Experimental asthma persists in IL-33 receptor knockout mice because of the emergence of thymic stromal lymphopoietin–driven IL-9$^+$ and IL-13$^+$ type 2 innate lymphoid cell subpopulations

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Background: IL-33 plays an important role in the development of experimental asthma.

Objective: We sought to study the role of the IL-33 receptor suppressor of tumorigenicity 2 (ST2) in the persistence of asthma in a mouse model.

Methods: We studied allergen-induced experimental asthma in ST2 knockout (KO) and wild-type control mice. We measured airway hyperresponsiveness by using flexiVent; inflammatory indices by using ELISA, histology, and real-time PCR; and type 2 innate lymphoid cells (ILC2s) in lung single-cell preparations by using flow cytometry.

Results: Airway hyperresponsiveness was increased in allergen-treated ST2 KO mice and comparable with that in allergen-treated wild-type control mice. Peribronchial and perivascular inflammation and mucus production were largely similar in both groups. Persistence of experimental asthma in ST2 KO mice was associated with an increase in levels of thymic stromal lymphopoietin (TSLP), IL-9, and IL-13, but not IL-5, in bronchoalveolar lavage fluid. Expectedly, ST2 deletion caused a reduction in IL-13$^+$ CD4 T cells, forkhead box P3–positive regulatory T cells, and IL-5$^+$ ILC2s. Unexpectedly, ST2 deletion led to an overall increase in innate lymphoid cells (CD45$^+$lin$^-$CD25$^+$ cells) and IL-13$^+$ ILC2s, emergence of a TSLP receptor–positive IL-9$^+$ ILC2 population, and an increase in intraepithelial mast cell numbers in the lung. An anti-TSLP antibody abrogated airway hyperresponsiveness, inflammation, and mucus production in allergen-treated ST2 KO mice. It also caused a reduction in innate lymphoid cell, ILC2, and IL-9$^+$ and IL-13$^+$ ILC2 numbers in the lung.

Conclusions: Genetic deletion of the IL-33 receptor paradoxically increases TSLP production, which stimulates the emergence of IL-9$^+$ and IL-13$^+$ ILC2s and mast cells and leads to development of chronic experimental asthma. An anti-TSLP antibody abrogates all pathologic features of asthma in this model. (J Allergy Clin Immunol 2017;■■■:■■■-■■■.)

Key words: *Asthma, type 2 innate lymphoid cells, IL-33 receptor, thymic stromal lymphopoietin, IL-9, mast cells*

Type 2 innate lymphoid cells (ILC2s) represent an important group of type 2 immune cells.[1,2] They are regulated by many growth factors. IL-33 is an important growth factor for ILC2s, especially lung ILC2s,[1,2] and promotes their differentiation, maturation, and type 2 cytokine expression. IL-33 signals through its receptor, suppressor of tumorigenicity 2 (ST2), also known as T1/ST2L.[3] ST2 signaling is critically important for IL-5 induction in T_H2 cells and is required for T_H2 memory.[4] ST2 signaling regulates many other cells, including regulatory T (Treg) cells,[5] dendritic cells,[6] mast cells,[7] basophils,[8] eosinophils,[9] and macrophages.[10] By regulating so many immune cells, the IL-33/ST2 axis plays a seminal role in host defense and allergic and chronic inflammatory diseases.[11]

The role of ST2 was previously investigated in various models of experimental asthma.[12-17] Some studies reported a complete or substantial resolution of airway hyperreactivity, inflammation, goblet cell hyperplasia, and other features of asthma.[12-15] A few studies observed persistence of experimental asthma in ST2 knockout (KO) mice.[16,17] Complete resolution of experimental asthma and lung inflammation was observed only when all 3 type 2–inducing epithelial cytokines, IL-25, IL-33 and thymic stromal lymphopoietin (TSLP), or their receptors were blocked.[17] Interestingly, most of these studies were performed in acute asthma models or in a model of chronic allergen exposure in which outcomes were measured within 1 to 3 days of the last allergen exposure.

We developed a mouse model in which experimental asthma persists for more than 6 months after the last allergen exposure.[18,19] The persistence of experimental asthma in this model was dependent on ILC2s but not T cells, although the latter contributed to the magnitude of airway inflammation. We investigated whether ST2 played an essential role in this chronic asthma model.

