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Targeting ST2L Potentiates CpG-Mediated Therapeutic Effects in a Chronic Fungal Asthma Model

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IL-33 and its soluble receptor and cell-associated receptor (ST2L) are all increased in clinical and experimental asthma. The present study addressed the hypothesis that ST2L impairs the therapeutic effects of CpG in a fungal model of asthma. C57BL/6 mice were sensitized to *Aspergillus fumigatus* and challenged via i.t. instillation with live *A. fumigatus* conidia. Mice were treated with IgG alone, anti-ST2L monoclonal antibody (mAb) alone, CpG alone, IgG plus CpG, or anti-ST2L mAb plus CpG every other day from day 14 to day 28 and investigated on day 28 after conidia. Lung ST2L and toll-like receptor 9 protein expression levels concomitantly increased in a time-dependent manner during fungal asthma. Therapeutic blockade of ST2L with an mAb attenuated key pathological features of this model. At subtherapeutic doses, neither anti-ST2L mAb nor CpG alone affected fungal asthma severity. However, airway hyperresponsiveness, mucus cell metaplasia, peribronchial fibrosis, and fungus retention were markedly reduced in asthmatic mice treated with the combination of both. Whole lung CXCL9 levels were significantly elevated in the combination group but not in the controls. Furthermore, in asthmatic mice treated with the combination therapy, dendritic cells generated significantly greater IL-12p70 with CpG *in vitro* compared with control dendritic cells. The combination of anti-ST2L mAb with CpG significantly attenuated experimental asthma, suggesting that targeting ST2L might enhance the therapeutic

efficacy of CpG during allergic inflammation. (*Am J Pathol* 2011, 179:104–115; DOI: 10.1016/j.ajpath.2011.03.032)

Allergic asthma is characterized by a type 2 helper T-cell (Th2)–dominated inflammatory response that drives long-term physiological and structural remodeling events in the lung.¹ Recent strategies directed at the treatment of allergic asthma have focused on limiting the Th2 inflammatory process by targeting the cellular participants in the initial recognition and maintenance phase of this immune response.¹ Dendritic cells (DCs) are key players in both phases of a Th2 immune response, in part because of their ability to recognize pathogen-associated molecular patterns present in allergens through pathogen recognition receptors, such as the toll-like receptors (TLRs). Subsequent innate and adaptive immune responses are promoted through TLR-activated DCs, leading to recruitment of allergic effector cells (ie, eosinophils, basophils, and T cells) to the asthmatic lung.²

Not all TLR activation events in DCs precipitate allergic inflammation in the lung.³ For example, TLR9 activation by hypomethylated CpG oligodeoxynucleotides or CpG shifts immune responses away from Th2-type toward Th1-type immune responses⁴; this shift at least partially contributes to the inhibitory effect of CpG in experimental allergic asthma models.^{5–8} Unlike the findings in experimental models, promoting TLR9 activation with CpG has had limited therapeutic success in clinical asthma.⁹ An explanation for the disparity between CpG effects in experimental and clinical disease presumably involves multiple factors, such as species differences in the cellular distribution of TLR9¹⁰ and differential effects of mouse-versus human-specific CpG. However, we hypothesized

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that the negative regulators of TLR signaling might be other endogenous factors that limit the immunotherapeutic effect of CpG in humans.^{11,12} Among the many negative regulators of TLR signaling that have been described,¹³ the transmembrane receptor for IL-33 (ST2L) attracted our attention because of its strongly induced expression on several cells of hematopoietic origin during Th2-type conditions.^{14–16} When activated, ST2L sequesters MyD88 adaptor protein, thereby preventing TLR-mediated signaling, including that initiated by CpG.¹⁷

With this background, we hypothesized that the enhanced expression of ST2L during Th2-driven allergic inflammatory responses impeded the TLR9-dependent therapeutic effects of CpG in a model of fungal asthma, which was previously determined to be responsive to local (ie, lung), but not systemic, CpG therapy.⁸ Although the therapeutic effects of targeting ST2L,^{18–22} increasing soluble ST2 (sST2) levels,^{23,24} or targeting IL-33²⁵ activity in experimental asthma have been previously examined, it is not known whether the therapeutic effect of targeting IL-33/ST2L is related to enhanced TLR9 activation in this setting. In the present study, the combination of systemic CpG and an anti-ST2L monoclonal antibody (mAb) markedly reduced the severity of all features in this chronic fungal asthma model, whereas the administration of either treatment alone had no therapeutic effect at the selected doses. The combination therapy was associated with enhanced Th1-type mediator generation by DCs. Thus, targeting ST2L activity appears to enhance the therapeutic effects of CpG during experimental fungal asthma.

