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Endogenous IL-33 and Its Autoamplification of IL-33/ST2 Pathway Play an Important Role in Asthma

Jenna M. Magat,^{*,1} Joanna L. Thomas,^{†,1,2} Justin P. Dumouchel,^{*} Fiona Murray,^{*,3} Willis X. Li,^{*} and Jinghong Li^{*}

IL-33 and its receptor ST2 are contributing factors to airway inflammation and asthma exacerbation. The IL-33/ST2 signaling pathway is involved in both the onset and the acute exacerbations of asthma. In this study, we address the role of endogenous IL-33 and its autoamplification of the IL-33/ST2 pathway in Ag-dependent and Ag-independent asthma-like models. Wild-type, IL-33 knockout, ST2 knockout mice were either intratracheally administrated with 500 ng of rIL-33 per day for four consecutive days or were sensitized and challenged with OVA over 21 d. In wild-type mice, IL-33 or OVA induced similar airway hyperresponsiveness and eosinophilic airway inflammation. IL-33 induced its own mRNA and ST2L mRNA expression in the lung. IL-33 autoamplified itself and ST2 protein expression in airway epithelial cells. OVA also induced IL-33 and ST2 protein expression. In IL-33 knockout mice, the IL-33- and OVA-induced airway hyperresponsiveness and eosinophilic airway inflammation were both significantly attenuated, whereas IL-33-induced ST2L mRNA expression was preserved, although no autoamplification of IL-33/ST2 pathway was observed. In ST2 knockout mice, IL-33 and OVA induced airway hyperresponsiveness and eosinophilic airway inflammation were both completely diminished, and no IL-33/ST2 autoamplification was observed. These results suggest that endogenous IL-33 and its autoamplification of IL-33/ST2 pathway play an important role in the induction of asthma-like phenotype. Thus an intact IL-33/ST2 pathway is necessary for both Ag-dependent and Ag-independent asthma-like mouse models. *The Journal of Immunology*, 2020, 204: 000–000.

Interleukin-33 was identified as the functional ligand for the IL-1R family member ST2 (1). ST2 has two forms. One is the transmembrane ST2 (ST2L), which transduces the IL-33 signaling. The other one is a soluble ST2 molecule (sST2), caused by alternative splicing (2). sST2 acts as a decoy receptor for IL-33 (3). Polymorphisms of human IL-33 and ST2 genes are associated with asthma and increased numbers of eosinophils (4, 5). IL-33 is a contributing factor to airway inflammation and asthma exacerbation (6, 7). The serum concentrations of sST2 are higher in asthma patients during acute exacerbation, suggesting that the host tries to attenuate IL-33 signaling (8, 9). An IL-33 loss-of-function mutation is associated with reduced blood eosinophil counts, and people with this mutation are protected from asthma (10).

IL-33 was originally characterized as a nuclear protein (11). It has a dual function of a potent cytokine and a nuclear transcription factor (12, 13). IL-33 binds to its receptor ST2 to augment the production of Th2 cytokines IL-4, IL-5, and IL-13 in vitro and in vivo (1, 14). It has been shown that epithelial derived IL-33 is a potent stimulator to induce IL-13-producing type 2 innate lymphoid cells (ILC2) (15, 16). ILC2 contribute to Ag-dependent and Ag-independent airway hyperactivity (15, 16). The role of IL-33 nuclear localization and chromatin association was not well established (17). IL-33 was found to sequester nuclear NF- κ B and dampen proinflammatory signaling (13). We have previously shown that the intratracheal administration of IL-33 for 4 d induced an asthma-like phenotype mouse model with increased production of Th2 cytokines (18). The IL-33/ST2 signaling pathway may work in an autocrine and paracrine manner to exacerbate airway inflammation. In addition, recent data showed multiple feedback circuits involving IL-33 are required for asthma persistence (19).

In this study, we address the role of autoamplification of IL-33/ST2 pathway in Ag-dependent and Ag-independent asthma-like mouse models. In IL-33 knockout mice, we found that exogenous IL-33 could activate the IL-33/ST2 pathway but no IL-33 autoamplification happened. As a result, no robust airway hyperresponsiveness or eosinophilic inflammation were elicited. In ST2 knockout mice, no IL-33/ST2 autoamplification was observed and no airway hyperresponsiveness and eosinophilic inflammation were found. Endogenous IL-33 and its autoamplification of IL-33/ST2 pathway play an important role in the induction of asthma-like phenotype.

