig. 6b**). Thus IL-33, but not TSLP, drives experimental asthma exacerbation, enhances airway inflammation and increases viral load in lung, resulting in a more severe response to influenza in HDM-sensitized mice. In contrast, TSLP did not markedly contribute to inflammatory cells numbers, but reduced AHR synergistically with IL-33. Unexpectedly, TSLP appeared to direct immune trafficking in lymphoid organs (**Supplementary Fig. 6a, b**).

IL-33 drives early and late, innate and adaptive airways inflammation in influenza-induced exacerbation of experimental asthma

Previously we have shown that the early (14h to 2 days p.i.) immune response of allergic mice infected with influenza was clearly distinct from that of controls¹². This led us to focus on IL-33-dependent events shortly after viral challenge (**Supplementary Fig. 1d**). α ST2 treatment was sufficient to significantly reduce the AHR at days 3 and 6 p.i. (**Fig. 4a**), but not at day 7 p.i. (**Fig. 3a**), and resulted in reduced numbers of BAL total cells, eosinophils, neutrophils, alveolar macrophages, total lymphocytes (data of latter not shown), CD4+ T cells and iNKT cells at most early time points (**Fig. 4b**). Biphasic reduction, at 14h to 1 day and again at 6 to 8 days p.i., was seen for BAL basophils, NCR+ILC3, LTi, and NK cells. Interestingly, the ILC2 population was not affected at early time points (**Fig. 4b**). In mLNs, α ST2 lowered numbers of myeloid and lymphoid cell populations in a specific biphasic kinetic response (1 day and 6 days p.i.) in comparison to controls (**Supplementary Fig. 7a**), as opposed to an early, short cellular decrease in lung parenchyma (**Supplementary Fig. 7b**). We confirmed the results for days 6 and 8 p.i., as reported in **Fig. 3**. The resolution of airway inflammation and pathology (i.e. mucus over-

production) by blocking IL-33 signalling were also strikingly evident by histological examination of lungs (8 days p.i.) (**Fig. 4c**) (**Supplementary Fig. 8**).

IL-33 halts innate and adaptive anti-viral immunity, boosts NETosis but does not affect Th-2 response in influenza-induced asthma exacerbation

We examined whether cellular responses were paralleled by changes in inflammatory mediator and cytotoxic profiles within lung parenchyma and BAL. Treatment with α ST2 resulted in an early significant reduction in BAL for the cytokine IL-12p40 (14 hours and day 1 p.i.) and later (day 4 p.i.) for the chemokines MIP-1 α , MIP-1 β and cytokine IL-6 (day 6 p.i.), however the Th2 cytokine IL-5 was not significantly reduced (**Supplementary Fig. 7c**). In lung homogenates IL-33 was markedly reduced in the early phase (14h to day 1 p.i.) after α ST2 (**Fig. 4d**). Notably, α ST2 increased concentrations of IL-1 α (data not shown) and IL-1 β , IL-23, IL-22 (cytokines involved in lung tissue repair processes), Th1 cytokines IL-21, IL-12p70, IFN- γ (day 2 p.i.), IL-12p40 (day 8 p.i.) and RANTES (14 hours and day 4 to 6 p.i.) (**Fig. 4d**) (**Supplementary Fig. 7d**). This inhibitory effect of IL-33 on Th1 cytokines, IL-22 and IL-23 is also confirmed by the negative correlations in exacerbating mice (**Supplementary Fig. 7e**). Importantly, IL-5 and IL-13, together with IL-9 (involved in Th2 inflammation and lung remodelling) were not affected by α ST2 whereas IL-31, involved also in limiting the Th2 inflammation³⁶ was significantly reduced (day 6 p.i.). The chemokines MCP-1/CCL2, MIP-1 α , MIP-1 β , KC (all at day 6 p.i.) and eotaxin (days 1 to 2 p.i.), were significantly induced by α ST2 compared to controls (**Supplementary Fig. 7d**). Strikingly, intracellular IL-13 production was not reduced by α ST2 in CD4⁺ nor CD8⁺ T cells from lungs of exacerbating mice, whereas Th1 cytokines levels (IFN- γ and IL-21) were elevated in both cell-subsets (day 6 p.i.) (**Supplementary Fig. 7f**). Together these data demonstrate that, in exacerbating mice, IL-33 halted innate and adaptive Th-1 responses and did not promote Th2-like responses. Although IL-33 blockade significantly reduced the numbers of Th2-polarized CD4⁺T cells (ST2+CD4⁺T cells) in BAL (over time) and in lung (14h and day 1 p.i.) (**Supplementary Fig. 9**), it did not attenuate Th2 mediators and thus did not drive Th2 airway inflammation in influenza-induced asthma exacerbation. As IL-33 exerts differential effects in HDM-sensitized mice (**Fig. 1**) and influenza-challenged HDM-sensitized mice, the effect of IL-33 thus appears to depend on the local environment where it is released.

IL-33 suppresses the antiviral immunity to influenza by instructing EC and DCs of HDM-sensitized mice

We hypothesized that IL-33 silences Th1 cell-mediated immunity via the induction of OX40L on DCs and the reduction in IL-12p35 production, involved in Th2 cells polarization³⁷ and in promoting Th1 immunity^{38, 39}, respectively. Importantly, we demonstrated that in exacerbating

mice with increased IL-33 levels, flow-sorted lung moDCs and CD11b⁺ cDCs up-regulated OX40L and the costimulatory molecule CD86 (days 4 and 6 p.i.) compared to DCs from mice that received α ST2 (**Fig. 4e**). On the contrary, IL-33 down-regulated IL-12p35 mRNA in flow-sorted lung moDCs (**Fig. 4f**). Thus, blocking IL-33 signalling was able to reduce OX40L and restore IL-12p35 expression. These data demonstrate that the α ST2/IL-33 axis determines the skewing of the Th2/Th1 polarization of the immune response promoted by DCs.