METHODS

The protocol for this study was approved by the National Jewish Health Institutional Animal Care and Use Committee. We obtained ST2 KO mice from Dr Andrew McKenzie (Cambridge, United Kingdom).[12] These mice were backcrossed to BALB/C mice for more than 8 generations. We used BALB/C mice as wild-type (WT) controls (Jackson Laboratory, Bar Harbor, Me).

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Abbreviations used
APC: Allophycocyanin
BAL: Bronchoalveolar lavage
FITC: Fluorescein isothiocyanate
FoxP3: Forkhead box P3
ICOS: Inducible costimulator
ILC: Innate lymphoid cell
ILC2: Type 2 innate lymphoid cell
KLRG1: Killer cell lectin-like receptor subfamily group 1
KO: Knockout
MCP1: Mast cell protease 1
nTreg: Natural regulatory T
PE: Phycoerythrin
ST2: Suppressor of tumorigenicity 2 (also serum stimulation 2)
Treg: Regulatory T
TSLP: Thymic stromal lymphopoietin
TSLPR: Thymic stromal lymphopoietin receptor
WT: Wild-type

Mucosal sensitization of mice

The allergen extract used in this experiment was a mixture of 3 allergens: dust mite *(Dermatophagoides farinae)*, ragweed *(Ambrosia artemisiifolia)*, and *Aspergillus fumigatus* (Greer Laboratories, Lenoir, NC). We used the following protein concentrations of the allergen extracts: *D farina*, 5 μg (LPS content, 3-35 EU by means of LAL assay); ragweed, 50 μg (LPS content, 5 EU); and *Aspergillus* species, 5 μg (LPS content, 0.1 EU). The allergen dose was based on the results of a survey of previous publications (a total of 7 publications) indicating successful sensitization and elicitation of allergic inflammation in the lungs.[18] This allergen mixture was delivered intranasally in 15-μL aliquots in saline. Chronic experimental asthma was developed by means of intranasal delivery of the triple-allergen mixture twice a week for 6 consecutive weeks in female mice 8 to 12 weeks of age, as described previously.[18,19] A timeline of manipulations and interventions for the chronic asthma protocol with allergens is shown in Fig 1, *A*.

Antibody, pharmacologic, and genetic interventions

For TSLP blockade experiments, an anti-TSLP antibody (#MAB555; R&D Systems, Minneapolis, Minn; 20 μg/20 μL in saline administered intranasally) was delivered for 3 consecutive days 3 days before analysis in week 10. An isotype rat IgG_{2a} (#MAB006) antibody was used as a control.

Airway hyperreactivity measurement

Measurement methodologies have been explained in depth elsewhere.[18] Briefly, mice were anesthetized with ketamine (180 mg/kg), xylazine (9 mg/kg), and acepromazine (4 mg/kg). After the loss of the foot-pad pinch reflex, a tracheotomy was performed, and the mouse was attached through an 18-gauge cannula to a small-animal ventilator with a computer-controlled piston (flexiVent Fx; SCIREQ, Montreal, Quebec, Canada).

After performing initial calibrations (cylinder pressure channel and nebulizer calibration), we conducted dynamic tube calibration and used a default program called QuickPrime 3 (version 7) for measurement of airway resistance in response to methacholine. This program uses prime perturbations, which are a family of complex forced oscillation perturbations at a frequency greater than and less than the subject's ventilation frequency (1-20.5 Hz). The amplitude of the oscillatory signal is preset to a volume that is slightly smaller than the subject's tidal volume (0.2 mL). Volume and pressure signal are recorded during a measurement, and the flow signal is derived from the volume. The foregoing allows calculation of Newtonian resistance, tissue damping, tissue elastance, and hysteresivity. Resistance measurements were taken to establish the baseline for total lung resistance and at each methacholine dose. Group averages were expressed as the fold increase over baseline resistance (mean ± SEM).

