IL-33 is more potent than IL-25 in provoking IL-13–producing nuocytes (type 2 innate lymphoid cells) and airway contraction

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Background: IL-25 and IL-33 belong to distinct cytokine families, but experimental mouse studies suggest their immunologic functions in type 2 immunity are almost entirely overlapping. However, only polymorphisms in the IL-33 pathway (*IL1RL1* and *IL33*) have been significantly associated with asthma in large-cohort genome-wide association studies.
Objective: We sought to identify distinct pathways for IL-25 and IL-33 in the lung that might provide insight into their roles in asthma pathogenesis and potential for therapeutic intervention.
Methods: IL-25 receptor–deficient ($Il17rb^{-/-}$), IL-33 receptor–deficient (ST2, $Il1rl1^{-/-}$), and double-deficient ($Il17rb^{-/-}Il1rl1^{-/-}$) mice were analyzed in models of allergic asthma. Microarrays, an *ex vivo* lung slice airway contraction model, and $Il13^{+/eGFP}$ mice were then used to identify specific effects of IL-25 and IL-33 administration.
Results: Comparison of IL-25 and IL-33 pathway–deficient mice demonstrates that IL-33 signaling plays a more important *in vivo* role in airways hyperreactivity than IL-25. Furthermore, methacholine-induced airway contraction *ex vivo* increases after treatment with IL-33 but not IL-25. This is dependent on expression of the IL-33 receptor and type 2 cytokines. Confocal studies with $Il13^{+/eGFP}$ mice show that IL-33 more potently induces expansion of IL-13–producing type 2 innate lymphoid cells, correlating with airway contraction. This predominance of IL-33 activity is enforced *in vivo* because IL-33 is more rapidly expressed and released in comparison with IL-25.
Conclusion: Our data demonstrate that IL-33 plays a critical role in the rapid induction of airway contraction by stimulating the prompt expansion of IL-13–producing type 2 innate lymphoid cells, whereas IL-25–induced responses are slower and less potent. (J Allergy Clin Immunol 2013;132:933-41.)

Key words: *Nuocytes, type 2 innate lymphoid cells, IL-13, IL-25, IL-33, asthma, contraction*

Recent genome-wide association studies incorporating 10,365 asthmatic patients and 16,110 unaffected control subjects in several different countries identified only 8 asthma-associated alleles.[1] These included *IL1RL1* (IL-33 receptor), *IL33*, and *IL13*, highlighting the critical importance of type 2 response–associated genes in patients with allergic asthma. Although the action of IL-13 has been well characterized in patients with allergic asthma,[2-4] relatively little is known about the mechanisms through which IL-33 acts *in vivo* on specific aspects of the asthmatic response, such as bronchoconstriction.

IL-33 is a potent type 2–inducing cytokine invoking IL-4, IL-5, and IL-13 production and downstream effects, including eosinophilia and mucus secretion.[5] IL-33/ST2 signaling has been implicated in the pathogenesis of airway inflammation,[6-10] fibroproliferative disease,[11] and rheumatoid arthritis.[12,13]

IL-25, a member of the IL-17 family, has also been shown to induce type 2 pathology.[14-18] Blocking IL-25 reduced airways hyperreactivity (AHR) and airways inflammation in an ovalbumin (OVA) allergic airways model. Notably, both IL-25 and IL-33 stimulate lineage-negative, inducible costimulator (ICOS)–positive type 2 innate lymphoid cells (ILC2s), which produce IL-5 and IL-13 and are critical for the initiation of type 2 gut responses.[19] ILC2s have also been shown to be sufficient for the induction of lung allergy, even in the absence of IL-13–producing T cells.[20-22] IL-25 has also been reported to induce an IL-17RB^{+} steroid-resistant myeloid cell population that exacerbates allergy.[23]

Although the immunologic functions of IL-25 and IL-33 appear to almost entirely overlap, genetic analysis has indicated a more pronounced role for IL-33 in asthmatic patients.[1] To address this paradox, we analyzed AHR in mice deficient in the signaling pathways of IL-33, IL-25, or both in 2 separate models of allergic asthma. In agreement with human genetic data, the IL-33 pathway was more important for AHR and in an *ex vivo* contraction model. This contraction correlated with a more rapid