Consistent with previous human studies that demonstrated a negative correlation between innate interferons level and asthma exacerbation^{40, 41}, in epithelial cells from chronically HDM-exposed mice we found a deficient IFN- β response upon influenza infection (day 4 p.i.) as compared to controls (**Supplementary Fig. 10**). Surprisingly, inhibition of IL-33 signalling restored IFN- β expression in EC and CD11b⁺cDCs sorted from lung of exacerbating mice (**Fig. 4g**). Therefore, we concluded that IL-33 halts the innate anti-viral response not only in immune cells but also in structural lung cells.

IL-33 also significantly enhanced levels of mediators involved in recruitment of lymphocytes and neutrophils (RANTES, MCP-1, MIP-1 α , MIP-1 β , KC, IL-1 β), which can exert cytotoxic anti-microbial functions when activated^{18, 42}. IL-33 attenuated anti-viral responses of lymphocytes and neutrophils of HDM/X31 mice, as reflected by reduced levels of granzyme A (GrzA), elastase and myeloperoxidase (MPO) (day 6 p.i.) in lung parenchyma (where viral replication occurs). IL-33, however, boosted GrzA and elastase in BAL from exacerbating mice, suggestive of enhanced cytotoxic activity in the airway lumen, potentially harmful for the mucosal barrier integrity (**Fig. 4h**). This observation is of particular interest because neutrophil-derived elastase and MPO in airway lumen are reported to be associated with NETosis-dependent airflow obstruction and asthma exacerbation^{43, 44} (**Fig. 4i**). NETs were abundantly present in the lungs of exacerbating mice at day 4 p.i.. Treatment with α ST2 abolished NET and MPO/Cit-H3 proteins detection at the mucosal surface. Together these findings indicate that IL-33 reduced anti-viral responses of lymphocytes and neutrophils in the parenchyma and promoted NETosis and lytic activity at the mucosal surface^{45, 46}. Further mechanistic *in vitro* studies, however, are required to substantiate these findings.

IL-33 drives innate and adaptive airway inflammation and suppresses anti-viral immunity to influenza in naïve mice

To address whether IL-33 also attenuated anti-viral and Th1 responses during acute influenza infection in naïve mice, α ST2 was given 3 days before influenza infection and every 3 days thereafter. Control mice received isotype control antibodies and all mice were analysed at days 4, 6 and 8 p.i. (**Supplementary Fig. 1e**). No difference in body-weight loss was noted between α ST2-treated and control mice (**Supplementary Fig. 11a**) and AHR did not differ (data not

shown), but α ST2-treated mice had markedly reduced viral loads (**Fig. 5a**). Furthermore, total BAL cell numbers (day 6 p.i.) and eosinophils, neutrophils (days 4 to 6 p.i.), alveolar macrophages (day 6 p.i.), T lymphocytes (day 8 p.i.), NK and iNKT cells (days 6 to 8, p.i.), NCR+ILC3 (days 4 to 8 p.i.) (**Fig. 5b**), basophils (data not shown), but not ILC2 (data not shown) were reduced by α ST2. In lung parenchyma, α ST2 significantly reduced ILC2, NK, iNKT and neutrophil numbers (day 8 p.i.) compared to that in control animals (**Supplementary Fig. 11b**). In parallel, α ST2 increased levels of IL-21, IL-22, IL-23, IL-31 and IFN- γ in lung parenchyma (**Fig. 5c**) and significantly reduced lymphocyte- or neutrophil-dependent cytotoxic enzymes concentrations in BAL (**Fig. 5d**). These findings correspond with our observations for α ST2 in HDM-sensitized mice infected with X31 (**Fig. 4**) in that IL-33 dampens anti-viral responses, resulting in prolonged, aggravated airway inflammation underlying the asthma exacerbation.

DISCUSSION

Despite evidence that the viral load correlates with disease severity, virus-induced asthma exacerbations are considered mostly driven by amplified Th2 responses rather than inefficient Th1 antiviral responses^{10, 44}. Our findings clearly indicate that IL-33 drives virus-induced asthma exacerbations mainly by dampening innate and adaptive Th1-like and cytotoxic responses and not by enhancing Th2-like responses. IL-33 reduced Th1-like innate cell populations and significantly reduced innate Th1 cytokines/chemokines levels that determine Th1 competence of lymphocytes (IL-12, IL-21, RANTES, IL-23, IFN- γ) and of myeloid cells^{47, 48} (MIP-3 α , MIP-1 β , KC, IFN- γ , IL-12) (**Fig. 4d**) (**Supplementary Fig. 7c, d**). IL-33 also suppressed IFN- β expression in both structural cells and CD11b+cDCs, which provide the earliest response to a viral challenge (**Fig. 4g**). Finally, IL-33 attenuated anti-microbial activities of cytotoxic T cells and neutrophils in lung tissue and induced NETosis in the lumen. In line with these attenuated innate and adaptive Th1-like and cytotoxic responses, the influenza load increased in lung tissue of HDM-sensitized and naïve mice in an IL-33-dependent manner (**Fig. 3c, Fig. 5a**).