Materials and Methods

IL-33-induced and OVA-induced asthma-like phenotype in mouse models

All studies were performed according to National Institutes of Health Guidelines for the Care and Use of Laboratory Animals and approved by the

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Abbreviations used in this article: BAL, bronchoalveolar lavage; F, forward; IHC, immunohistochemistry; ILC2, type 2 innate lymphoid cell; qPCR, quantitative PCR; R, reverse; sST2, soluble ST2 molecule.

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Institutional Animal Care and Use Committee of the San Diego VA Healthcare System. Mouse strains studied included C57BL/6 from Jackson Laboratories (Bar Harbor, ME), IL-33 knockout from Dr. R. Lee (Brigham and Women's Hospital, Harvard Medical School); and ST2 knockout from Dr. S. Akira (Research Institute for Microbial Disease, Osaka University, Japan). Asthma-like phenotype mouse models were previously described (18). In brief, for the IL-33 model, 6–8-wk-old mice were intratracheally administered with 500 ng of rIL-33 per day for four consecutive days. For the OVA model, 6–8-wk-old mice were immunized and sensitized to OVA using a 21-d protocol. Mice were immunized by i.p. injection of 50 µg OVA and 1 mg alum in PBS on day 0 and 7, followed by four intratracheal injections of 20 µg OVA in 50 µl of PBS on days 17, 18, 19, and 20. On day 5 for the IL-33 model or on day 21 for the OVA model, the mice were intubated with a 20 gauge i.v. catheter and placed on a computer-controlled small animal ventilator (Flexivent; SCIREQ, Montreal, Canada) delivering 2% isoflurane continuously (20). Baseline airway resistance was measured. The animals were then challenged with an ultrasonic aerosol of 1.5, 3, 6, 12, and 24 mg/ml methacholine. The peak airway resistance with each dose was obtained.

BAL cell counts, real-time quantitative PCR, and ELISA

Following the methacholine challenges, the mice were euthanized and bronchoalveolar lavage (BAL) fluid were obtained with 0.5 ml of PBS repeated for three times. The total cell counts and differentiation of the cells in BAL were studied. One lung was removed for RNA extraction and protein extraction. The other lung was fixed for histological analysis and immunohistochemistry (IHC). Total RNA was extracted from lung tissue by using TRIzol (Invitrogen, Carlsbad, CA). cDNA was synthesized and real-time quantitative PCR (qPCR) were performed with SYBR Green (Invitrogen). Expression is shown relative to the reference gene GAPDH. Primers are mouse IL-33 forward (F): 5'-ACTCCAAGATTTCCGCCG-3'; mouse IL-33 reverse (R): 5'-CATGCAGTAGACATGGCAGAA-3'; mouse ST2L F: 5'-GTGATAGTCTTAAAAGTGTCTTGG-3'; mouse ST2L R: 5'-TCAAAAGTGTTCAGGTCTTAAGCA-3'; mouse sST2 F: 5'-ACGCTCGACTTATCCTGTGG-3'; mouse sST2 R: 5'-CAGCTCAATTGTGGACACG-3'. ELISA for IL-33 and sST2 were performed on BAL samples with mouse IL-33 and ST2 ELISA kits (R&D Systems, Minneapolis, MN) according to the manufacturer's instructions.

Immunohistochemistry and lung inflammation score

Formalin-fixed paraffin-embedded specimens were serially sectioned at 5-µm intervals and mounted on glass slides. IHC for IL-33 and ST2 were performed using the Avidin-Biotin Complex Kits (Vector Laboratories, Burlingame, CA). Rabbit anti-mouse ST2 Ab (Abcam, Cambridge, MA), mouse anti-mouse IL-33 Ab (Abcam), and rabbit and mouse IgG isotype controls (Vector Laboratories) were used. Lung inflammation score was performed based on the degree of hypertrophy of the airway epithelium and peribronchial/perivascular cellular inflammation on a scale of 0–5 by a lung pathologist blinded to the experimental groups. Peribronchial/perivascular infiltrates were as follows: 0 (not present); 1 (<20% of the airways affected); 2 (20–40%); 3 (40–60%); 4 (60–80%); and 5 (>80%).

Western blots

Total lung proteins were prepared with RIPA buffer. Proteins were separated in 10% SDS-PAGE and transferred to nitrocellulose membrane. The membrane was blocked for 1 h in blocking buffer for fluorescent Western blot (Rockland, Gilbertsville, PA). Proteins were detected by Western blot by using anti-IL-33 Ab (Abcam). Fluorescent-labeled secondary Ab (Li-Cor Biosciences, Lincoln, NE) was used to detect the protein bands. An Odyssey imaging system imager was used to capture the images (Li-Cor Biosciences).

Statistical analysis

ANOVA was performed using GraphPad Prism software (La Jolla, CA). Statistical analyses of data were performed using paired and unpaired Student *t* tests or using one-way ANOVA. For qPCR results, log fold changes were used for *t* tests. Statistical significance is defined by *p* < 0.05.