Materials and Methods

Asthmatic Patient Plasma Samples

Plasma samples from patients with asthma and controls were analyzed. Inclusion and exclusion criteria and the criteria for the severity of asthma were based on established guidelines that have been previously described.²⁶ Patients with asthma were stratified according to medication requirements for asthma control. Mild asthma was defined as the use of ≤ 200 $\mu\text{g/day}$ of fluticasone, or the equivalent, to achieve symptom control. Moderate asthma was defined as the use of 200 to 800 $\mu\text{g/day}$ of fluticasone, or the equivalent, to achieve control. Patients with severe asthma fulfilled the definition of severe asthma adopted by the American Thoracic Society Workshop on Refractory Asthma.²⁷ A human investigation committee at Yale University, New Haven, CT, and the University of Michigan, Ann Arbor, approved these studies. All subjects gave written informed consent before blood draw.

Individual and Combination CpG and Anti-ST2L mAb Therapies during Fungal Asthma

Female C57BL/6 mice (aged 6 to 8 weeks) were purchased from Taconic Farms (Germantown, NY) and sensitized with *Aspergillus fumigatus* antigens.²⁸ After sensitization, mice were challenged via i.t. instillation with live

A. fumigatus conidia.²⁸ Prior approval for mouse use was obtained from the University Committee on Use and Care of Animals at the University of Michigan.

Beginning on day 14 after conidia challenge, mice received one of the following treatments: i) CpG (5 μg per dose; HyCult Biotechnology, Uden, the Netherlands), ii) control IgG alone (10 or 100 μg per dose; anti-keyhole limpet hemocyanin; Centocor, Inc., Radnor, PA), iii) anti-ST2L mAb alone (10 or 100 μg per dose; CNTO3914; Centocor, Inc.), iv) CpG (5 μg per dose) plus IgG (10 μg per dose), or v) CpG (5 μg per dose) plus anti-ST2L mAb (10 μg per dose). The anti-ST2L mAb used is a chimeric antibody containing a mouse IgG1 Fc, and the IgG isotype control is a mouse anti-keyhole limpet hemocyanin; IgG1; details regarding the generation and characterization of the parent rat IgG antibody CNTO3914 from which CNTO3914 was derived are published elsewhere.²⁹ Individual and combination therapies were dissolved in a total volume of 200 μL of distilled water for i.p. instillation every other day until day 28 after conidia. The CpG-oligodeoxynucleotide used in the present study was the following sequence: 5'-TCCATGACGTTCTGATGCT-3'; it contained a motif that mimics the immunostimulatory effects of bacterial DNA. Mouse airway physiological, inflammatory, and remodeling parameters were analyzed as previously described.³⁰

Bone Marrow–Derived DC Culture, Isolation, and Activation

Bone marrow–derived DCs were prepared from control or allergic mice at various times before and after conidia challenge. Bone marrow cells were cultured for 6 days with granulocyte-macrophage colony-stimulating factor (20 ng/mL; R&D Systems, Rochester, MN), and DCs were sorted for CD11c⁺ expression using magnetic-activated cell sorting (Miltenyi Biotec, Bergisch Gladbach, Germany). Sorted DCs were cultured with medium, CpG (2 nmol/L), Pam3Cys (2.5 $\mu\text{g/mL}$), or poly(I-C) (50 $\mu\text{g/mL}$) for 6 or 24 hours before RNA or protein determination, respectively.

Whole Lung Histological Analysis

Whole lungs from *A. fumigatus*–sensitized C57BL/6 mice before and at various times after *A. fumigatus* conidia challenge were fully inflated with 10% formalin, dissected, and placed in fresh 10% formalin for 24 hours. Routine histological techniques were used to paraffin embed the entire lung, and 5- μm sections of whole lung were analyzed by immunohistochemical (IHC) techniques, as described later, or stained with H&E or Gomori methenamine silver to detect *Aspergillus* conidia or Masson trichrome to detect collagen.