Histology and immunofluorescence staining

Paraffin-embedded lungs were sectioned and stained with hematoxylin and eosin for morphometric analysis, periodic acid–Schiff for mucin staining, Masson trichrome for collagen deposition, and toluidine blue for mast cell staining. Sections for immunofluorescence staining were permeabilized with 0.01% saponin in PBS, blocked with 2% BSA, and stained with a primary antibody against mouse TSLP (rabbit polyclonal; #ab115700; Abcam, Cambridge, United Kingdom); visualized with an Alexa Fluor 594–conjugated secondary antibody, as described previously[19]; and counterstained with 4′-6-diamidino-2-phnylindole dihydrochloride for nuclear staining.

Images were acquired on a Nikon Eclipse TE2000-U microscope using ×20 dry lenses at room temperature through a Diagnostics Instruments camera model #4.2 using Spot Software 5.0 (Diagnostic Instruments, Sterling Heights, Mich). Hematoxylin and eosin–stained sections were mounted with Permount Medium (Thermo Fisher Scientific, Waltham, Mass). Images were adjusted for brightness and contrast to improve viewing.

Lung digests

Lungs were perfused with saline, and single-cell suspensions of lung cells were acquired by using mechanical mincing of the lungs followed by digestion at 37°C for 45 minutes in RPMI with 10% FBS, 1% penicillin/streptomycin, and collagenase Type I (1 mg/mL; #LS004197; Worthington Biochemical, Lakewood, NJ), as described previously.[19] Cell suspensions were agitated at room temperature for 10 minutes in RPMI with 100 U/mL DNAse I before filtration through 40-μm filters and red blood cell lysis. Single-cell suspensions were cultured subsequently in RPMI with 10% FBS and 1% penicillin/streptomycin at 37°C in a CO_2 incubator overnight. The next day, cells were used for flow cytometric staining and analysis.

Flow cytometric analyses of innate lymphoid cells and T cells

We stained live cells first for cell-surface receptors. Then we fixed the cells with 4% paraformaldehyde for 15 minutes at 4°C, washed them with staining buffer (PBS plus 1% BSA), and then incubated with permeabilization buffer (PBS [pH 7.4] plus 1% BSA plus 0.1% saponin) at 4°C for 20 minutes. After centrifugation, we resuspended the cells in permeabilization buffer and incubated with antibodies against the intracellular proteins (cytokines and transcription factors) at 4°C for 30 minutes. We washed the cells twice with the permeabilization buffer, resuspended in the staining buffer, and maintained at 4°C until flow analysis. For intercellular cytokines, we added monensin (2 μmol/L) to the cells and cultured for 4 hours before staining. Most of the fluorophore-conjugated antibodies were purchased from BioLegend (San Diego, Calif) and some were purchased from eBioscience (San Diego, Calif) or R&D Systems, unless otherwise stated.

Mouse lung single-cell suspension was blocked with an anti-FcR blocking reagent (#130-092-575; Miltenyi Biotec, Bergisch Gladbach, Germany) before staining. Innate lymphoid cells (ILCs) were stained with fluorescein isothiocyanate (FITC)–labeled anti-CD45.2 (clone 104), Pacific blue–labeled lineage marker antibodies (CD3, Ly-6G/Ly-6C, CD11b, CD45R/B220, TER-119/erythroid cells with added antibodies against NK1.1 and FcεRIα), peridinin-chlorophyll-protein complex (PerCP)-Cy5.5–conjugated anti-CD25 (clone PC61.5; eBioscience), allophycocyanin (APC)–labeled anti–IL-9 and anti–IL-5 (TRFK5), and phycoerythrin (PE)-Cy7–labeled anti–IL-13 (clone eBio13A; eBioscience). ILC2s were characterized initially by the addition of Brilliant Violet 605–conjugated anti-KLRG1 (MAFA; clone: 2F1/killer cell lectin-like receptor subfamily group 1 [KLRG1]), Brilliant Violet 510–conjugated anti–inducible costimulator (ICOS; CD278; clone: C398.4), PE-conjugated anti–thymic stromal lymphopoietin receptor (TSLPR; #FAB5461P; Systems), and APC-conjugated anti–IL-17RB (IL-25 receptor; #FAB10402A; R&D Systems), followed by Pacific blue–labeled lineage, FITC-conjugated anti-CD45.2, and PerCP-Cy5.5 conjugated anti-CD25