

Endogenous IL-33 enhances T_H2 cytokine production and T-cell responses during allergic airway inflammation

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Abstract

IL-33 is an IL-1-related cytokine which has been implicated in T_H2-associated biology and allergic diseases in humans and mice. IL-33 stimulates T_H2 cells, mast cells, eosinophils, basophils, iNKT cells and circulating CD34⁺ stem cells to proliferate and produce pro-allergic cytokines such as IL-5 and IL-13. IL-33 mediates its cytokine effects through a receptor consisting of ST2 and IL-1RAcP. Whereas IL-1RAcP is ubiquitously expressed, ST2 expression is cell-type restricted and determines responsiveness to IL-33. Studies employing ST2-deficient mice have reported variable results on the role of this receptor, and consequently IL-33, with regards to allergic lung inflammation. In this study, we demonstrate that IL-33 is important for allergic lung inflammation. Intra-nasal administration of IL-33 triggered an immediate allergic response in the airways, and more importantly, we show that endogenous IL-33 contributes to airway inflammation and peripheral antigen-specific responses in ovalbumin-induced acute allergic lung inflammation using IL-33-deficient mice. Our results suggest that IL-33 is sufficient and required for severe allergic inflammation in the lung and support the concept of IL-33 as a therapeutic target in allergic lung inflammation.

Keywords: allergy, cytokines, IL-33, lung, T_H2 cells

Introduction

IL-33 (C9ORF26, DVS27, NF-HEV, IL-1F11) is an IL-1-related protein. Similar to other cytokines in the IL-1 family, IL-33 exists as a nuclear precursor. Major sources of IL-33 expression include epithelial tissues and vascular endothelial cells, and IL-33 is thought to be released from necrotic cells (1, 2). Once outside the cell, IL-33 mediates its cytokine-like effects via signaling through a heterodimeric receptor consisting of ST2 (T1, Fit-1, DER-4, IL-1RL1, IL-1R4) and IL-1RAcP (IL-1R3) (3, 4). Repeated systemic administration of recombinant IL-33 *in vivo* induces eosinophilia, splenomegaly, production of T_H2 cytokines and increased IgE serum levels in an ST2-dependent fashion (3, 5, 6). *In vitro*, IL-33 directly stimulates several T_H2-associated cell types including antigen-experienced T_H2 cells, mast cells, eosinophils, basophils, iNKT cells and CD34⁺ stem cells (7–13). The T_H2 bias frequently observed in response to IL-33 stimulation in various settings *in vitro* and *in vivo* has consolidated the

concept of IL-33 as a pro-inflammatory mediator in allergic inflammation. In agreement with this concept, preventative administration of a rabbit polyclonal antibody against mIL-33 during ovalbumin (OVA)-induced asthma modulated pulmonary inflammation and T_H2 cytokine production (5).

Several studies have investigated the contribution of ST2 to allergic inflammation with conflicting results and the role of ST2 appears somewhat unclear. Depending on the experimental setting, ST2 has been shown to be not associated, positively associated or negatively associated with the regulation of T_H2 responses during OVA-induced lung inflammation (6,14–16). As such, the experiments described herein using intra-nasal administration of IL-33 protein and IL-33 KO mice were performed to clarify the role of IL-33 in a model of acute allergic airway inflammation. We demonstrate that IL-33 contributes to production of T_H2 associated cytokines, to bronchoalveolar lavage (BAL) eosinophil infiltrates and to

histopathologic changes in the lung. Moreover, IL-33 deficiency leads to reduced OVA-dependent accumulation of T_H2 cells in the lung-draining lymph nodes. These results validate IL-33 as a therapeutic target candidate for treatment of allergic lung inflammation.

Methods

Mice and in vivo experimental protocols

BALB/c mice were purchased from the Jackson Laboratory. IL-33 KO mice were generated by Caliper Biosciences on the C57BL/6 (Taconic, Hudson, NY, USA) genetic background. Briefly, the mRNA of murine IL-33 is encoded in a 7-exon-6-intron gene located on mouse chromosome 19. The entire 7-exon-6-intron stretch of genomic DNA was replaced with a Neomycin cassette, which was flanked by two flip recombinase target sites. After Neomycin-based selection of transformed ES cells, the Neomycin cassette was excised using recombinant flip recombinase. On either side of the deletion, ~6 kb of genomic sequence were used for 5'- and 3'-homology recombination arms. A PCR-based strategy was used to discriminate between wildtype (WT) and mutant alleles (WT amplicon ~ 280 bp, using PCR primers 5'-ggcattaacactaagactactcagcctcag-3' and 5'-cggtatgttggttggtgcga-3'; mutant amplicon ~ 450 bp, using PCR primers 5'-ggcattaacactaagactactcagcctcag-3' and 5'-cggggaaatctggagttggaataact-3'). For the OVA-induced asthma model, mice were sensitized intra-peritoneally (i.p.) with 50 µg of OVA from chicken egg white (Sigma-Aldrich, St. Louis, MO, USA) complexed to 2 mg of Imject Alum (Pierce, Rockford, IL, USA) in 0.15 M saline (Sigma-Aldrich) on day 0 and day 14 and primed on day 28 and 29 with saline or nebulized OVA (10 mg ml⁻¹) for 45 min per session. Mice were given a final challenge of saline or OVA (25 mg ml⁻¹) on day 30 and sacrificed on day 31. Where indicated, mice received 1 mg of BrdU i.p. 12 h before final sacrifice. In IL-33-treatment experiments, BALB/c mice received 5 µg of carrier-free recombinant mouse IL-33 (R&D Systems, Minneapolis, MN, USA) intra-nasally, and BAL fluid and lung tissues were harvested 24 or 48 h later. Six to ten animals, sex and age matched, were used per treatment group in the experiments described. Each experiment was performed two to four times. Handling of mice and experimental procedures were conducted in accordance with the guidelines of the AAALAC-accredited Schering-Plough Biopharma Animal Care and Use Committee.