ST2L and TLR9 IHC

Paraffin-embedded whole lung samples were analyzed using routine IHC techniques for the presence of either

Figure 1. Cell-associated ST2L and sST2 expression in experimental and clinical asthma. Whole lung tissue sections from *Aspergillus*-sensitized mice on days 0 (A and E), 7 (B and F), 14 (C and G), and 28 (D and H) after i.t. conidia challenge were stained using routine IHC techniques. Panels are representative of whole lung sections from mice stained with IgG control (A–D) or anti-ST2L antibody (E–H). Receptor expression stained brown with this IHC procedure. Original magnification, $\times 200$ (all photomicrographs). Serum sST2 (I) and whole lung ST2L (J) from *Aspergillus*-sensitized mice on days 0 to 28 after conidia challenge. sST2 levels in human plasma samples from control, chronic obstructive pulmonary disease (COPD), and mild, moderate, and severe asthmatic groups (K). ELISA analysis was used to determine ST2L and sST2 levels in mouse and human samples. Data are expressed as the mean $\pm$ SEM ($n = 5$ per group per time point for the chronic fungal asthma model). The number of patient samples analyzed and the number of samples less than the LOD ($\#$ below LOD) are indicated.

ST2L or TLR9. Rabbit anti-mouse IL-33, ST2L, and TLR9 polyclonal antibodies were obtained from Alexis Biochemicals (San Diego, CA), Abcam (Cambridge, MA), and Imgenex (San Diego), respectively. Briefly, 5- μ m histological sections were dewaxed with xylene, rehydrated in graded concentrations of ethanol, and stained with a mouse horseradish peroxidase–diaminobenzidine cell and tissue staining kit, according to the manufacturer's instructions (R&D Systems). Other histological samples were immunostained with control antibodies (IgG isotype controls and horseradish peroxidase substrate).

TaqMan Analysis

Total RNA was isolated from DCs or homogenized mouse lungs using TRIzol reagent (Invitrogen/Life Technologies, Carlsbad, CA). Purified RNA was treated with DNase, and 0.2 μ g of RNA was reverse transcribed into cDNA using TaqMan reverse transcription reagents (Foster City, CA). TaqMan gene expression assays were used to quantify ST2L, IL-33, IL-4, IL-12p40, Muc5ac, Gob5, and TLR9, per manufacturer's instructions (Applied Biosystems, Carlsbad, CA). *Aspergillus* conidia content in RNA from paraffin-embedded whole lung tissue sections was analyzed, as previously described in detail.³¹ Mouse glyceraldehyde-3-phosphate dehydrogenase was used as the internal control for all mouse genes, whereas *Aspergillus* 18S was used as the internal control for *Aspergillus* conidia content. Gene expression was normalized to either glyceraldehyde-3-phosphate dehydrogenase or *Aspergillus* 18S before the fold change in gene expression was calculated. The fold changes in transcript expression were calculated by comparing the gene expression in whole lung samples from mice with chronic fungal asthma with that in whole lung samples from naïve mice, which were assigned a value of 1.

ELISA Analysis of Whole Lung Tissue, Plasma, and DC Supernatants

Murine ST2, IL-33, IL-4, IL-12p70, and monokine-induced by γ -interferon (MIG)/CXCL9 were determined in 50- μ L samples from serum, whole lung homogenates, and/or lysed DCs using a standardized sandwich enzyme-linked immunosorbent assay (ELISA) technique or a bead-based multiple target sandwich ELISA system (BioPlex; BioRad Laboratories, Hercules, CA).³⁰ Recombinant murine or human proteins (R&D Systems) were used to generate standard curves. The limit of ELISA detection for each cytokine was consistently >50 pg/mL for sandwich ELISA and 1 pg/mL for BioPlex. The cytokine, chemokine, and ST2 levels in each sample were normalized to total protein levels, measured using the Bradford assay.

Statistical Analysis

All results are expressed as the mean $\pm$ SEM. A Student's *t*-test or an analysis of variance and a Student-Newman-Keuls multiple comparison test were used to determine statistical significance between groups. $P < 0.05$ was considered statistically significant.