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Abbreviations used
AHR: Airways hyperreactivity
BAL: Bronchoalveolar lavage
Cit: Citrine
ICOS: Inducible costimulator
ILC2: Type 2 innate lymphoid cells
OVA: Ovalbumin
RWP: Ragweed pollen
Tom: Tomato

accumulation of IL-13–producing ILC2s in the submucosal area around the airway. IL-33 expression also preceded the IL-25 response during allergic asthma. Thus IL-33 is a more potent inducer than IL-25 of type 2 cytokine–induced lung AHR.

METHODS

Mice

Mice (6-12 weeks old) were housed in pathogen-free, environmentally controlled conditions. BALB/c mice were obtained from Charles River (Burlington, Mass). *Il13*$^{+/eGFP}$,[19] *Il13*$^{+/Tomato\ (Tom)}$,[21] *Il17rb*$^{-/-}$,[19] *Il1rl1*$^{-/-}$,[24] *Il17rb*$^{-/-}$*Il1rl1*$^{-/-}$, *Il4*$^{-/-}$*Il5*$^{-/-}$*Il9*$^{-/-}$*Il13*$^{-/-}$,[25] and *Rag2*$^{-/-}$[26] mice were generated on or backcrossed to the BALB/c background. *Il13*$^{+/Tom}$ mice were crossed with *Il33*$^{+/Citrine\ (Cit)}$ mice[27] to create *Il33*$^{+/Cit}$*Il13*$^{+/Tom}$ compound reporter mice. All experiments were undertaken with approval of the United Kingdom's Home Office.

IL-25 and IL-33 treatment and measurement of allergic lung inflammation

Two micrograms of recombinant mouse IL-25 (low endotoxin reagent at 0.032 EU/mg; Janssen, Radnor, Pa) or 0.5 μg of recombinant mouse IL-33 (supplied at 0.1 EU/mg; BioLegend, San Diego, Calif) was administered in PBS intranasally. Acute OVA-induced allergic lung responses were induced, as previously described.[28] Chronic lung inflammation required challenge with 1% OVA for a further 2 consecutive weeks. Alternatively, mice were challenged with 40 μg of ragweed pollen (RWP; Greer Laboratories, Lenoir, NC) in 40 μL of PBS administered intranasally for 5 consecutive days. This was repeated for 2 further consecutive weeks. Control mice received PBS in both models. Airways resistance, contraction, and tissue analysis were undertaken 24 hours after the final challenge. Airways resistance was assessed by using a restrained whole-body plethysmograph (EMMS, Hants, United Kingdom), as described previously.[28] All baseline readings were found to be similar between experimental groups, and thus average pulmonary resistance after drug treatment was then divided by the baseline average for each mouse to determine fold induction. Lung sample preparation and flow cytometry were performed, as previously described.[21]

Preparation of lung slices

Live mouse lung slices were generated, as previously described.[30] Mice were killed, and tracheas were cannulated (20-gauge Intima; Becton Dickinson, Franklin Lakes, NJ). Lungs were inflated to 95% capacity with 2% agarose, after which a pulse of air was injected to clear the airways of agarose. The lungs were cooled, excised, and placed in ice-cold HBSS. Lung slices (130 μm) were cut with a tissue slicer (Vibratome, model EMS-4000; Electron Microscope Sciences, Hatfield, Pa) and transferred to Dulbecco modified Eagle medium containing 2 mmol/L L-glutamine, penicillin-streptomycin, and indicated cytokines and incubated at 37°C/5% CO_2 for 24 hours.