Results

IL-33 autoamplifies the IL-33/ST2 pathway by inducing its own and ST2 expression

We have previously shown that intratracheal administration of rIL-33 can induce airway hyperresponsiveness and eosinophilic

airway inflammation in wild-type mice (18). In the IL-33 models, we found IL-33 induced its own mRNA and ST2L mRNA levels in the lung tissue (Fig. 1A). We also examined the BAL IL-33 and sST2 protein levels by ELISA. We found that the endogenous IL-33 levels were increased and the sST2 levels were nearly abolished in BAL (Fig. 1B, 1C). In the OVA models, although the IL-33 and ST2 mRNA levels were not significantly changed in the lung tissue (Fig. 1A), the BAL IL-33 levels were increased and

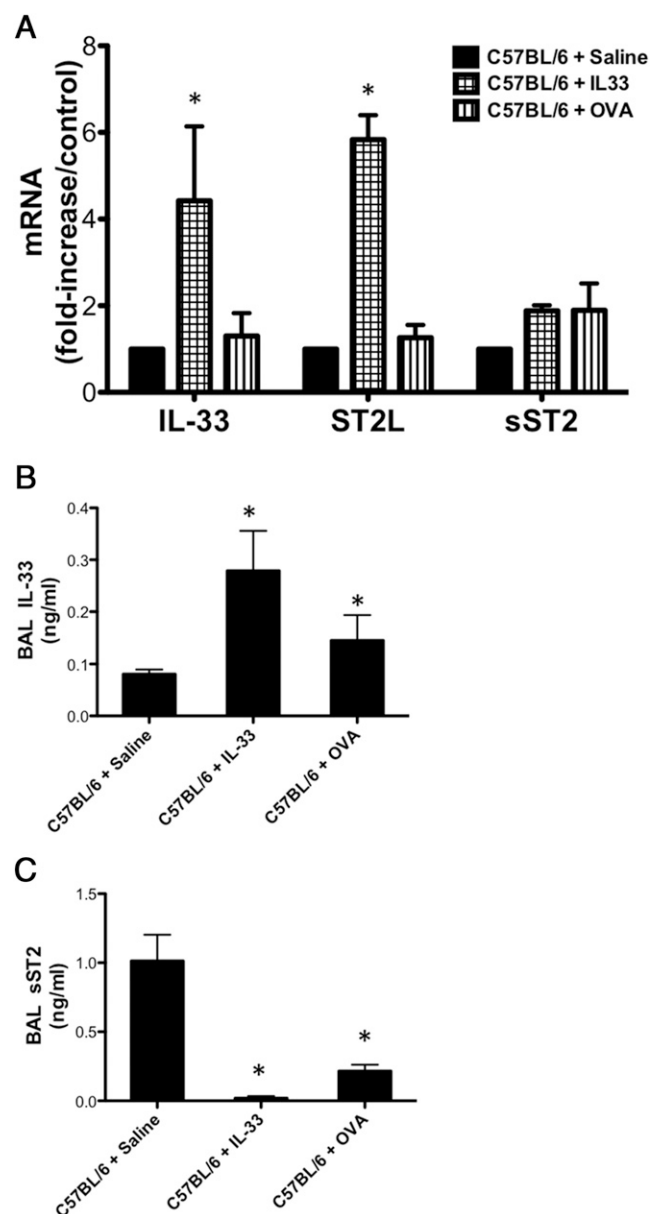


FIGURE 1. IL-33 autoamplifies the IL-33/ST2 pathway by inducing its own and ST2 expression. **(A)** rIL-33 induces endogenous IL-33 and ST2L mRNA in the lung tissue. Wild-type mice were treated with saline, IL-33, or OVA. Real-time qPCR was performed by using IL-33, ST2L, and sST2 specific primers. In the IL-33 model, a 4-fold increase of IL-33 was observed compared with saline controls (*n* = 6). A 5.8-fold increase of ST2L was observed compared with saline controls (*n* = 6). Student *t* test was used to calculate the significance of differences in log fold-changes of the qPCR results, which are normally distributed. **(B)** IL-33 induces IL-33 in BAL, compared with saline controls (*n* = 6). OVA has moderate increase of IL-33, compared with saline controls (*n* = 6). **(C)** IL-33 and OVA decreases sST2 in the BAL, compared with saline controls (*n* = 6). **p* < 0.01 compared with saline controls.

sST2 levels were decreased with similar trends of lesser magnitude compared with the IL-33 models (Fig. 1B, 1C).