Results

Cell-Associated ST2L and sST2 Expression in Experimental and Clinical Asthma

ST2-positive Th2 cells have been identified in experimental asthma models,^{18,21} but the effect of allergic inflammation on the expression of ST2L by other lung cell types has not been previously examined. By using both IHC staining of whole lung tissue sections and ELISA analysis of whole lung homogenates, ST2L expression was determined immediately before (ie, day 0) and on days 7, 14, and 28 after i.t. administration of conidia into mice (Figure 1, A–H and J). Both tech-

Figure 2. IL-33 expression in experimental and clinical asthma. Whole lung tissue sections from *Aspergillus*-sensitized mice immediately before (A and E) and on days 7 (B and F), 14 (C and G), and 28 (D and H) after conidia challenge were stained using routine IHC techniques. Panels are representative of whole lung sections from *A. fumigatus*-sensitized mice before and after conidia challenge stained with IgG control (A–D) or anti-IL-33 antibody (E–H). Ligand expression stained brown with this IHC procedure. Original magnification, $\times 200$ (all photomicrographs). ELISA analysis of whole lung (I) IL-33 levels from *Aspergillus*-sensitized mice from day 0 to day 28 after conidia challenge. ELISA analysis of IL-33 levels in human plasma samples from the control, chronic obstructive pulmonary disease (COPD), and mild, moderate, and severe asthmatic groups (J). Data are expressed as the mean $\pm$ SEM ($n = 5$ per group per time point for the chronic fungal asthma model). * $P \leq 0.05$, ** $P \leq 0.01$ versus control plasma IL-33 levels. The number of patient samples analyzed and the number of samples less than the lower LLOD (# below LLOD) are indicated.

niques revealed that the greatest expression of ST2L was present in whole lung samples on day 14 after conidia (Figure 1G). Although absent at earlier times in the model, strong ST2L staining was present in bronchial and alveolar epithelial cells on days 14 and 28 after conidia (Figure 1, A–H). Serum levels of sST2 were similarly increased on days 7, 14, and 28 after conidia compared with serum levels in mice immediately before the conidia challenge in this fungal asthma model (Figure 1I). ST2L levels in whole lung samples are shown in Figure 1J; it was apparent that levels of this receptor were markedly increased after the conidia challenge at all times examined. Thus, ST2L is significantly elevated in a temporal dependent manner in this fungal asthma model and its expression appeared to be markedly induced on epithelial cells in whole lung histological sections.

Previous studies by Oshikawa and colleagues³² revealed that circulating human sST2 protein levels were elevated in asthmatic patients after an acute exacerbation of their disease. In the present study, plasma samples from nonasthmatic and asthmatic patients were analyzed for the presence of sST2; and these data are summarized in Figure 1K. Of the control plasma samples, 60% contained sST2 levels that were less than the lower limit of detection (LLOD), whereas 30% of the samples from the chronic obstructive pulmonary disease group were less than the LLOD (Figure 1K). Higher levels of sST2 were present in the plasma samples from the asthma group compared with the control and chronic obstructive pulmonary disease groups, but between 42% and 54% of the asthma plasma samples contained sST2 levels that were less than the LLOD (Figure 1K). Among the asthma groups, plasma sST2 was greatest in the patient group with moderate asthma. Together, these data suggested that sST2 was present in 40% to 70% of the plasma samples analyzed and that greater levels of sST2 were

detected in asthmatic samples compared with the control and chronic obstructive pulmonary disease samples.

IL-33 Expression in Experimental and Clinical Asthma

IL-33 is produced by various cells and potently drives allergic inflammation through its effects on several hematopoietic cells.³³ IHC studies of whole lung samples from the chronic fungal asthma model revealed that IL-33 immunoreactivity was present in smooth muscle and epithelial cells at all times after conidia challenge (Figure 2, A–H). However, unlike ST2L expression, IL-33 levels appeared to diminish with time after the conidia challenge (Figure 2, A–H). The greatest whole lung staining for IL-33 was present on day 7 after conidia, and this finding was consistent with the ELISA analysis of whole lung homogenates from asthmatic mice, which showed the greatest amount of IL-33 at this time after conidia (Figure 2I). Surprisingly, IL-33 levels in serum from asthmatic mice were less than the LLOD of the mouse-specific ELISA used (data not shown). Together, these data indicate that IL-33 is induced in this model of experimental asthma but that its temporal expression profile differs from that of ST2L.