Pulmonary function (non-invasive plethysmography)

Murine pulmonary responses to the non-specific bronchoconstrictor methacholine chloride were measured using non-invasive Whole Body Plethysmography (Buxco Research Systems, Wilmington, NC, USA). Unrestrained mice were placed in individual chambers and exposed to increasing doses of nebulized methacholine (0, 12.5, 25, and 50 mg ml⁻¹) for 1 min, and responses were recorded for the following 3 min. Penh was calculated to quantify lung function.

Organ harvest and processing

BAL fluid was isolated by gently washing the lung (through the trachea) with 1 ml of PBS. Lavage fluid was kept on ice and centrifuged at 400 g for 5 min. The cell pellet was resuspended in 1 ml of PBS for cell differentials by cytopspin. Slides were air-dried, fixed with 95% ethanol, and stained with Wright-Giemsa (Sigma-Aldrich). A minimum of 200 cells were counted under the microscope per slide for cell differentials. Lung tissue was snap-frozen for mRNA analysis by quantitative real-time reverse transcription-PCR (qRT-PCR) as described (17) or paraffin embedded, sectioned and stained with hematoxylin and eosin or Periodic Acid-Schiff (PAS). Stained lung sections, blinded for experimental group, were scored for the degree of hypertrophy of the airway epithelium and peribronchiolar/perivascular cellular inflammation on a scale of 0–5 (0 lacking any pathology and 5 exhibiting severe pathology) by a board-certified pathologist. Single cell suspensions were prepared from pooled lung-draining lymph nodes (mediastinal). Cells (2 × 10⁵) in 200 µl were grown in culture medium [consisting of RPMI 1640 (Cellgro, Manassas, VA, USA) supplemented with 10% Fetal Bovine Serum (FBS) and 100 U ml⁻¹ penicillin/streptomycin] in the presence or absence of OVA (100 µg ml⁻¹) (Sigma-Aldrich) for 3 days in a humidified incubator at 37°C with 5% CO₂, and subsequently 100 µl of culture supernatant was harvested for cytokine analysis. One microcurie of ³H-thymidine (GE Healthcare, Piscataway, NJ, USA) was added to the remaining 100 µl of culture suspension per well, incubated for 6 h in humidified incubator at 37°C with 5% CO₂, and harvested onto a glass fiber filter (Perkin Elmer, Waltham, MA, USA) using the Micro96 harvester (Molecular Devices, Sunnyvale, CA, USA). Filter was dried and ³H-thymidine incorporation was assessed with a MicroBeta Trilux (Perkin Elmer) in the presence of scintillation fluid. Mouse cytokines were assayed using Luminex multi-plex kits (Millipore, Billerica, MA, USA) or ELISAs (R&D Systems).

Lung histology and immunohistochemistry

Murine lungs were perfused with 10 ml of PBS via the right ventricle of the heart. The single left lung lobe was excised, fixed in 10% neutral buffered formalin, paraffin embedded, sectioned and stained with hematoxylin and eosin as per standard procedures. Lung tissue was scored for hypertrophy of the airway epithelium and peribronchiolar/perivascular cellular inflammation on a scale of 0–5 by a board-certified pathologist. Immunohistochemical studies for IL-33 were performed on 5 µm lung tissue sections using a 1–400 dilution of a mouse mAb against anti-human IL-33 (Nessy-1) (Alexis Biochemicals, San Diego, CA, USA), followed by incubation with Mouse Envision-Plus (Dako Corporation, Carpinteria, CA, USA). Slides were developed with DAB-Plus (Dako Corporation), counterstained in Modified Mayer's Hematoxylin, blued in 0.3% ammonia water, and mounted in a routine manner. Zeiss Mirax Midi scanner and software (Carl Zeiss Microimaging, Thornwood, NY, USA) were used to acquire images using identical settings for all comparisons.

Flow cytometric analysis of mediastinal lymph node leukocytes

Mediastinal lymph node (MLN) cells were isolated and labeled with antibodies specific for CD4 and TCR β . Intracellular staining for BrdU was performed as per the BrdU Flow Kit instruction manual. For intracellular staining protocols, single cell suspensions were prepared from pooled lung-draining (mediastinal) lymph nodes. Cells (2×10^5) in 200 μ l were grown in culture medium [consisting of RPMI 1640 (Cellgro) supplemented with 10% FBS and 100 U ml $^{-1}$ penicillin/streptomycin] in the presence or absence of OVA (100 μ g ml $^{-1}$) (Sigma-Aldrich) for 6 h in a humidified incubator at 37°C with 5% CO $_2$. BD Golgi Plug (containing Brefeldin A) was added after 2 h of stimulation. Following 6 h of stimulation, cells were removed from culture, labeled externally with antibodies specific for CD4 and TCR β , fixed and labeled internally with antibodies specific for IL-4 and IL-5, as per the BD Cytofix/Cytoperm™ Kit protocol. All antibodies and staining kits were purchased from BD Biosciences (San Jose, CA, USA). Data were acquired using a BD FACS-Canto II flow cytometer (BD Biosciences) and analyzed using FlowJo software (Tree Star, Inc, Ashland, OR, USA).

Statistical analyses

The unpaired two-tailed *t*-test was performed using GraphPad Prism version 4.02 (GraphPad Software, La Jolla, CA, USA) to determine average $\pm$ SEM. *P* < 0.05 was considered statistically significant. **P* < 0.05, ***P* < 0.01, ****P* < 0.001.