Airway epithelial cells are the major source of endogenous IL-33 production in autoamplification of IL-33/ST2 pathway

To investigate the changes in the IL-33/ST2 pathway at the cellular level, we performed IHC studies (Fig. 2A). In the saline controls (Fig. 2A, left panel), IL-33 was found in the airway epithelial cells, whereas ST2 was minimally detected in the airway epithelium. In the IL-33 models (Fig. 2A, middle panel), IL-33 was found in the airway and alveolar epithelial cells; interstitial cells, including lymphocytes; macrophages; and the nucleus of type II epithelial cells. ST2 was found in epithelial cells and

interstitial cells as well. In the OVA models (Fig. 2A, right panel), in addition to the airway and alveolar epithelial cells and interstitial cells, IL-33 was also found in smooth muscle cells and the cytoplasm of alveolar macrophages. ST2 was found in the epithelium and interstitial cells. Lung inflammation scores were performed by a lung pathologist blinded to the experimental groups (Fig. 2B). Both IL-33 and OVA groups showed significant increased lung inflammation scores compared with saline control group. Examples of lung tissue IL-33 protein levels are shown by Western blots (Fig. 2C). rIL-33 (exogenous) and endogenous IL-33 were distinguishable in sizes. Autoamplified endogenous IL-33 protein levels in IL-33 models were confirmed.