Previous studies have shown that IL-33 levels are increased in biopsy specimens from patients with asthma, most notably in bronchial smooth muscle cells³⁴ and epithelial cells.³⁵ Analysis of plasma levels of IL-33 was undertaken in the present study, and this analysis revealed that circulating levels of this cytokine in most samples were less than the LLOD of the human-specific ELISA used (Figure 2J). Specifically, 70% of the control, 90% of the chronic obstructive pulmonary disease, 100% of the mild asthmatic, 60% of the

Figure 3. DCs from asthmatic mice expressed greater ST2L and IL-33 compared with naïve DCs. TaqMan analysis of ST2L (A) and IL-33 (C) transcript expression in DCs isolated before and on days 7, 14, and 28 after conidia introduction into *Aspergillus*-sensitized mice compared with DCs isolated from naïve mice. ELISA analysis of sST2 present in cell-free supernatants from asthmatic DCs derived at various times after the induction of fungal asthma (B). Data are expressed as the mean $\pm$ SEM ($n = 5$ per group per time point). LPS, lipopolysaccharide.

moderate asthmatic, and 82% of the severe asthmatic samples contained IL-33 at levels that were less than the LLOD (Figure 2J). However, statistical analysis of patient plasma IL-33 levels revealed that the levels of this cytokine in patients with moderate asthma were significantly greater than IL-33 levels in all other patient groups (Figure 2J). Thus, circulating levels of IL-33 are present in a few patients with asthma; and detectable circulating levels of IL-33 appear to be absent in patients with mild asthma.

DCs from Asthmatic Mice Express Greater ST2L Transcript and Protein than Naïve DCs

IL-33 promotes granulocyte-monocyte colony-stimulating factor production by DCs,³⁶ and IL-33-activated

DCs induce an atypical Th2-type response characterized by increased IL-5 and IL-13 but not IL-4.³⁷ Because DCs exert a prominent effect on the development and maintenance of allergic inflammation, we examined the expression of ST2L and IL-33 in naïve and asthmatic DCs at various times after the conidia challenge. ST2L transcript levels in bone marrow-derived DCs from asthmatic mice were induced above levels in naïve DCs, most notably on days 14 and 28 after the conidia challenge (Figure 3A). At these later points after conidia challenge, CpG, poly(I:C), and lipopolysaccharide (but not Pam3Cys) enhanced ST2L transcript expression (Figure 3A). sST2 protein levels were detected in cultures of DCs from asthmatic mice on day 28 after conidia. Most notably, poly(I:C), but not other TLR agonists, promoted the release of sST2 pro-

Figure 4. Therapeutically targeting ST2L alone significantly attenuated airway inflammation, methacholine (Mch)-induced hyperreactivity, and remodeling in fungal asthma. Whole lung tissue sections from *Aspergillus*-sensitized mice treated with 100 μ g of IgG (A–D) or anti-ST2L mAb (E–H) on alternate days from day 14 to day 28 after conidia challenge. Tissues were stained with H&E (A and E), trichrome (B and F), PAS (C and G), and Gomori methenamine silver (D and H). Airway resistance analysis (I), ELISA analysis of serum IgE (J), and BAL eosinophil counts (K) of these groups are also shown. TaqMan analysis was used to determine lung Muc5ac (L), Gob5 (M), and *Aspergillus* conidia content in lung sections (N). Peak increases in airway resistance or hyperresponsiveness were determined at each time point after the i.v. injection of Mch. Data are expressed as the mean $\pm$ SEM ($n = 5$ per group per time point). * $P \leq 0.05$ (airway resistance versus the appropriate baseline measurement or the indicated treatment group after Mch challenge). Original magnification, $\times 200$ (all photomicrographs).

tein from asthmatic DCs (Figure 3B). DCs from asthmatic mice exhibited marked increases in IL-33 transcript levels compared with naïve DCs, with the greatest increases in these levels on either day 14 or day 28 after conidia (Figure 3C). All of the TLR agonists tested further induced IL-33 transcript expression by asthmatic DCs compared with naïve DCs (Figure 3C). However, unlike sST2, IL-33 protein was not detected in the cell-free supernatants of DCs under these *in vitro* conditions (data not shown). Thus, ST2 and IL-33 levels increased in a time-dependent manner in this model; and TLR activation promoted ST2 transcript and protein expression in asthmatic DCs.