Results

We have previously shown that repeated systemic administration of IL-33 for 7 days induced mRNA expression of IL-5 and IL-13 in the lung and protein production of these cytokines in the serum (3). To examine the acute local effects of the cytokine in the lung, a single dose of 5 μ g of recombinant IL-33 was administered intra-nasally to BALB/c mice, and BAL fluid and serum were collected 24 h later for cytokine and qRT-PCR analysis. Lung tissue was also collected at 48 h for histological analysis. Administration of IL-33 induced immediate IL-5 and IL-13 protein production (Fig. 1A) and mRNA expression (Fig. 1B) in the lung, as assessed by ELISA of BAL fluid and qRT-PCR of lung tissue, respectively. IL-4 expression was not modulated at the protein or mRNA level (data not shown). IL-33 instillation also increased serum levels of T $_H$ 2-associated chemokines Eotaxin (CCL11) and Thymus and activation-regulated chemokine (CCL17) (Fig. 1C). In lung tissue, elevated mRNA expression of *Ccl11*, *Ccl17* and also Macrophage-Derived Chemokine (*Ccl22*) was found (Fig. 1D), although up-regulation of *Ccl1*, a chemokine ligand for CCR8 on T $_H$ 2 cells and implicated in T $_H$ 2 cell chemotaxis, was not observed at these time points (data not shown). In addition, delivery of IL-33 led to mucus production by the airway epithelium (Fig. 1E, arrows) and peribronchiolar cuffing (Fig. 1E, arrowheads) at 48 h. BAL eosinophils were not present at the 24-h time point but began to appear 2 days following treatment (data not shown). These results show that intra-nasal delivery of IL-33

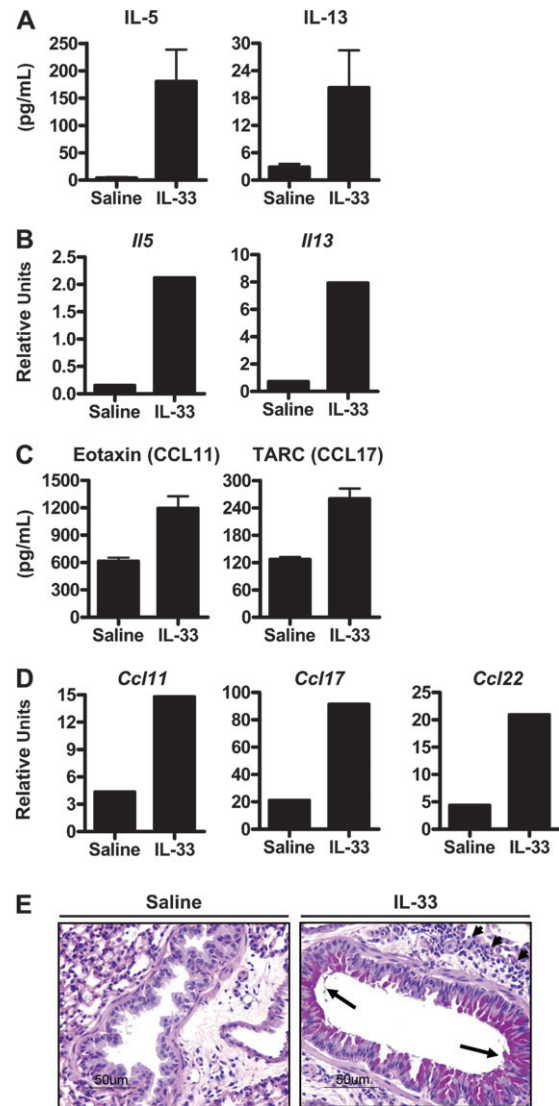


Fig. 1. Intra-nasal instillation of IL-33 induces T $_H$ 2 responses. BALB/c mice received a single 5 μ g dose of recombinant murine IL-33 intra-nasally, and BAL fluid, lung tissue and serum were collected 24 h later for mRNA and protein analysis. Lung tissue was also collected for histology at 48 h following treatment. IL-5 and IL-13 levels in the BAL fluid (A) or lung tissue (B) were assessed by ELISA and qRT-PCR of pooled lung mRNA, respectively. Eotaxin (CCL11) and TARC (CCL17) were assayed in the serum by ELISA (C). qRT-PCR was performed for *Ccl11*, *Ccl17* and *Ccl22* on pooled lung tissue from saline- or IL-33-treated animals (D). PAS staining of lung tissue was performed (E) to observe mucus production (arrows) and cellular infiltration (arrowheads).

leads to acute and severe allergic inflammation in the lung of non-sensitized mice.

We next sought to examine the expression of IL-33 and its receptors in the OVA-induced asthma model to ensure that endogenous IL-33 is expressed and potentially functional during allergic inflammation. C57BL/6 mice were primed and challenged with OVA, and lungs were harvested 24 h after the final challenge. C57BL/6 mice mount a reduced but qualitatively similar allergic response to OVA as BALB/c mice (data not shown). Compared with saline-challenged

mice, mRNA for both IL-33 and its membrane-bound receptor ST2 were considerably up-regulated in lung tissue of OVA-challenged C57BL/6 mice (Fig. 2). Relative mRNA levels encoding soluble ST2 were essentially undetected (relative levels <1, qRT-PCR cycle number >35), either after saline or OVA treatment, which suggest that soluble ST2 mRNA is absent in either condition and that the difference between saline- and OVA-treated animals cannot be considered meaningful. Similar results were obtained in sa-

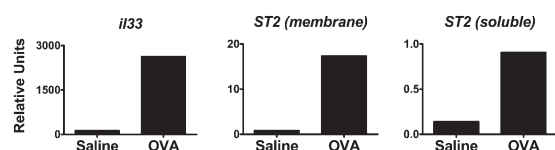


Fig. 2. IL-33 and its receptor are expressed during asthmatic responses. qRT-PCR for *Il33* or *ST2 (membrane)* or *ST2 (soluble)* was performed on pooled mRNA from the lung tissue of saline- or OVA-challenged C57BL/6 mice (7–10 mice per group). Results are presented normalized to ubiquitin expression.