Some of the mechanisms by which IL-33 dampens the anti-viral responses in this asthma exacerbation model are elucidated here. We found that IL-33 severely impaired IL-12p35 expression, the predominant cytokine driving Th1 cell development, whereas it upregulated the Th2-driving OX40L expression^{38, 39}. Type-I interferons like IFN- β stimulate the expression of many genes, which induce an anti-viral state to limit viral replication. IFN- β also enhances the antiviral mechanism and cytolytic activity of T-cells by inducing granzyme expression⁴⁹. Several reports have indicated that both bronchial epithelial cells and circulating immune cells from asthma patients are defective in innate interferon production^{40, 50}. These observations, however, are disputed as other groups failed to find defective innate interferon production⁵¹. Our findings could provide an explanation for this as the effect of IL-33 on cells may be attenuated after prolonged *in vitro* culturing of the sampled cells. In a very recent article it was proposed that IL-33 could predispose to RV-induced exacerbation in cockroach-sensitized and pneumonia virus-infected mice. Although no data was presented that RV indeed induced an asthma exacerbation with its fundamental characteristics (increases of AHR, lung pathology, lymphocytes infiltrate and viral load), treatment with an anti-IL-33 antibody resulted in reduced *ifn- α* and *ifn- λ* expression⁵².

We propose a broad indirect effect of IL-33 on IFN- β -dependent immune events. Inhibiting IL-33 restored IFN- β expression in structural and immune cells, with in parallel increased IFN- γ production by T-cells and increased granzyme-A expression in lung parenchyma. The exact mechanism by which IL-33 inhibits IFN- β awaits further studies. The IL-33-mediated reduced cytotoxic anti-viral processes in lung parenchyma may also relate to the reduced IFN- β

expression. It is unclear whether the increased cytotoxicity in BAL and NETosis relate directly to IL-33 or to the enhanced viral loads (**Fig. 4i**).

IL-33 release is enhanced very early after viral challenge and drives all cellular increases in BAL, lung tissue and mLNs within two days p.i.. Interestingly, the high levels of IL-33 release are also paralleled by *de novo* *il-33* transcription. Despite this release, four days after the viral challenge α ST2 did not influence the recruitment of all cell populations in lung parenchyma with the same magnitude. This indicates that 4 days p.i. also other mechanisms than IL-33 contribute to exacerbations, which suggests that intervention therapies aimed at IL-33 may be optimal only shortly after infection.

α ST2 reduced IL-33 levels, which is suggestive of autocrine IL-33 production. IL-33 was released predominantly in cell-dense areas, reflecting areas of acute inflammation (**Fig. 2c, d**)²⁹. The enhanced expression of IL-33 in asthma and after viral inoculation was shown in mice and asthma patients, although the source of IL-33 in patients appeared to be ciliated epithelial cells with some positive IL-33 staining within basal cells, whereas in mice IL-33 was found in alveolar type II cells. These findings contrast with IL-33 staining in basal cells in COPD patients²⁹, which suggest that the pathogenic role of structural cells differs between asthma and COPD.

Our studies show that in experimental asthma IL-33 promotes Th2-like responses, but upon influenza infection there was no increase, nor decrease in Th2-like responses despite enhanced levels of IL-33. ILC2 are considered to amplify Th2 airway inflammation in response to IL-33, but ILC2 were not increased during X31-induced exacerbations and importantly did not correlate with IL-33 concentration and exacerbation severity. We demonstrate for the first time that ILC2 and NCR+ILC3 were the only prednisone-resistant subsets in experimental chronic asthma. Therefore, we speculate that ILC2 still release Th2 mediators that maintain Th-2 inflammation during asthma exacerbation. It would be of interest to further clarify this *in vivo*. ILC2 and ILC3 are plastic subsets and they could potentially turn into ILC1 upon influenza infection, but these were not increased either^{53, 54}. To address these issues it would be of interest to assess the kinetics of cytokine production by ILCs subsets *ex vivo*. Despite reduction of Th2-promoting OX40L expression on CD11b+cDCs and moDCs, and reduced numbers of Th2-polarized CD4+T cells by α ST2, the Th2-cytokine levels in the airway were not affected until day 8 p.i.. So, the Th2-like responses appear quite stable as neither an increase nor a reduction of IL-33 changed Th2-like responses. We consider it likely that a reduction of Th2-like responses could occur at later time points, e.g. once the IL-33-dependent DCs programming has been reset. In another study with a different mouse strain (BALB/c) however, blocking IL-33 signalling in HDM-sensitized mice was unsuccessful as it did not affect lung inflammation. This failure perhaps is due to the very high HDM doses and the prototypical Th2-type mouse strains used in that study²⁶.

Because IL-33 and TSLP are known to synergise we addressed also this in the present study^{22, 23}. IL-33 was necessary and sufficient for driving increased AHR, viral replication and the innate and adaptive leukocyte responses. In this model, TSLP was redundant in worsening airway inflammation, but it synergised with IL-33 to drive AHR only at a late time point (day 8 p.i.) (**Fig. 3, Fig. 1 and Fig. 4a**). TSLP-activated DCs promote differentiation of naïve CD4⁺ T cells into a Th2 phenotype and the expansion of CD4⁺ Th2 memory cells in an OX40L-dependent manner^{55, 56}. Given our findings, we speculate that the marked α ST2-induced OX40L downregulation on DCs dampened a clear effect, at least on CD4⁺ T cells, by α ST2⁺ and α TSLP. Whereas a previous study showed that OX40L signalling did not affect T cell homing⁵⁷, more recently, in an atopic dermatitis-like model⁵⁸ and in sarcoma models⁵⁹, TSLP derived from structural cells and DCs-OX40L were found to orchestrate trafficking of innate leukocytes and CD4⁺ T cells towards the lymph nodes. Whether this can explain the accumulation of cells in LN, as observed by blocking TSLP, remains to be determined.