Therapeutically Targeting ST2L Alone Significantly Attenuates Airway Inflammation, Remodeling, and Hyperreactivity in Fungal Asthma

Given that selective antibody-directed targeting of ST2 has been an effective therapeutic approach in other experimental asthma models,^{18,21} we first examined whether targeting this receptor alone was efficacious in experimental fungal asthma. An mAb directed against mouse ST2 or an appropriate control IgG (both at a 100- μ g dose) was administered to asthmatic mice via i.p. instillation every other day from day 14 to day 28 after conidia challenge. Histological analysis of whole lung sections from both groups of asthmatic mice revealed that the anti-ST2L antibody treatment markedly reduced lung inflammation (Figure 4E), peribronchial fibrosis (Figure 4F), goblet cell metaplasia (Figure 4G), and fungal material retention (Figure 4H) compared with the IgG-treated control group (Figure 4, A–D). Furthermore, the anti-ST2L antibody therapy significantly reduced the methacholine-induced airway resistance in asthmatic mice compared with the IgG group on day 28 after conidia (Figure 4I). Statistically significantly decreased serum IgE levels (Figure 4J), BAL eosinophil counts (Figure 4K), and mucus-associated genes [ie, Muc5ac (Figure 4L) and Gob5 (Figure 4M)] were also observed in the anti-ST2L antibody-treated group compared with the IgG-treated group. The anti-ST2L antibody therapy significantly decreased fungal counts (as assessed by quantitative PCR) in the lungs of asthmatic mice (Figure 4N).

As shown by TaqMan analysis, whole lung IL-12p40 (Figure 5A) and the Th1-type chemokine MIG/CXCL9 (Figure 5C) transcript levels were elevated approximately sixfold and 10-fold, respectively, higher than levels in naïve lung in the anti-ST2L antibody treatment group; however, transcript levels for these cytokines were not increased in the IgG-treated group. Whole lung IL-4 transcript levels were present in the IgG-treated group but not in the anti-ST2L antibody-treated group (Figure 5E). Whole lung transcript levels for IL-33 were similar in both groups of asthmatic mice compared with transcript levels in whole lung samples from naïve mice (Figure 5F).

Figure 5. Anti-ST2L mAb therapy modulated whole lung cytokine levels in mice with fungal asthma. TaqMan analysis of IL-12p40 (A), MIG/CXCL9 (C), IL-4 (E), and IL-33 (G) in whole lung. IL-12p70 (B), MIG/CXCL9 (D), IL-4 (F), and IL-33 (H) were measured in whole lung samples using a standard ELISA protocol or BioPlex. Samples were obtained from *Aspergillus*-sensitized mice treated with 100- μ g IgG or 100- μ g anti-ST2L mAb on alternate days from day 14 to day 28 after *Aspergillus* conidia challenge. Data are expressed as the mean $\pm$ SEM ($n = 5$ per group per time point). * $P \leq 0.05$.

By using ELISA analysis, MIG/CXCL9 protein levels were significantly increased approximately fourfold (Figure 5D), whereas IL-4 protein levels were significantly reduced approximately eightfold (Figure 5F) and IL-33 protein levels were significantly reduced greater than twofold (Figure 5H) in the anti-ST2L mAb-treated group compared with the IgG-treated mice. Whole lung protein levels of IL-12 (Figure 5B) and IL-33 (Figure 5G) did not differ between the treatment groups. Thus, therapeutic blockade of ST2L using an mAb approach reversed many of the key inflammatory pathological features of experimental fungal asthma.