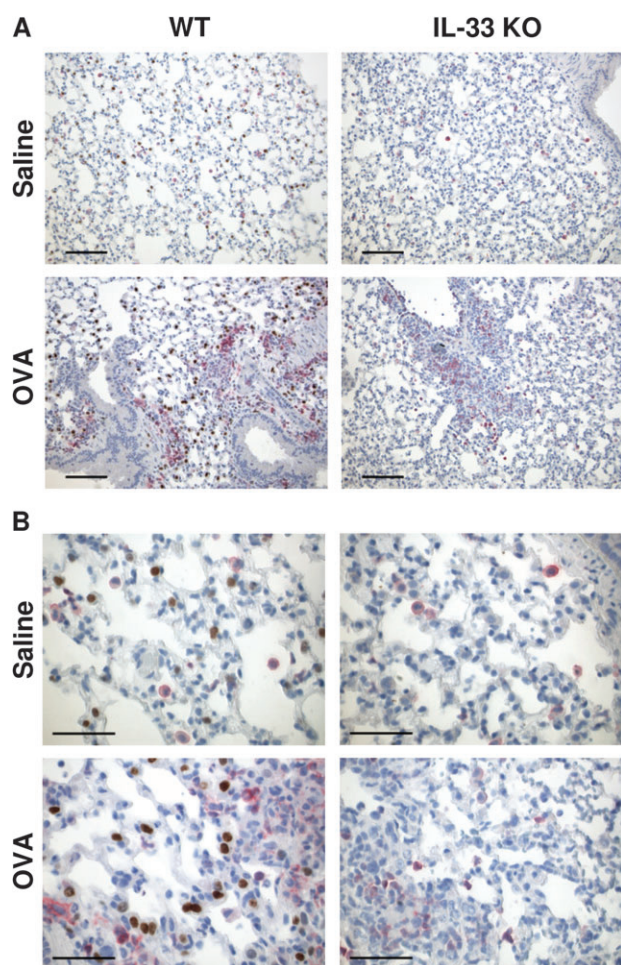


Fig. 3. IL-33 exhibits nuclear expression in F4/80+ cells of lung alveoli from saline- or OVA-challenged mice. IHC was performed for IL-33 (brown) and F4/80 (pink) on lung tissue sections from WT or IL-33 KO mice challenged with either saline or OVA at lower (A) or higher (B) magnifications. Scale bars represent 0.1 mm (A) and 0.05 mm (B).

line- and OVA-challenged BALB/c mice (IL-33 increase from 82.12 to 1655.26 relative units and membrane-bound ST2 increase from 4.77 to 63.03 relative units, respectively). Therefore, these data suggest that expression as well as regulation of soluble ST2 in lung tissue may be absent or minimal in this model (Fig. 2).

We next used immunohistochemistry to examine the location of IL-33 in lung tissue. As shown in Fig. 3, IL-33 protein was present in the lungs of saline-challenged mice. Expression of IL-33 was localized to the nuclei of large cells in the alveolar epithelium, distinct from F4/80+ alveolar macrophages. Following OVA challenge, F4/80 staining was markedly increased and was present on macrophages and eosinophils, both of which were negative for IL-33 protein. IL-33 staining was also increased following OVA challenge and appeared to localize to the same cell type as in saline-challenged mice (Fig. 3). IL-33 staining was not different in mice that received OVA priming and subsequently saline challenge compared with naïve mice (data not shown). The increase observed in pulmonary IL-33 protein levels upon OVA treatment is consistent with elevated IL-33 mRNA (Figs 2 and 5D), and the increase in F4/80+ eosinophils is consistent with the BAL cell counts (Fig. 5B). Taken together, expression of the IL-33/ST2 pathway constituents and the acute biological activities of IL-33 in the lung suggest a role for IL-33 in the regulation of allergic inflammation in the lung.

In order to revisit the contribution of the ST2/IL-33 pathway to allergic inflammation from a different angle, IL-33-deficient mice were created on the C57BL/6 background and examined in the OVA-induced allergic lung inflammation model. IL-33-deficient mice were generated by deletion of the genomic region encoding the IL-33 mRNA and corresponding introns (Fig. 4). IL-33^{-/-} mice breed normally, have normal appearance and weight, are devoid of apparent behavioral abnormalities and do not require special nutrition or housing conditions. Systematic histological examination of multiple tissues did not reveal morphological differences in IL-33^{-/-} mice compared with WT littermate controls (data not shown). As expected, IL-33 was not detected in lung tissue of IL-33 KO mice (Fig. 3). Genetic IL-33 deficiency had no detectable impact on the development of B cells in the bone marrow or on thymic T-cell development. In secondary lymphoid organs, the percentages and absolute numbers of T cells (CD3⁺CD4⁺ or CD3⁺CD8⁺), B cells (CD19⁺B220⁺), NK cells (NK1.1⁺DX5⁺CD3⁻) and dendritic cells (CD11c⁺) were comparable between WT and IL-33 KO mice (data not shown). Also, numbers and relative composition of lymphocytes and granulocytes circulating in the blood were comparable between WT and IL-33 KO mice (data not shown). In conclusion, gross examination did not reveal any obvious developmental abnormalities in non-challenged IL-33^{-/-} mice.