Surprisingly, α TSLP treatment alone resulted in a further aggravated AHR. We propose that TSLP-induced STAT3 phosphorylation-dependent activation in airway smooth muscle cells (ASM) synergizes with IL-33-mediated STAT5 activation, leading to increased ASM contraction⁶⁰. Nevertheless, mechanisms by which TSLP controls AHR remain unexplained. In addition, TSLP exerted a robust chemo-attractant function for many myeloid and lymphoid populations postulating a role for TSLP in regulating hematopoietic-cell egress from lymphoid organs and re-circulation for homeostatic clearance in the bone marrow (**Fig. 3e**) (**Supplementary Fig. 6**). These findings unveiled the role of TSLP *per se* in the context of experimental asthma exacerbation. We cannot exclude, however, that blocking TSLP in association with other treatments (i.e. ICS) result in the abrogation of airway inflammation or normalizes AHR. Recently it was demonstrated that blocking TSLP in asthmatic patients treated with long-acting beta-agonists and high doses of ICS, resulted in lower rates of clinically significant asthma exacerbations than those who received placebo⁶¹.

In conclusion, we have shown that IL-33 drives virus-induced asthma exacerbations mainly by dampening innate and adaptive Th1 and cytotoxic responses. This leads to exaggerated inflammatory responses reflected by increased levels of mediators and numbers of inflammatory cells and NETosis. It is unclear whether higher or prolonged viral loads underlie these exaggerated inflammatory responses, but we have excluded a role for Th2-like inflammatory responses. The IL-33/ST2 axis represents a potential pharmacological target to control severe virus-induced asthma exacerbations. Because of the fundamental role of IL-33 in resolution of sepsis, viral and helminthic infections, angiogenesis, vasculature and cardiac repair, central nervous system injury recovery^{18, 62-65}, short-duration rather than prolonged

558 interventions are an option. Given the acute IL-33 responses during virus-induced exacerbation,
559 local short-term treatment in the airways in stable asthma to reduce IL-33 levels or acute
560 therapy after the very first signs of infection should be contemplated.

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FIGURE LEGENDS

Figure 1. IL-33 sustains airway hyperresponsiveness and Th2 innate and adaptive immunity during chronic allergic airway inflammation. (A) Airway hyperresponsiveness (AHR) to increasing doses of Methacholine assessed at day 37 of the sensitization schedule measured as Penh. (B) Total cell and differential immune cell counts in BAL and in lung parenchyma. Data are representative of 2 independent experiments with 4 mice per group. (A) Data represent mean $\pm$ SEM (2-way ANOVA followed by Bonferroni's post-test); * p <0.05, ** p <0.01, *** p <0.001 for the comparison between indicated groups. (B) Data represent mean $\pm$ SEM (t-test); * p <0.05, ** p <0.01, *** p <0.001 for the comparison between indicated groups.

Figure 2. Epithelial IL-33 expression and dynamics of innate immune cells driven by viral challenge. (A) Representative staining of IL-33 protein in PBEC from a mild asthma patient before (*i, iii*) and 6 days after RV16 challenge (*v, vii*) compared to that of a healthy control (*ii, iv* and *vi, vii*, respectively); magnification 4X and 1.5X zoom. *iii, iv, vii, viii* are overlaid with bright-field images. (B) IL-33 mRNA expression in PBEC from mild asthma patients ($n=17$) relative to that from healthy volunteers ($n=4$). (C) (*i*) IL-33 immunoreactivity (left) in lung parenchyma from HDM/X31 mice, which is in type II alveolar cells (ATII; right). (*ii*) Overview (left) of cell dense areas (CDA) from HDM/X31 mice or controls (*iii and v*) and corresponding areas (right) with IL-33-immunoreactive cells (red = high IL-33 expression). (*iv*) Detail from CDA from HDM/X31 mice (left) or X31-infected naïve mice (right). (D) Quantification of IL-33 release (means $\pm$ SEM (t-test); * p <0.05). (E) Kinetics of BAL and lung parenchyma ILC2 in HDM/X31 mice compared to controls; data represent means $\pm$ SEM (2-way ANOVA and Bonferroni's post-test); * p <0.05, ** p <0.01, *** p <0.001 for comparison between HDM/X31 and NaCl/X31; + p <0.05, ++ p <0.01, +++ p <0.001 for comparison between HDM/X31 and HDM.

Figure 3. IL-33 drives influenza-induced exacerbation of experimental asthma. (A) AHR (PenH) at day 7 p.i. (B) α ST2 but not α TSLP protects from severe response to X31 infection as indicated by their body weight loss (0% and 13%, respectively) at 8 days p.i. (C) Influenza viral load in lung homogenates at days 6 and 8 p.i. (D) Total cell and differential immune cell counts in BAL on days 6 and 8 p.i.. (E) mLN sizes at day 8 p.i.. Data are representative of 4 replicates of independent experiments for groups of 4 or 6 mice. (A, B) Data represent mean $\pm$ SEM (2-way ANOVA followed by Bonferroni's post-test); * p <0.05, ** p <0.01, *** p <0.001 for the comparison between indicated groups. (C, D) Data represent means $\pm$ SEM (t-test); * p <0.05, ** p <0.01, *** p <0.001 for the comparison between indicated groups.