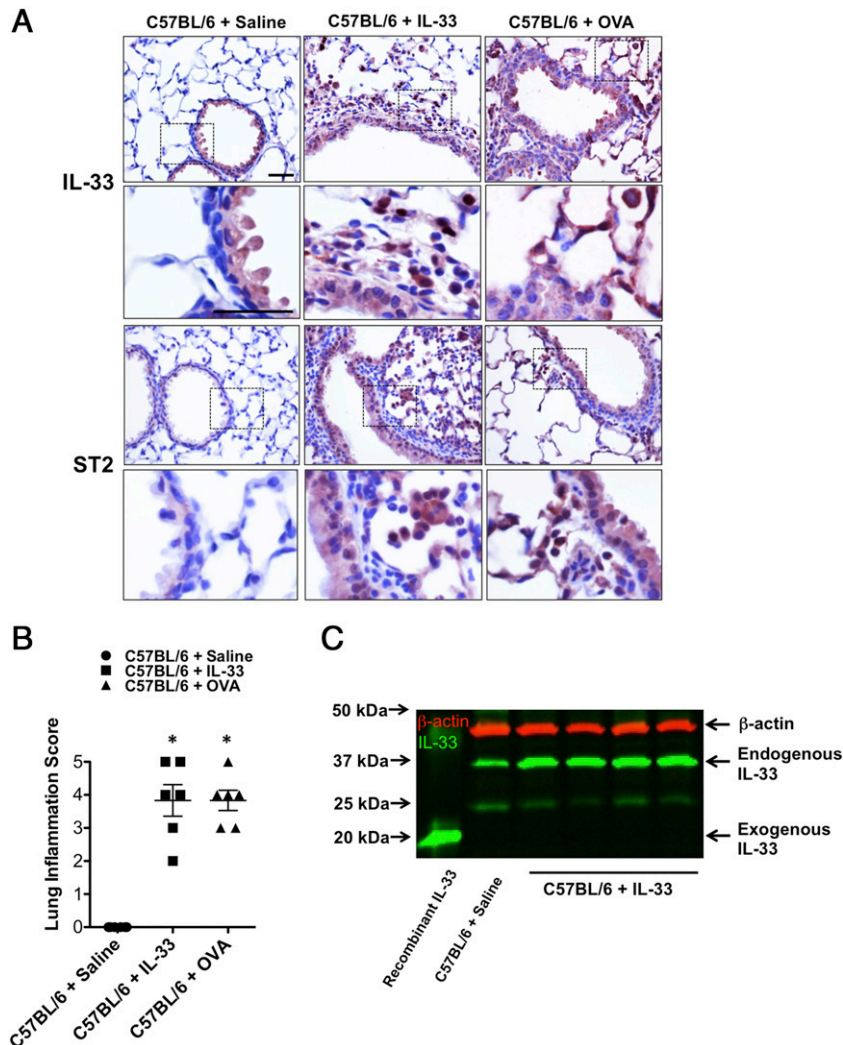


FIGURE 2. Airway epithelial cells are the major source of endogenous IL-33 production in autoamplification of IL-33/ST2 pathway. **(A)** Representative images of IHC lung specimens were shown ($n = 6$). Scale bar, 100 μm at lower or 50 μm at higher magnifications. Higher magnification images are shown for regions indicated by dashed boxes. Left panel, Wild-type mice with saline control. IL-33 was mostly found in the airway epithelial cells (top two rows), much less was found in the alveolar epithelial cells. ST2 was minimally detected in the airway epithelium (bottom two rows). Middle panel, Wild-type mice with IL-33 models, IL-33 was found in the airway and alveolar epithelial cells, interstitial cells, including lymphocytes, macrophages, and the nucleus of Type II epithelial cells. ST2 was found in epithelial cells and interstitial cells as well. Right panel, Wild-type mice with OVA models, in addition to the airway and alveolar epithelial cells and interstitial cells, IL-33 was also found in smooth muscle cells and the cytoplasm of alveolar macrophages. ST2 was found in the epithelium and interstitial cells. **(B)** Lung inflammation score was performed based on the degree of hypertrophy of the airway epithelium and peribronchial/perivascular cellular inflammation. Both IL-33 and OVA models showed significant increased lung inflammation scores compared with saline control group. $*p < 0.01$ compared with saline controls. **(C)** Examples of lung tissue IL-33 protein levels are shown by Western blots. Lung tissue proteins from the indicated mice were subject to SDS-PAGE, the resulting membrane was blotted with anti-IL-33 (green) and anti- β -actin (red), and were analyzed with an Odyssey imaging system imager. Multiple Western blots were done and representative images are shown. From left: lane 1, rIL-33 protein (exogenous, molecular mass 18.1 kDa). Lane 2, wild-type mice with saline control. Lane 3–6, wild-type mice with IL-33 models. The endogenous IL-33 (lane 2–6) has a molecular mass of 35 kDa. In IL-33 group, the endogenous IL-33 is upregulated compared with the saline control group. In all the mice groups, no detectable rIL-33 was found.

Endogenous IL-33 is necessary for Ag-dependent and Ag-independent asthma-like phenotype in vivo

To understand if endogenous IL-33 is necessary for Ag-dependent and Ag-independent asthma-like phenotype, we used the IL-33 knockout mice. In IL-33 knockout mice, rIL-33 induced airway hyperresponsiveness and eosinophilic airway inflammation were significantly attenuated compared with wild-type mice (Fig. 3A, 3B). Exogenous rIL-33 was not sufficient to restore the asthma-like phenotype in IL-33 knockout mice, despite the intact ST2 and other components of downstream IL-33/ST2 pathway. In IL-33 knockout mice, OVA induced airway hyperresponsiveness and eosinophilic airway inflammation were significantly attenuated as well (Fig. 3A, 3B).

Exogenous IL-33 can induce ST2L mRNA expression but is not sufficient to induce the endogenous autoamplification of IL-33/ST2 pathway

To further understand why exogenous IL-33 was not sufficient to restore the asthma-like phenotype in IL-33 knockout mice, we examined the ST2L and sST2 mRNA in lung tissue and IL-33 and sST2 protein levels in BAL. In IL-33 models, we found the exogenous IL-33-induced ST2L mRNA expression was preserved in lung tissue (Fig. 4A), whereas the effects of IL-33 on increasing IL-33 protein levels and decreasing sST2 in the BAL were diminished (Fig. 4B, 4C). In OVA models, OVA-induced ST2L mRNA expression was attenuated (Fig. 4A). The effects of OVA on increasing IL-33 protein levels and decreasing sST2 protein levels in the BAL were NS (Fig. 4B, 4C).