TLR9 Expression and CpG Responsiveness Do Not Correlate during Fungal Asthma

TLR9 expression is required for the containment of *Aspergillus* conidia in *A. fumigatus*-sensitized mice³⁰; TLR9 activation using intranasal, but not systemic, CpG therapy (composed of 50 μ g per dose administered on alternate days from day 14 to day 28 after conidia challenge) attenuated all features of chronic fungal asthma.⁸ These

Figure 6. Whole lung and DC TLR9 protein expression during the experimental fungal asthma and naive and asthmatic DC generation of IL-12p70. Whole lung tissue sections from *Aspergillus*-sensitized and challenged mice immediately before (A and E), and on days 7 (B and F), 14 (C and G), and 28 (D and H) after conidia challenge were stained using routine IHC techniques. Panels are representative of whole lung sections from mice stained with IgG control (A–D) or anti-TLR9 antibody (E–H). Receptor expression stains red with this IHC procedure. Original magnification, $\times 200$ (all photomicrographs). TLR9 transcript expression (I) and IL-12 generation (J) by DCs derived from bone marrow removed before and on days 7, 14, and 28 after conidia challenge. DCs were also derived from naive mice. In all experiments, DCs were cultured either in medium alone or with CpG (2 nmol/L) for 6 (RNA) or 24 (protein) hours before analysis. TaqMan was used to analyze TLR9 transcript expression, and ELISA was used to detect IL-12p70 protein in cell-free supernatants. Data are expressed as the mean $\pm$ SEM ($n = 5$ per group). * $P \leq 0.05$, ** $P \leq 0.001$ versus the appropriate control group.

previous observations raised the following question: What prevents the therapeutic effects of systemic CpG therapy in fungal asthma? We hypothesized that the negative regulatory effect of ST2L on TLR9 activation might be one explanation and first examined TLR9 protein expression in whole lung sections from asthmatic mice at various times after the conidia challenge (Figure 6). TLR9 was strongly expressed on days 14 and 28 after conidia challenge compared with the earlier points in this asthma model (ie, days 0 and 7) (Figure 6, A–H). Alveolar and bronchial epithelial cells were strongly stained for TLR9, an expression pattern that was similar to that observed for ST2L (Figure 1). Thus, TLR9 protein was markedly elevated in whole lung sections on days 14 and 28 after conidia but not at earlier times in this chronic fungal asthma model.

The expression of TLR9 in cultured DCs from naive and asthmatic mice was investigated next because DCs mediate the therapeutic effects of CpG in allergic asthma models.^{38,39} As shown in Figure 6I, TLR9 transcript levels in untreated DCs derived from asthmatic mice before and on days 7, 14, and 28 after conidia were unchanged from TLR9 levels in DCs derived from naive mice. The addition of CpG to cultures of DCs derived on day 7 after conidia challenge increased TLR9 transcript levels approximately 2.5-fold higher than TLR9 levels in naive DCs. However, TLR9 transcript levels in asthmatic DCs, derived on either day 14 or day 28 after conidia, were significantly lower compared with those DCs derived on day 7 after conidia (Figure 6I). The effect of CpG on IL-12p70 generation by cultured DCs is shown in Figure 6J. The greatest constitutive IL-12 generation was observed in cultures of naive DCs, and IL-12 levels were less than the limits of detection in cultures of DCs derived from asthmatic mice on day 14 after conidia challenge (Figure 6J). CpG strongly induced IL-12 generation by cultured DCs, but the levels of IL-12 generated in cultures containing DCs derived from asthmatic mice on days 7 and 28 after conidia were significantly lower compared with naive DCs (Figure 6J). Together, these data indicated that TLR9 expres-

sion by asthmatic DCs varied during fungal asthma, but these changes in TLR9 expression did not appear to explain the impaired IL-12 generation by these cells after CpG activation.