Figure 4. IL-33 drives innate and adaptive, early and late airways inflammation after influenza infection of HDM-sensitized mice and halts Th1 and antiviral immune responses. (A) AHR (PenH) at 3 and 6 days p.i.. (B) Total and differential immune cell counts in BAL. (C) H&E (top panel) and PAS (bottom panel) staining of lung sections of HDM/X31 (left) and HDM/X31+ α ST2 mice (right) 8 days p.i.. (D) Levels of cytokines and chemokines in lung homogenate. (E) Absolute numbers of CD11b+cDCs and moDCs in lung parenchyma and their MFI for OX40L or CD86. mRNA expression levels of (F) IL-12p35 and (G) IFN- β in tracheal epithelial cells (EC), CD11b+cDCs and moDCs. (H) Levels of cytotoxic enzymes in BAL and lung homogenate. (I) Visualization of NETs and effect of α ST2; 40X and 1.5X zoom. Data are representative of 4 (A-D) or 2 (E-I) replicates of independent experiments with 4 mice/group. (A-B) Data represent mean $\pm$ SEM (2-way ANOVA and Bonferroni's post-test); * p <0.05, ** p <0.01,

*** $p < 0.001$ for the comparison between HDM/X31+ α ST2 and HDM/X31 groups. (D-H) Data represent mean $\pm$ SEM (t-test); * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$ for the comparison between indicated groups.

Figure 5. IL-33 drives innate and adaptive airways inflammation and dampens Th1 immunity upon infection of naïve mice with influenza. (A) Influenza viral load in lung homogenates over time. (B) Total cell and differential immune cell counts in BAL at days 4, 6 and 8 p.i. (C) Cytokines in lung homogenate. (D) Cytotoxic enzymes in BAL. (A-D) Data are representative of 2 replicates of independent experiments with 4 mice/group and represent mean $\pm$ SEM (t-test); * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$ for the comparison between indicated groups.

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Supplementary Material

Material and Methods

Treatments. Anti-mouse ST2 and anti-mouse TSLP mAbs were generated and selected by Amgen Inc. after extensive *in vitro* and *in vivo* testing. Previously unpublished tests for the efficacy of anti-TSLP included a bone marrow-derived DC bioassay measuring the inhibition of TSLP-induced CCL17/TARC (thymus and activation regulated chemokine) production and an assay measuring the inhibition of TSLP-induced proliferation of a pro-B cell line stably transduced with murine TSLP receptor. Neutralization of IL-33 and TSLP signaling was achieved by administering 150 or 350 µg of antibody in 100 µl, respectively, via intraperitoneal injection when indicated. As proper control for the neutralizing antibodies administered with single and double blocks, a group of mice received 250 µg of mouse IgG1 in the absence of anti-ST2 and 250 µg of rat IgG1 in the absence of anti-TSLP.

Specimens collection and primary cell isolation. Blood (0.5-1 ml) was collected via cardiac puncture (preferably right ventricle) in the presence of heparin as anti-coagulant. Blood samples were subjected to density gradient separation on Ficoll Paque Plus (ratio 1:1) (GE Healthcare Life Sciences, Buckinghamshire, UK) and centrifuged. After centrifugation, the PBMC layer was collected and washed in Hank's Balanced Salt Sodium (HBSS) with phenol red without calcium and magnesium (Lonza). For bronchoalveolar lavage (BAL) collection, mice were cannulated through the trachea and the lavage was performed by instilling and retrieving 1 mL of sterile saline into the right lung. Recovered BAL was centrifuged at 260 g for 10 min at 4°C and supernatants were stored at -80°C until further use, whereas the pellet was immediately resuspended in 0.2 mL of sterile saline. Cell counts were then determined for each BAL sample by Trypan Blue staining, and differential cell counts were performed on cytospin preparations stained with modified Giemsa stain. For each BAL sample the cellular subset phenotypes were determined by flow cytometry analysis.

To remove the intra-vascular pool of cells, lungs were perfused with 5 mL of sterile saline via the pulmonary circulation through catheterization of the right heart ventricle. Then the left lung (not subjected to BAL), spleen and the mLNs were removed. To prepare a lung homogenate, left lung was minced using iridectomy scissors; lungs and mLNs were enzymatically digested for one hour at 37°C with Collagenase type III (200 U/ml, Worthington Biochemical Corp. Lakewood, NJ) and DNase I (100 U/ml, grade II from bovine pancreas, Sigma) in RPMI 1640 medium. Pulmonary, splenic and lymph node cell suspensions were obtained by grinding the tissue through 70 µm nylon sieves and red blood cells were lysed using ammonium chloride buffer. Cell counts were then performed before staining for flow cytometry analysis. The cell suspension was lysed using Greenberger buffer (75 mM NaCl, 7.5 mM Tris, 0.5 mM MgCl₂, 0.5 mM CaCl₂, 0.5% Triton X-100, 4 µg/ml 4-(2-aminoethyl) benzenesulfonyl fluoride, 50 µg/ml Na₂-EDTA, 10 ng/ml Pepstatin A, 10 ng/ml Leupeptin, pH 7.4) for 30 min on ice, followed by centrifugation at 680 g for 10 min. Supernatants were stored at -80°C until further use.

Mouse trachea epithelial cell transcriptome analysis. Samples were analyzed for IL-33, TSLP, IFN-β expression by the Affymetrix GeneChip® Mouse Gene 2.1 ST array (Affymetrix, CA, USA). The raw data were quality assessed and normalized by robust multi-array average normalization and differential gene expression analysis performed with the Array Studio software (Omicsoft Corporation, Research Triangle Park, NC). IL-33 values are expressed as log₂ transformed intensity values and fold-change (FC) between indicated groups with statistical comparison based on a general linear model. The false discovery rate (FDR) was used to address multiple test correction for genes with a fold-change of greater than 2 or less than 2. Some genes had an FDR of less than 0.05 but did

not reach a fold-change of greater than 2 or less than 2. In the GSVA analysis 1-way ANOVA was used to analyze the ES differences among group means and post hoc analysis by using the Tukey test. g:Profiler was used for pathway analysis of differential gene and protein expression. Statistically significant differences between groups ($FDR < 0.05$) are indicated.