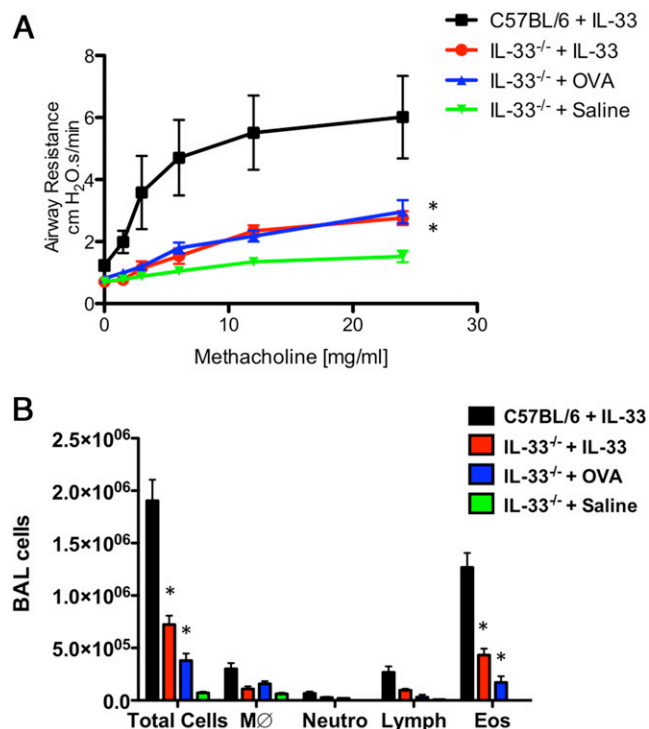


FIGURE 3. Endogenous IL-33 is necessary for Ag-dependent and Ag-independent asthma-like phenotype in vivo. **(A)** IL-33 knockout mice were treated with saline, IL-33 or OVA. IL-33 induced airway hyperresponsiveness was significantly attenuated ($n = 6$). OVA induced airway hyperresponsiveness was significantly attenuated ($n = 6$). $*p < 0.01$ compared with wild-type mice IL-33 model. **(B)** In IL-33 knockout mice, IL-33 induced eosinophilic infiltration was significantly attenuated ($n = 6$). OVA induced eosinophilic infiltration was significantly attenuated ($n = 6$). $*p < 0.01$ compared with wild-type mice IL-33 model.

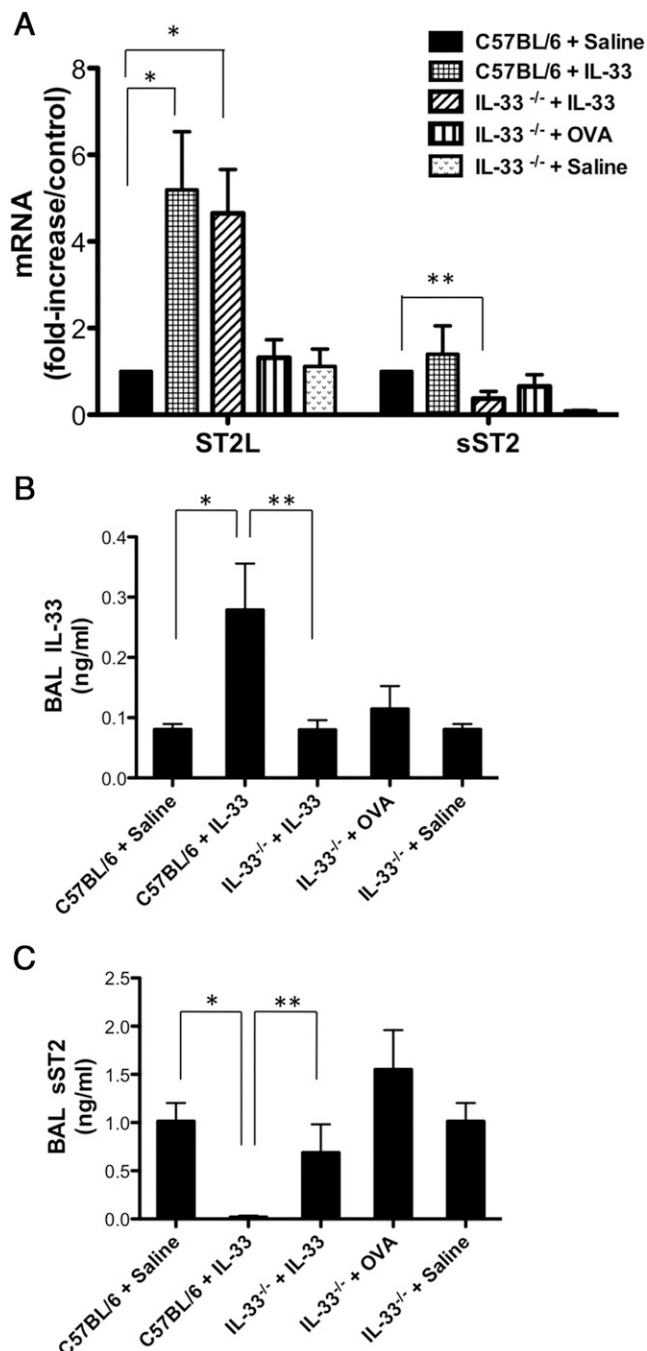


FIGURE 4. Exogenous IL-33 can induce ST2L mRNA expression but is not sufficient to induce the endogenous autoamplification of IL-33/ST2 pathway. **(A)** In IL-33 knockout mice, IL-33-induced ST2L mRNA expression was preserved in the lung ($n = 6$), comparable with wild-type mice saline control. $*p < 0.01$ compared with wild-type mice saline controls. OVA-induced ST2L mRNA expression was attenuated ($n = 6$), NS compared with wild-type mice saline controls. IL-33 decreased sST2 mRNA expression in the lung ($n = 6$), comparable with wild-type mice saline control. $**p < 0.05$ compared with wild-type mice saline controls. **(B)** In IL-33 knockout mice, the effects of IL-33 on increasing IL-33 protein levels in the BAL were diminished ($n = 6$), compared with wild-type mice IL-33 model. $**p < 0.01$ compared with wild-type mice IL-33 model. The effects of OVA on increasing IL-33 protein levels in the BAL were NS compared with saline controls ($n = 6$). **(C)** The effects of IL-33 on decreasing sST2 protein levels in the BAL were diminished ($n = 6$), compared with wild-type mice IL-33 model. $**p < 0.01$ compared with wild-type mice IL-33 model. The effects of OVA on decreasing sST2 protein levels in the BAL were NS, compared with saline controls ($n = 6$).