WT and IL-33 KO mice were sensitized to OVA by i.p. injection, primed with two daily doses of aerosolized saline or OVA and then challenged on the third day with saline or a higher dose of OVA to assess systemic and pulmonary inflammation. The degree of inflammation in the lungs of WT and IL-33 KO mice in response to saline or OVA was assessed histologically, judged on a scale of 0–5 based on the degree of epithelial hypertrophy and peribronchiolar/

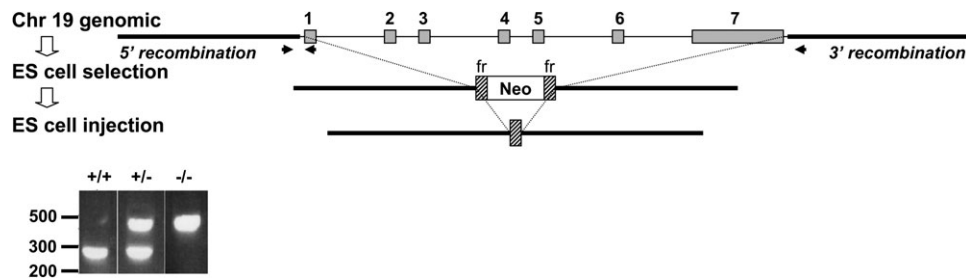


Fig. 4. Schematic representation of targeting construct design for generation of IL-33 KO mice. Seven exons and their corresponding introns were replaced by a Neomycin cassette (white box) flanked by flip recombinase (fr) sites (hatched boxes) for ES cell selection. After successful ES cell selection recombinant flip was used to excise the Neomycin cassette. Exons are shown as gray boxes, recombination arms in bold. The arrowheads indicate hybridization sites for the genotyping primers. A typical genotyping result for IL-33^{+/+}, IL-33^{+/-}, and IL-33^{-/-} mice is shown.

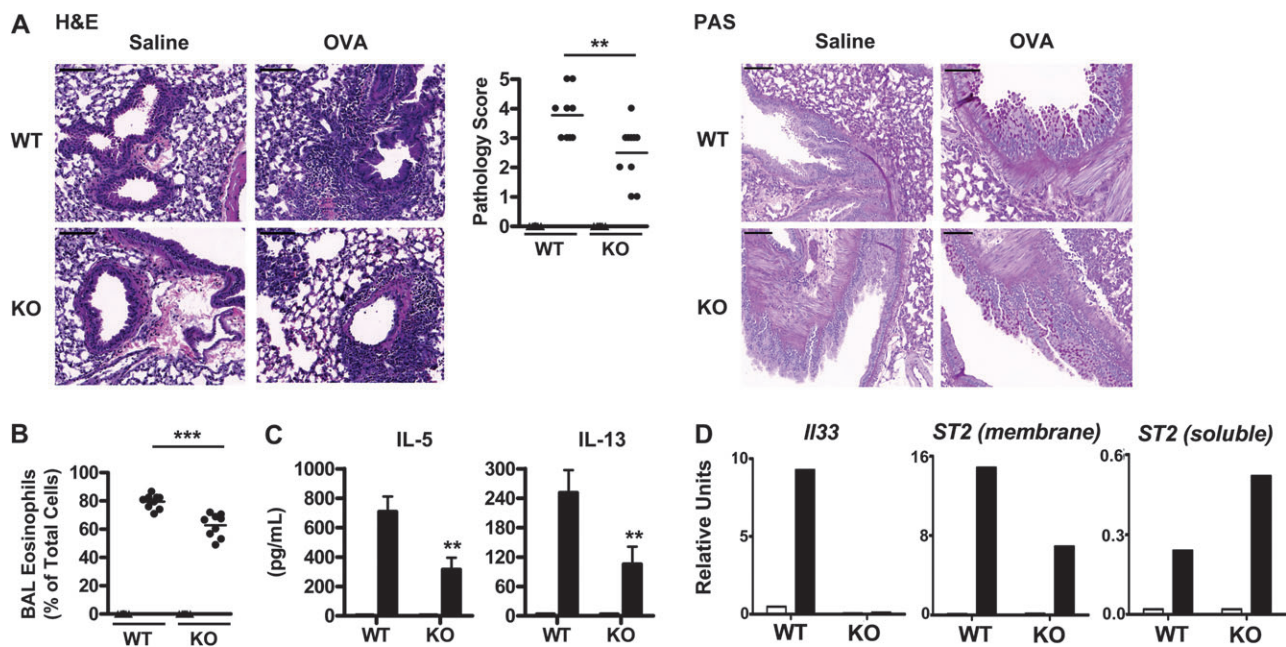


Fig. 5. IL-33 KO mice have reduced lung responses during OVA-induced asthma. (A) Representative images of H&E and PAS stained lung tissue from saline-treated and OVA-treated WT mice (top row) and IL-33 KO mice (bottom row). Scale bars represent 0.1 mm. Lung tissue from WT or IL-33 KO mice challenged with saline (triangles) or OVA (circles) was scored for the degree of hypertrophy of the airway epithelium and peribronchiolar/perivascular cellular inflammation on a scale of 0–5. Average pathology score was 3.8 for WT and 2.6 for IL-33 KO mice. (B) The percentage of eosinophils in the BAL of WT or IL-33 KO mice challenged with saline (triangles) or OVA (circles) was assessed by manual counting of Wright–Giemsa-stained BAL cells. A minimum of 200 cells were counted. (C) IL-5 and IL-13 levels in BAL fluid were assessed by ELISA. (D) qRT-PCR for *IL33*, *ST2* (membrane) or *ST2* (soluble) was performed on pooled mRNA from the lung tissue of saline-treated (white bar) or OVA-treated (black bar) WT. Results are presented normalized to ubiquitin expression. ** $P < 0.01$, *** $P < 0.001$.

perivascular cellular infiltrate. Epithelial thickening and pulmonary inflammation were significantly reduced in OVA-challenged IL-33 KO mice, with an average pathology score of 2.5, compared with 3.8 for WT challenged mice (Fig. 5A). The airway epithelium of IL-33 KO exhibited reduced production of mucus, assessed by PAS staining (Fig. 5A). Consistent with the lung histology, the proportion of BAL eosinophils was also significantly reduced in IL-33 KO mice after challenge (Fig. 5B), whereas other cell constituents were unaffected and the total number of cells recovered from BAL of WT and IL-33 KO mice was not significantly different (data not shown). The levels of IL-5 and IL-13 were also reduced in the BAL fluid of IL-33 KO mice (Fig. 5C), although IL-4 expression was not significantly altered (data

not shown). OVA-specific serum IgE levels were measured; concentrations were in the order of 0.1 ng ml^{-1} with no significant differences between treatment groups. mRNAs for IL-33 and ST2 receptor were up-regulated upon OVA challenge, and again, pulmonary expression of soluble ST2 mRNA was minimal after saline or OVA treatment (Fig. 5D; compare with Fig. 2). Overall, these findings extend the results obtained upon intra-nasal administration of recombinant IL-33 protein (Fig. 1) and suggest that recombinant IL-33 triggers pathways comparable to those activated by endogenous IL-33 in this model.