Human primary bronchial epithelial cells transcriptome analysis. Human PBEC were collected by bronchial brush during bronchoscopy and dissolved in 1ml of TRIzol™ (Invitrogen, ThermoFischer scientific) and RNA was isolated according to manufacturer instructions and purified by Nucleospin® RNA XS extraction kit (Macherey-Nagel). Quality and concentration of the samples were assessed by fragment analyzer. Sample preparation was performed according to the protocol “NEBNext Ultra Directional RNA Library Prep Kit for Illumina” (NEB #7420S/L). Briefly, oligo-dT magnetic beads were used to isolate mRNA from total RNA. cDNA synthesis was performed after fragmentation of mRNA. This was used for ligation with sequencing adapters followed by PCR amplification of the resulting product. The quality and yield after sample preparation was measured with a fragment analyzer. The size of the resulting products was consistent with the expected size distribution between 300-500 bp. To evaluate the quality of the library preparation and kits used, the raw data was sampled and mapped to annotated genomic reference. Mapping positions were classified as intragenic, exonic, intergenic, intronic and rRNA. Clustering and DNA sequencing was performed according to the manufacture’s protocol using the Illumina NextSeq 500. A concentration of 1.6pM was used as input. The reads were trimmed for adapter sequences using Trimmomatic v0.30 before the alignment. Presumed adapter sequences were removed from the read when the bases matched a sequence in the adapter sequence set (TruSeq adapters) with 2 or less mismatches and an alignment score of at least 12. The reference of ‘Homo_sapiens.GRCh37.75’ was used to align the reads. The mapping to the reference sequence was done using a short reader aligner based on Burrows-Wheeler transform. The default of mismatch rate of 2% (3 mismatches in a read of 150 bases) was used.

The frequency of the reads mapped on the transcript was determined as counts, which is used as an input for downstream analysis. Additionally, RPKM and FPKM (reads/fragments per kilobase of exon per million reads mapped) values were calculated. The read counts were loaded into the DESeq package v 1.26.0, a statistical package within the R platform to determine the differentially expressed genes. The R platform is specifically for small sample size and over-dispersion and uses an expression curve model based on a negative binomial distribution and local regression to estimate the relationship between the mean and variance of each gene. Image analysis, base calling and quality check was performed with illumine data analysis pipeline RTA v2.4.11 and Bcl2fastq v2.17. The entire data set will be published separately (A. Ravi).

IL-33 Immuno-staining. Paraffin-embedded lung tissue was cut into 3-μm sections and subjected to heat-induced epitope retrieval (HIER) in a pre-treatment module (PT-link; Dako Cytomation) with citrate buffer (pH = 6) before immunohistochemical staining. The staining was performed at room temperature in an automated IHC robot (AutostainerPlus; Dako Cytomation). For IL-33 staining, sections were sequentially blocked with endogenous enzyme block (EnVision FLEX Peroxidase-Blocking Reagent; Dako) and serum-free protein block (Dako) before incubation with a primary goat anti-mouse IL-33 antibody (R&D system). Next, sections were incubated with a Polymer/HRP-linked secondary antibody (FLEX K8010, Dako), followed by incubation with 3,3'-diaminobenzidine (DAB) substrate-chromogen solution for 10 minutes and counterstaining with Mayer’s hematoxylin (blue nuclei). Finally, sections were dehydrated through ethanol series, cleared in xylene and mounted with Pertex (HistoLab). High resolution digital images of the stained sections were generated by a combined bright field and epi-fluorescence digital slide scanner unit (Olympus VS120). The IL-33 immunoreactivity was analyzed by automated computerized image analysis (CCV software, Medetect

AB, Lund). Briefly, the brown DAB-stained IL-33 immunoreactivity was captured by pre-set color segmentation threshold values. The tissue outline and total area of lung tissue was calculated (excluding alveolar air spaces and luminal compartment of blood vessels and conducting airways). Next, lung IL-33 levels were calculated and expressed as the proportion of firm lung tissue occupied by positive IL-33 staining. Alterations in IL-33 expression was also investigated by individual cell data plots where dots (x,y coordinates) for each individual IL-33-immunoreactive cell was plotted on the tissue section graph and color-coded according the mean intensity of IL-33 staining.