ST2 is necessary for Ag-dependent and Ag-independent asthma-like phenotype in vivo

ST2 is currently the only known receptor for IL-33 signaling. In ST2 knockout mice, both IL-33- and OVA-induced airway hyperresponsiveness or eosinophilic airway inflammation were completely diminished. There were no significant differences in airway hyperresponsiveness and total cell count and cell differentials compared with saline controls (Fig. 5A, 5B). In ST2 knockout mice, IL-33-induced autoamplification of IL-33 was completely diminished (Fig. 5C). OVA-induced IL-33 was also completely diminished (Fig. 5C).

Discussion

IL-33 is expressed in higher levels in endobronchial biopsies from human asthma patients (7). The elevated IL-33 expression was particularly evident in severe asthmatic subjects (6, 7). IL-33 functions both extracellularly as a cytokine through activation of the ST2L receptor and intracellularly as a nuclear transcription factor (12, 13). IL-33 can increase ST2 expression in eosinophils (21). In animal models, multiple feedback circuits involving IL-33 are required for asthma persistence (19). In our IL-33 models, we found parallel increases of IL-33 and ST2L mRNA levels in the lung. In both IL-33 and OVA models, we found increased IL-33 and decreased sST2 in BAL. We found that epithelial cells are the major source of endogenous IL-33 and ST2 production. These data suggest the autoamplification of IL-33/ST2 pathway. OVA-induced IL-33 and ST2 expression may have a different mechanism. We found enlarged mediastinal lymph nodes in the OVA models, and ST2 was significantly increased in the lymph nodes (data not shown). Our data are consistent with the findings that dust mites and other allergens can induce IL-33 (22, 23) and elevate serum sST2 levels (24). Autoamplification of IL-33/ST2 pathway is necessary for the induction of asthma-like phenotype in both IL-33 models and OVA models.

We used IHC to examine the major source of IL-33 in lung tissue. IL-33 was localized to the nuclei of airway and alveolar epithelial cells. IL-33 induced IL-33 and ST2 expression localizing at the same epithelial cells and interstitial cells. It was reported that IL-33 accumulated in the nucleus of producing cells and binds to histones and chromatin (25, 26). The function of IL-33 as an NF was not well established. A recent study showed the only identified protein modulated by IL-33 as an NF was IL-33 itself (27). Our data provided the evidence that IL-33 autoamplified itself and ST2 protein expression in airway epithelial cells. It was reported that multiple feedback and feed-forward circuits between epithelial cells and ILC2 were identified (19). IL-33 may regulate IL-33/ST2 pathway in several manners, such as autocrine and paracrine regulation as a cytokine, and transcriptional regulation or epigenetic regulation at the chromatin level as an NF.

In IL-33 knockout mice, we showed that exogenous rIL-33 induced a modest airway hyperresponsiveness and mild eosinophilic airway inflammation. Exogenous IL-33-induced ST2L mRNA expression was preserved in lung tissue. However, because of the lack of endogenous IL-33 and its autoamplification of IL-33/ST2 pathway, BAL IL-33 levels and sST2 levels were not changed, leading to only moderate asthma phenotype. These data indicate that exogenous IL-33 was able to induce ST2L mRNA expression based on the intact ST2 and downstream pathway. The IL-33/ST2 signaling was successfully initiated but failed to maintain because of lack of endogenous IL-33 and its autoamplification. We conclude that multiple feedback loops of continuous autoamplification of IL-33/ST2 pathway are necessary for maintaining the asthma-like phenotype in vivo. It was previously