Pulmonary function measurements were also performed in this acute model of allergic airway inflammation using non-invasive methods. No significant difference in Penh was

found upon metacholine challenge of IL-33 KO compared with WT mice following OVA challenge (Penh of 3.2 ± 1.8 and 3.0 ± 1.5 , respectively), indicating that the absence of endogenous IL-33 did not alter lung function in this model.

We next examined the lung-draining lymph nodes of saline- and OVA-challenged mice for T_H2 cell responses. MLN cells were isolated and cultured for 3 days in media alone or with OVA re-stimulation. No differences were observed in the cell numbers recovered from the MLN of WT or IL-33 KO mice (data not shown). Compared with WT mice, total MLN cells from OVA-challenged IL-33 KO mice exhibited reduced proliferation either in media alone or upon OVA re-stimulation (Fig. 6A). Production of IL-5 and IL-13 were also reduced in MLN cells from IL-33 KO mice upon OVA re-stimulation (Fig. 6B). Additional analytes were measured; the results for all analytes are summarized in Supplemental Table 1, available at *International Immunology* Online.

To complement the cytokine data for IL-5 and IL-13 measured in the cellular supernatants, intra-cellular flow cytomet-

ric staining was performed on MLN cells for IL-4 and IL-5. Compared with WT mice, IL-33 KO mice had lower proportions of $CD4^+$ T cells producing either cytokine in response to *ex vivo* OVA re-stimulation (Fig. 6C). Flow cytometry was also performed to assess the percentage of activated $CD4^+$ T cells in the MLN following *in vivo* OVA challenge. Saline-treated WT and IL-33 KO mice had similar percentages of $TCR\beta^+CD4^{hi}$ cells in the MLN *ex vivo*; in contrast, IL-33 KO mice had lower proportions of activated $TCR\beta^+CD4^{int}$ cells following challenge with OVA (32.7 versus 15.7%) (Fig. 6D). To rule out a possible proliferative impairment of activated $TCR\beta^+CD4^{int}$ MLN cells in the IL-33 KO mice, *in vivo* incorporation of BrdU in $TCR\beta^+CD4^{int}$ MLN cells was investigated. BrdU incorporation was comparable between WT and IL-33 KO (Fig. 6E), suggesting that the reduced *in vitro* proliferation and cytokine production was due to a lower number of activated $CD4^+$ T cells present in the MLN cell cultures from IL-33 KO mice (compare Fig. 6A and B).