Flow cytometry analysis and sorting. For phenotypic analysis of eosinophils (CD11b⁺ CD11c⁻ SiglecF⁺ Ly6G⁻), neutrophils (CD11b⁺ CD11c⁻ SiglecF⁻ Ly6G⁺), alveolar macrophages (CD11b⁻ CD11c⁺ SiglecF⁺ MHCII⁺), CD11b+cDC (MHCII⁺ CD11b⁺ CD11c⁺ SiglecF⁻ CD103⁻ CD64⁻), B cells (CD45⁺ CD3⁻ CD4⁻ CD8⁻ CD19⁺), CD4⁺T-cells (CD45⁺ CD3⁺ CD4⁺ CD8⁻ ST2^{-/+} CD19⁻), CD8⁺T-cells (CD45⁺ CD3⁺ CD4⁻ CD8⁺ CD19⁻), ILC2 (CD45⁺ CD3⁻ CD4⁻ CD8⁻ CD19⁻ CD11b⁻ CD11c⁻ NKp46⁻ NK1.1⁻ Ly6A⁺ CD117⁺ CD127⁺ ST2⁺)²⁹⁻³¹, NK cells (CD45⁺ CD3⁻ CD4⁻ CD8⁻ CD19⁻ CD11b⁻ CD11c⁻ NKp46⁺ NK1.1⁺ Ly6A⁺ CD117⁻ CD44⁺ CD127^{-/+})²⁹⁻³¹, iNKT (α GalCer-loaded CD1d tetramer⁺ CD3⁺ CD4^{-/+} CD127⁻ NKp46⁺ NK1.1⁺ CD44⁺)^{E1}, basophils (CD127⁻ CD3⁻ CD4⁻ CD8⁻ CD19⁻ CD11b⁻ CD11c⁻ NKp46⁻ NK1.1⁻ Ly6A⁻ CD117⁻ CD200R3⁺)^{E2}, LTi (CD45⁺ CD3⁻ CD4⁻ CD8⁻ CD19⁻ CD11b⁻ CD11c⁻ NKp46⁻ CD4⁺ CD127⁺ Ly6A⁻ CD117⁺)²⁹⁻³¹, NCR+ILC3 (CD45⁺ CD3⁻ CD4⁻ CD8⁻ CD19⁻ CD11b⁻ CD11c⁻ NKp46⁺ CD4⁻ CD127⁺ CD117⁺)²⁹⁻³¹, by flow cytometry, data were acquired on an LSRFortessa or FACSCanto II instrument (BD Bioscience) and analyzed with FlowJo software (TreeStar). For sorting by flow cytometry from lung cell samples, CD11b+cDC (MHCII⁺ CD11b⁺ CD11c⁺ SiglecF⁻ CD103⁻ CD64⁻) and moDC (MHCII⁺ CD11b⁺ CD11c⁺ SiglecF⁻ CD103⁻ CD64⁺) were positively selected on a FACS Aria (BD).

Muc5b gene expression assessment by qPCR. Total RNA was extracted from lung cell suspension with TRIzol, according to manufacturer instructions (ThermoFisher scientific), and 200 ng was reverse-transcribed for cDNA (complementary DNA) synthesis (SuperScript™, First-Strand Synthesis System for RT-PCR, Invitrogen, ThermoFisher scientific). Quantitative PCR was performed with both primers and probe map within a single exon for Muc5b (assay ID Mm00466391) according to manufacturer instructions (ThermoFisher scientific). Muc5b gene was normalized to HPRT (hpert, assay ID Mm03024075_m1, ThermoFisher scientific) and expressed as copies per μ L of cDNA reaction.

SUPPLEMENTARY FIGURE AND TABLE LEGENDS

Supplementary Figure 1. Experimental treatment schedules. (A) Experimental outline. C57BL/6J mice were chronically (i.n.) exposed for 5 days a week for 5 consecutive weeks to HDM and from day 26 onwards received α ST2 every 3 days. Control groups received isotype antibody or systemic Prednisone. Mice were sacrificed on day 38 for analyses. (B) C57BL/6J mice were chronically (i.n.) exposed for 5 days a week for 5 consecutive weeks to HDM and since day 26 of the sensitization schedule were administered with doses of α ST2 every 3 days. The control groups received an isotype control antibody or systemic Prednisone; on day 38 of the sensitization schedule mice were analysed. (C) C57BL/6J mice were chronically (i.n.) exposed for 5 days a week for 5 consecutive weeks to HDM and infected (i.n.) with X31 on day 29 of the sensitization schedule. Mice were administered with doses of anti-ST2 (α ST2) and anti-TSLP (α TSLP) 3 days before X31 infection and every 3 days in combination to achieve double blockade. The control group received isotype control antibodies; on day 4, 6 and 8 all mice were analysed. (D) C57BL/6J mice were chronically (i.n.) exposed for 5 days a week for 5 consecutive weeks to HDM and infected (i.n.) with influenza virus A/X31 H3N2 (X31) on day 29 of the sensitization schedule. Mice were administered with doses of α ST2 3 days before X31 infection and every 3 days. The control group received an isotype control antibody. (E) C57BL/6J mice were infected (i.n.) with influenza virus A/X31 H3N2 (X31) and administered with doses of α ST2 3 days before X31 infection and every 3 days. The control group received an isotype control antibody; on day 4, 6 and 8 all mice were analysed.

Supplementary Figure 2. IL-33 sustains Th2 innate and adaptive immunity during chronic allergic airway inflammation. Relative cell counts differentiated by intracellular cytokines in lung parenchyma. Data are representative of 2 independent experiments with 4 mice per group. Data represent mean $\pm$ SEM (t-test); * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$ for the comparison between indicated groups.

Supplementary Figure 3. Epithelial IL-33 expression and innate immune cells dynamics driven by viral challenge. (A) IL-33 expression in tracheal epithelial cells; FDR < 0.05 are indicated. (B) Kinetics of BAL and lung parenchyma innate leukocytes in HDM/X31 mice compared to controls. Data represent means $\pm$ SEM (2-way ANOVA and Bonferroni's post-test); * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$ for comparison between HDM/X31 and NaCl/X31; + $p < 0.05$, ++ $p < 0.01$, +++ $p < 0.001$ for comparison between HDM/X31 and HDM.

Supplementary Figure 4. TSLP protein and mRNA expression in severe influenza-induced exacerbation of experimental asthma. (A) TSLP protein in supernatant from cells isolated from the lung of HDM/X31 mice compared to controls. (B) TSLP mRNA expression in tracheal epithelial cells (EC) from HDM/X31 mice compared to that from controls. (A) Data represent mean $\pm$ SEM (t-test); * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$ for the comparison between indicated groups. (B) Data are normalized by robust multi-array average normalization and differential gene expression analysis.