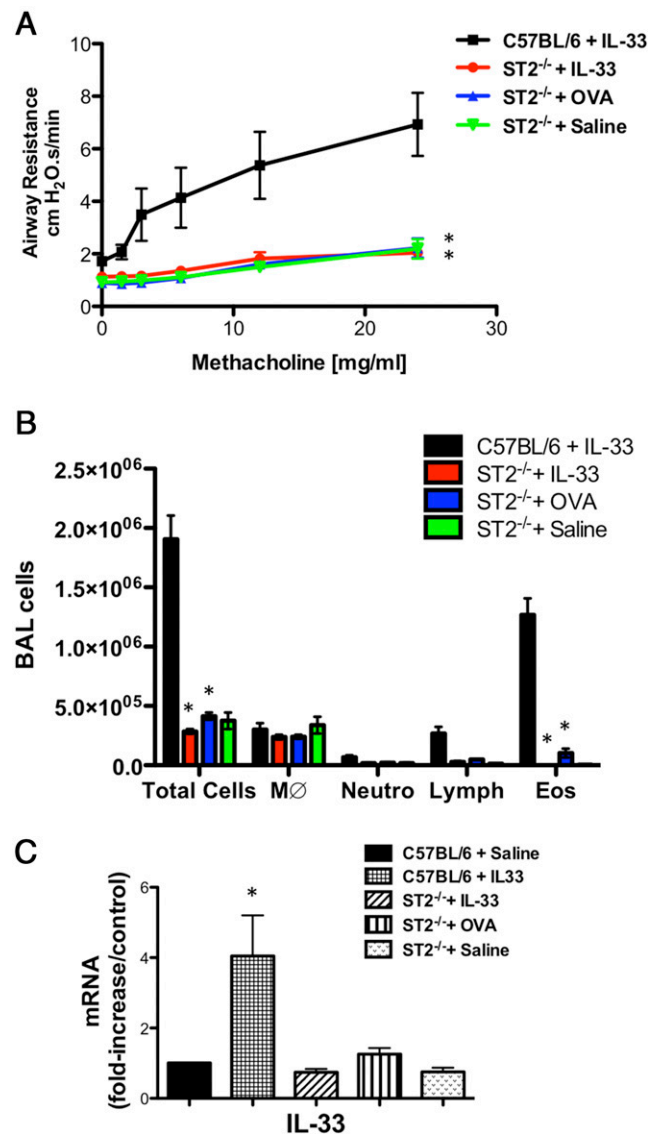


FIGURE 5. ST2 is necessary for Ag-dependent and Ag-independent asthma-like phenotype in vivo. **(A)** ST2 knockout mice were treated with saline, IL-33, or OVA. IL-33-induced airway hyperresponsiveness was completely diminished ($n = 6$). OVA induced airway hyperresponsiveness was completely diminished ($n = 6$). $*p < 0.01$ compared with wild-type mice IL-33 model. **(B)** In ST2 knockout mice, IL-33 induced eosinophilic infiltration was diminished ($n = 6$). OVA induced eosinophilic infiltration was diminished ($n = 6$). $*p < 0.01$ compared with wild-type mice IL-33 model. **(C)** In ST2 knockout mice, rIL-33 induces endogenous IL-33 mRNA in the lung tissue was completely diminished. OVA induced IL-33 mRNA in the lung tissue was also completely diminished. $*p < 0.01$ compared with wild-type mice saline controls.

reported that IL-33 knockout mice showed attenuated eosinophil and lymphocyte recruitment to the lung in OVA model (28, 29). We observed similar results. OVA can elicit a moderate response because of the intact adaptive immune response in IL-33 knockout mice. In both IL-33 and OVA models, the responses are reduced because of lack of endogenous IL-33. These data indicate that the endogenous IL-33 and its autoamplification of IL-33/ST2 pathway are necessary for Ag-dependent and Ag-independent asthma-like phenotype in vivo.

We showed that ST2 expression in the airway epithelium and inflammatory cell infiltrate were increased in both IL-33- and OVA-induced asthma models. In ST2 knockout mice, both IL-33- and OVA-induced airway hyperresponsiveness or eosinophilic airway

inflammation were completely diminished. ST2 knockout mice are partially protected from house dust mite induced asthma, especially in the peripheral lung (23). An intact IL-33/ST2 pathway is necessary for both Ag-dependent and Ag-independent asthma-like mouse models.

In summary, endogenous IL-33 and its autoamplification of IL-33/ST2 pathway play an important role in both Ag-dependent and Ag-independent asthma-like mouse models. Airway epithelial cells are the major source of endogenous IL-33 production in autoamplification of IL-33/ST2 pathway. IL-33 and sST2 are possible biomarkers for asthma exacerbation (24, 30) and also could be therapeutic targets to inhibit the IL-33/ST2 autoamplification. Novel treatment including IL-33 Abs, sST2, and vaccination against IL-33 are under investigation in asthma (3, 31–33). Our recent study of small molecule inhibitors of the IL-33/ST2 pathway showed beneficial results (18). Clinical trials with Abs targeting IL-33 have already been initiated (34).

Disclosures

The authors have no financial conflicts of interest.

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