Measurement of airway contraction

Lung slices were checked for beating cilia and a lumen clear of agarose and then sandwiched between a microscope slide and cover slip, with a nylon mesh holding the slices in place. Solutions were delivered by means of gravity-fed perfusion (Valvebank; Digitimer, Bonn, Germany) or through a peristaltic pump (PPS; Scientifica, Dresden, Germany). After establishing a relaxed baseline in PBS for 5 minutes, 10 μmol/L methacholine was continuously perfused over the lung slice for 5 minutes to induce contraction. Phase-contrast images were recorded every 2 seconds with a Nikon DIAPHOT 300 microscope with RT3 Slider camera and SPOT imaging software or a spinning disk confocal microscope (Zeiss Axio Observer D1, Hamatsu electron multiplier CCD camera C19100-13, with a Yokogawa Spinning disk system; Oberkochen, Germany) with compatible Volocity imaging software. Volocity analysis software (PerkinElmer, Waltham, Mass) was used to calculate airway area. Airway narrowing was expressed as the percentage decrease in airway area compared with the area of the fully relaxed airway in PBS.

Quantitative PCR analysis

RNA extraction (Nucleospin RNA XS kit; Macherey-Nagel, Düren, Germany) and cDNA synthesis (Superscript First Strand Synthesis kit; Invitrogen, Carlsbad, Calif) from snap-frozen lung tissue were carried out according to the manufacturers' instructions. All primers used are shown in Table E1 in this article's Online Repository at www.jacionline.org. Each sample, including RT and no template control, were run in triplicate by using 900 nmol/L forward and reverse primers and 250 nmol/L probe. Amplification was detected with the Mx3005P real-time thermal cycler (Stratagene, La Jolla, Calif) or the 7900HT PCR system (Applied Biosystems, Foster City, Calif), and values were calculated by using the Δ cycle threshold method.

Flow cytometry and ELISA

An assessment of cell-surface marker expression was made by using mAbs against the following molecules: CD45 (clone 30-F11) conjugated to PerCP-Cy5.5, EpCAM (clone G8.8) conjugated to allophycocyanin, CD3 (clone 145-2C11), and CD4 (clone GK1.5) conjugated to PE-Cy7. ILC2s were identified as lineage^{-}ICOS^{+}ST2^{var}, as previously described.[21] Isotype and single-stained controls were included. IL-25 measurement used the Meso-Scale Discovery platform with an in-house capture anti–IL-25 mAb[4] and a polyclonal anti–IL-25 detection antibody (biotinylated, BAF1399; R&D Systems, Minneapolis, Minn). IL-4, IL-5, and IL-13 levels were all measured with a MAGPIX multiplexing kit (Luminex, Merck Millipore, Temecula, Calif).

Microarray and analysis

Total RNA was extracted and assessed by using the RNeasy Kit (Qiagen, Hilden, Germany) and an Agilent Bioanalyser (Agilent, Victoria, Australia), according to the manufacturer's instructions. RNA was reverse transcribed and cDNA amplified, fragmented, and labeled by using the Ovation RNA Amplification and cDNA Biotin System (NuGEN, San Carlos, Calif). Labeled cDNA was hybridized to the Affymetrix GeneChip HT MG-430 PM 24-Array plates (Affymetrix, Santa Clara, Calif). After washing and staining, cell files were produced from scanned images (High Throughput Array Plate Scanner, Affymetrix) by using Affymetrix Control Console software. ArrayStudio Version 4.0 (OmicSoft, Cary, NC) was used for gene expression analysis. Data were normalized by using the robust multichip average method and $\log_2$ transformed and assessed based on probe set intensity, principle component analysis, hierarchic cluster analysis, and sample correlation. Genes of interest were those with a fold change of at least 2, a false discovery rate–adjusted P value of less than .05, and an estimated least squares mean intensity of greater than or equal to 5 for at least 1 group in the comparison.

Frozen lung section preparation and confocal microscopy

Lung tissue was fixed in 4% paraformaldehyde, embedded in 15% sucrose/7.5% gelatin, and stored at −80°C before sectioning. Sections (12 μm) were cut at −20°C with a Cryostat (CM3050 S; Leica, Solms, Germany). Gelatin was removed from the sections by incubating slides in PBS-buffered 0.1% BSA/0.05% Triton for 5 minutes at 37°C. One percent BSA/0.05% Triton was