Supplementary Figure 5. IL-33 blockade modulates inflammatory cells in lung parenchyma in influenza-induced exacerbation of experimental asthma. Differential immune cell counts and percentages in BAL (A) and in lung parenchyma (B, C). Data are representative of 4 replicates of independent experiments with 4 mice/group. Data represent mean $\pm$ SEM (t-test); * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$ for the comparison between indicated groups.

Supplementary Figure 6. IL-33 drives influenza-induced exacerbation of experimental asthma and orchestrates immune cells trafficking in lymphoid organs. (A) Total cell counts and

differential immune cell counts in mLN and (B) in spleen at days 6 and 8p.i. Data are representative of 4 replicates of independent experiments for groups of 4 or 6 mice and represent mean $\pm$ SEM (t-test); * p <0.05, ** p <0.01, *** p <0.001 for the comparison between indicated groups.

Supplementary Figure 7. IL-33 drives innate and adaptive, early and late airways inflammation after influenza infection of HDM-sensitized mice and halts Th1 and antiviral immune responses.

Total and differential immune cell counts in (A) mLN and (B) lung. Levels of cytokines and chemokines in BAL (C) and lung homogenate (D). (E) Correlations between peak levels of IL-33 and peak levels of indicated cytokines in lung parenchyma. (F) Relative T cell counts producing intracellular Th2 or Th1 cytokines. Data are representative of 4 replicates of independent experiments with 4 mice/group. (A, B) Data represent mean $\pm$ SEM (2-way ANOVA and Bonferroni's post-test); * p <0.05, ** p <0.01, *** p <0.001 for the comparison between HDM/X31+ α ST2 and HDM/X31 groups. (C, D, F) Data represent mean $\pm$ SEM (t-test); * p <0.05, ** p <0.01, *** p <0.001 for the comparison between indicated groups. (E) Spearman's correlation performed on HDM/X31 mice.

Supplementary Figure 8. Reduced muc5b mRNA in response to α ST2 blockade during influenza-induced exacerbation of experimental asthma.

Muc5b mRNA expression in lung homogenate from HDM/X31 treated with α ST2 mice compared to that from controls. Data are representative of 2 replicates of independent experiments with 4 mice/group. Data represent mean $\pm$ SEM (t-test); * p <0.05.

Supplementary Figure 9. Influenza-induced influx of type-2 (Th2) CD4⁺ T cells is resolved by α ST2.

Th2 lymphocyte (SSC^{low}CD3⁺CD4⁺ST2⁺) numbers in BAL (A) and lung parenchyma (B). Data are representative of 4 replicates of independent experiments with 4 mice/group and represent mean $\pm$ SEM (2-way ANOVA followed by Bonferroni's post-test); * p <0.05, ** p <0.01, *** p <0.001 for the comparison between HDM/X31+ α ST2 and HDM/X31 groups.

Supplementary Figure 10. Reduced innate interferon response to influenza in HDM-sensitized mice.

IFN- β 1 mRNA expression in tracheal epithelial cells from HDM/X31 mice compared to that from controls. Data are normalized by robust multi-array average normalization and differential gene expression analysis.

Supplementary Figure 11. IL-33 dampens Th1 immunity upon infection of naïve mice with influenza.

(A) Body weight loss curves representative of influenza-induced morbidity. (B) Differential immune cell counts in lung parenchyma at days 4, 6 and 8 p.i. Data are representative of 2 replicates of independent experiments with 4 mice/group. (A) Data represent mean $\pm$ SEM (2-way ANOVA followed by Bonferroni's post-test); * p <0.05, ** p <0.01, *** p <0.001 for the comparison between indicated groups. (B) Data represent mean $\pm$ SEM (t-test); * p <0.05, ** p <0.01, *** p <0.001 for the comparison between indicated groups.

Supplementary Table 1. Baseline characteristics of study subjects.

17 Mild asthmatic subjects' characteristics are reported in the table. PC20: airway responsiveness to methacholine bromide (provocative concentration resulting in a 20% drop of FEV1); FEV1, forced expiratory flow volume within 1 second; % pred: percent of predicted value; FVC: Forced Vital Capacity; FeNO: fractional exhaled nitric oxide; ACQ: asthma control questionnaire average value; M/F: male to female ratio; IgE: immunoglobulin E. Data shown are mean, SD and p value (Unpaired t-test or Fisher exact test is used), n=17. Healthy individuals met the following criteria: FEV1 >80% predicted, PC20 >16 mg/mL, skin prick test negative for 12 common aeroallergens.

References

- E1. Umetsu DT, DeKruyff RH. A role for natural killer T cells in asthma. *Nat Rev Immunol* 2006; 6:953-8.
- E2. Karasuyama H, Mukai K, Tsujimura Y, Obata K. Newly discovered roles for basophils: a neglected minority gains new respect. *Nat Rev Immunol* 2009; 9:9-13.

Characteristics	mean	sd	p value
PC20 log2	0.45	1.56	0.71
FEV1 pred (L)	3.95	0.96	0.17
FEV1%pred	103.76	8.54	0.84
FVC pred (L)	4.72	1.08	0.10
FVC % pred	107.06	8.54	0.93
FeNO (ppb)	58.15	34.88	0.96
ACQ	0.86	0.54	0.26
M/F	6/11		0.10
Age (yr)	23.00	4.54	0.68
IgE (kU/L)	372.31	569.84	0.38
Blood eosinophils (10e9/L)	0.43	0.40	0.24