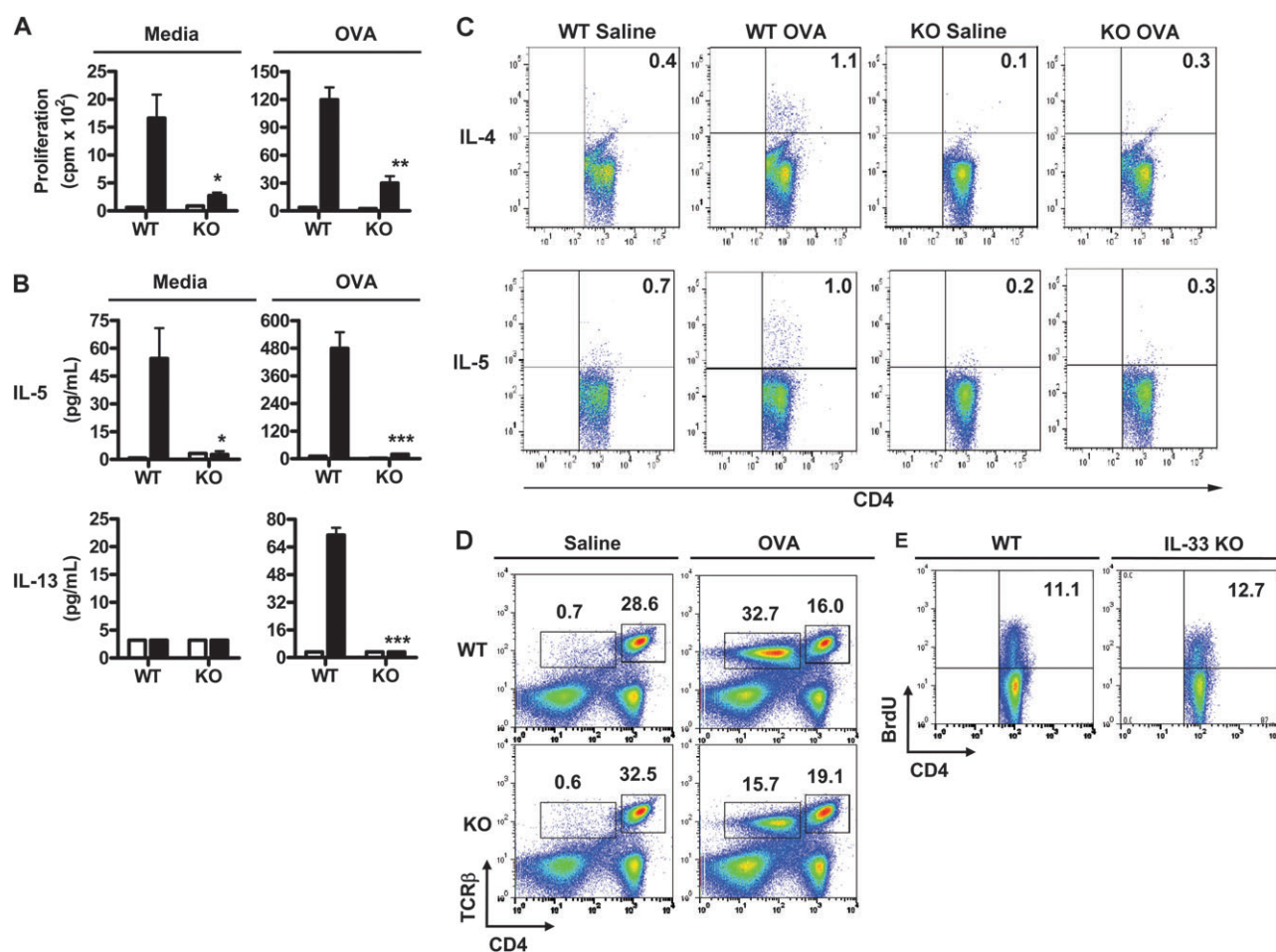


Fig. 6. IL-33 deficiency affects lung-draining lymph node responses during OVA-induced asthma. WT or IL-33 KO mice were challenged with saline (white bars) or OVA (black bars). Cells were isolated from the lung-draining MLNs and cultured in media alone (Media) or re-stimulated with OVA (OVA) for 3 days. At the end of this period, cells were assayed for (A) proliferation using a ³H-thymidine assay and (B) T_H2 cytokine production using a Luminex multi-plex assay. (C) Intra-cellular cytokine staining for IL-4 and IL-5 was performed on the $TCR\beta^+CD4^+$ subset of cells isolated from MLN and re-stimulated *ex vivo* with OVA. (D and E) Cells were isolated from the MLNs of saline- or OVA-challenged WT and IL-33 KO mice that received an i.p. injection of BrdU 12 h before harvest. MLN cells were double-stained with antibodies against CD4 and TCR β (D), and activated cells ($CD4^{int}TCR\beta^{int}$) were gated to observe the proportion of cells incorporating BrdU (E). * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$.

Discussion

The results presented here demonstrate for the first time that endogenous IL-33 plays a pro-inflammatory role during acute OVA-induced allergic inflammation of the lung: IL-33-deficiency results in milder lung pathology, reduced eosinophilic infiltrate and impaired amplification of the local CD4⁺ T-cell response.

Local administration of IL-33 into the lung resulted in peribronchiolar cuffing and production of mucus, cytokines and chemokines (Fig. 1). Both CCR3 and CCR4 have been implicated in the chemotaxis of eosinophils and T_H2 cells, respectively, and chemokines CCL-11, CCL-17 and CCL-22, which are ligands for CCR3 and CCR4, showed increased expression in the lung tissue of IL-33-treated mice. These findings indicate that intra-nasal administration of recombinant IL-33 triggers a respiratory tract-specific subset of T_H2-associated mediators. Our results are largely consistent with the findings of Kondo *et al.*, who reported ST2/MyD88-dependent airway hyperresponsiveness (AHR), goblet cell hyperplasia and cytokine production upon intra-nasal administration of IL-33 protein (18) and those of Zhiguang *et al.* in IL-33 transgenic mice (19). A repeated dosing scheme applied in those studies or constitutive expression, however, precludes direct comparison to our results, which focused on the acute effects of a single dose of IL-33.

Intra-nasal administration of IL-33 also resulted in the production of IL-5 and IL-13 but not IL-4 in BAL fluid, and similarly, OVA-challenged IL-33 KO mice had significantly reduced levels of IL-5 and IL-13 in the BAL fluid, whereas IL-4 production was not significantly altered, compared with challenged WT mice (Fig. 5). This result may be in agreement with the finding that in antigen-specific T_H2 cells IL-33 preferentially stimulates production of IL-5 and IL-13 but not IL-4 (8). Taken together, these results suggest that although IL-33 promotes T_H2 responses, the cytokine does not modulate innate or adaptive expression of IL-4 in the airways, consistent with the idea that IL-33 may be involved in IL-4-independent T_H2 cell differentiation (8).

In our experimental model, the IL-33 KO mice showed reduction in lung pathology, eosinophilic infiltrates and cytokine production (compare Fig. 5). Intriguingly, a possible link between IL-33 and eosinophilia, as suggested by our data, was recently described in humans: Kim *et al.* found a correlation of blood and pulmonary eosinophilia with elevated IL-33 in human serum (20). These results establish endogenous IL-33 as a pro-inflammatory mediator in acute airway inflammation. Of note, despite significant reduction in inflammatory parameters discussed above, pulmonary function measurements did not reveal significant differences between WT and IL-33 KO mice after Penh challenge. This suggests that, at least in the experimental setting of OVA-induced acute airway inflammation, absence of IL-33 does not impact pulmonary function. Intriguingly, in contrast to our data, a recent paper reported a differential response to Penh in WT and IL-33-deficient mice subjected to a similar OVA challenge (21). In those studies, groups of IL-33-deficient mice, that were challenged with saline or OVA, did not show a differential response to metacholin challenge, whereas corresponding groups of WT mice did. At this point

we can only speculate that the variable choice of experimental approaches for the metacholine challenge, non-invasive in our studies and invasive in the study published by Oboki *et al.*, may account for the differences between data sets. However, BAL eosinophils observed by Oboki *et al.* appear grossly similar to our findings (21).

Ongoing studies are investigating the AHR of IL-33 KO mice in the context of chronic airway inflammation. Of note, an anti-ST2 antibody was reported to modulate AHR in an OVA-induced model of allergic airway inflammation, although these data were generated using WT BALB/c mice (6), whereas the IL-33 KO mice are on the C57BL/6 background, a strain that tends to mount overall reduced allergic responses.

IL-33 protein consistently localized to F4/80⁺ cells of the alveolar epithelium, in both saline- and OVA-challenged mice (Fig. 3). The expression was nuclear, and vesicular or surface expression was not observed. Taken together with results from Cayrol *et al.* (2) and also Luthi *et al.* (1), that IL-33 is released from necrotic cells, the possibility exists that IL-33 is not actively secreted as a mature cytokine during challenge but is instead passively released by necrotic cells as a danger signal. In such a scenario, IL-33's role could be similar to the nuclear protein HMGB1 that has been shown to modulate cytokine production after being released from necrotic cells or during bacterial or viral infection (22–25). In a similar regard, released IL-33 could function as a chemoattractant to eosinophils or T cells (7) or could modulate proliferation or production of IL-5 and IL-13 in these cell types. Therefore, our data imply that the integrity of the alveolar epithelium is compromised in the context of OVA challenge. Furthermore, this supports the hypothesis that airway OVA challenge triggers T_H2-associated inflammation but in addition involves necrosis of lung epithelium and hence tissue repair responses. Our finding of parallel increases in mRNA for both IL-33 and the membrane form of ST2 upon OVA challenge (Fig. 2) suggests the possibility of an autocrine amplification loop of IL-33/ST2-dependent signals. This idea may be further supported by the observation that OVA-dependent up-regulation of mRNA for membrane ST2 is reduced in the IL-33 KO mice compared with WT (Fig. 5D).

In addition to the effects of endogenous IL-33 on lung pathology, cellular infiltrates and cytokine production, IL-33 also appears to impact the level of T-cell activation in the MLN. Clearly, MLN cells from IL-33 KO mice showed much reduced proliferation and cytokine production when cultured *ex vivo*. FACS analysis revealed a lower proportion of activated TCRβ⁺CD4^{int} T cells present in the MLN of IL-33 KO mice and BrdU labeling revealed that these cells have no principle defect in regards to proliferation *in vivo* (compare Fig. 6). Whether absence of endogenous IL-33 compromises TCRβ⁺CD4^{int} T-cell trafficking to MLN, reduces efficiency of antigen presentation and/or T-cell priming or limits viability of TCRβ⁺CD4^{int} T cells will be investigated in future studies.

IL-33 mediates its biological effects through a heterodimeric receptor complex of ST2 and IL-1RAcP. Whereas IL-1RAcP is ubiquitously expressed, ST2 expression is restricted and determines cellular responsiveness to IL-33 stimulation. A recent paper reports ST2 expression on lung epithelial and endothelial cells, suggesting that IL-33 could

directly activate those non-lymphoid cells through the ST2 receptor (26). Interestingly, increased serum levels of soluble ST2, a soluble receptor antagonist to IL-33, have been reported in human adult and pediatric asthma patients undergoing acute exacerbation episodes (27, 28). In addition, elevated levels of soluble ST2 in the serum of mice after allergen challenge in an allergic airway inflammation model have been reported (29, 30). These findings imply that soluble ST2-dependent regulation of IL-33 bioactivity may occur in the context of allergic lung inflammation. Our results suggest differential expression and regulation of membrane ST2 and soluble ST2 in the lung and show that membrane ST2 but not soluble ST2 may be expressed. However, our data cannot exclude extra-pulmonary expression of soluble ST2, which may impact IL-33-driven responses indirectly.

Experiments utilizing ST2-deficient mice or anti-ST2 antibodies have given varied results in OVA-induced asthma models. Treatment of mice with an anti-ST2 antibody reduced AHR and mucus production in the lung of BALB/c mice, although BAL fluid levels of IL-13 were not modulated (6). In a similar study, an anti-ST2 antibody reduced BAL eosinophilia and BAL fluid IL-5 and IL-4 (31). Using a T_H2 cell transfer model of *in vivo* lung inflammation, an anti-ST2 antibody reduced eosinophilia, IgE and cytokine levels (15), although a recent study investigating OVA-specific ST2^{-/-} T cells in a T-cell transfer model suggested a protective role for ST2 in allergic lung inflammation instead (16). Notably, ST2-deficient mice tested in the OVA-induced model of allergic airway inflammation reportedly show no difference with regards to IgE or cellular composition of the BAL compared with WT controls (14).

The results summarized above appear inconsistent; however, several considerations may help explain why these apparent differences were observed: (i) ST2^{-/-} cells are not only deficient in the membrane form of ST2 that functions as a signaling receptor for IL-33 but are also deficient in soluble ST2, the soluble receptor antagonist for IL-33. Varying contributions of soluble versus membrane ST2 forms, perhaps at distinct anatomic locations, in the context of the varying experimental settings may lead to confusing results; (ii) the anti-ST2 antibody reagents used in experiments typically recognize both ST2 forms which complicates the interpretation of observed results; (iii) as multiple T_H2 -associated cell types are capable of responding to IL-33 stimulation, some variability may be attributed to the relative involvement of certain cell types in the T_H2 response investigated in a given experimental model; (iv) the possibility that unknown ST2 binding proteins are also involved and contribute differentially in the various experimental settings cannot be excluded. Regardless of the ongoing debate concerning the exact role of ST2, our results clearly show that endogenous IL-33 acts as a pro-inflammatory mediator during acute allergic reactions in the lung, contributing to lung pathology, lung eosinophilia, cytokine production and expansion of antigen-specific CD4⁺ T cells.

Taken together, our data extend the understanding of the role of endogenous IL-33 in T_H2 -associated inflammation and support the concept of therapeutic modulation of the IL-33 pathway for treatment of allergic lung diseases.

Supplementary data

Supplementary data are available at *International Immunology* Online.

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