Targeting ST2L Potentiates CpG-Mediated Therapeutic Effects in Experimental Asthma

To address the hypothesis that ST2L negatively regulated the therapeutic effects of CpG in fungal asthma, we investigated whether the combination of CpG and anti-ST2L mAb therapy during this time markedly diminished disease features in the fungal asthma model. Although a previous study⁸ demonstrated that 50 μ g per dose of CpG administered via i.p. injection had no effect on methacholine-induced airway resistance or airway remodeling in the fungal asthma model, in the present study we used a 10-fold lower dose of CpG to further ensure that this agent was administered at a subtherapeutic dose. Indeed, 5 μ g per dose of CpG given systemically from day 14 to day 28 had no effect on methacholine-induced airway resistance (Figure 7A). A 100- μ g dose of anti-ST2L mAb alone given on alternate days from day 14 to day 28 after conidia significantly attenuated all features of fungal asthma (Figure 4, A–H); therefore, we speculated that the use of 10-fold less of this mAb alone would not exert a therapeutic effect in this model. This speculation was confirmed because no change in methacholine-induced airway resistance was observed between the anti-ST2L mAb alone (10 μ g per dose) and the IgG group (Figure 7B). However, the combination of CpG (5 μ g per dose) plus anti-ST2L mAb (10 μ g per dose) significantly reduced methacholine (420 μ g/mL)-induced airway resistance compared with the group that received CpG plus IgG (Figure 7B). ELISA analysis of serum samples revealed that IgE levels were not different among the treatment groups (Figure 7C). Compared with the IgG group, BAL eosinophil counts were significantly lower in the anti-ST2L antibody alone, CpG plus IgG, and CpG plus anti-ST2L antibody combination groups (Figure 7D).

Figure 7. CpG plus anti-ST2L mAb combined therapy significantly attenuated methacholine (Mch)-induced airway hyperreactivity. Airway resistance analysis in saline or CpG (5 µg per dose)-treated asthmatic mice (A) and IgG-, anti-ST2L mAb-, CpG plus IgG-, or CpG plus anti-ST2L mAb-treated asthmatic mice on alternate days from day 14 to day 28 after conidia challenge (B). Peak increases in airway resistance or hyperresponsiveness were determined at each time point after the i.v. injection of Mch. Serum IgE (C), BAL eosinophil counts (D), and ELISA analysis of IL-12 (E), MIG/CXCL9 (F), IL-4 (G), and IL-33 (H) in whole lung samples from various treatment groups on day 28 after conidia. Data are expressed as the mean $\pm$ SEM ($n = 5$ per group per time point). * $P \leq 0.05$, *** $P \leq 0.001$ versus the appropriate baseline measurement or the indicated treatment group after Mch challenge.

Through the use of either ELISA or BioPlex analytic approaches on whole lung samples, there was a trend toward increased IL-12 (Figure 7E) and a significant increase in MIG/CXCL9 in the CpG plus anti-ST2L mAb group compared with the CpG plus IgG group (Figure 7F). Whole lung IL-4 (Figure 7G) and IL-33 (Figure 7H) levels were either significantly decreased (compared with the anti-ST2L antibody alone group) or significantly increased in the CpG plus anti-ST2L mAb group (compared with the combination control group). Thus, these data demonstrated that the combination of subtherapeutic doses of CpG and anti-ST2L mAb significantly inhibited methacholine-induced airway hyperresponsiveness in asthmatic mice, possibly through the modulation of Th1- and Th2-type mediator generation in the lung.

The effect of the combination of CpG plus anti-ST2L mAb therapy was also investigated in histological lung sections (Figure 8). Whole lung sections from mice removed on day 28 after conidia challenge were treated with IgG (Figure 8, A, F, K, and P), anti-ST2L mAb (Figure 8, B, G, L, and Q), CpG (Figure 8, C, H, M, and R), CpG plus IgG (Figure 8, D, I, N, and S), or CpG plus anti-ST2L mAb (Figure 8, E, J, O, and T) were stained with H&E (Figure 8, A–E), trichrome (Figure 8, F–J), PAS (Figure 8, K–O), and Gomori methenamine silver (Figure 8, P–T). Lungs from mice treated with CpG plus anti-ST2L mAb exhibited little airway inflammation, lung remodeling, mucous cell metaplasia, and fungal material retention compared with all other control treatment groups. Quantification of the fungal burden in lung samples using TaqMan analysis revealed that the combination of CpG plus anti-ST2L mAb caused the greatest clearance of fungus from the lungs compared with all other treatment groups of asthmatic mice (Figure 9). Thus, these data demonstrated that the combination of CpG plus anti-ST2L mAb attenuated the lung pathological features in mice with fungal asthma, whereas the administration of these agents alone had no effect when doses were at subtherapeutic levels.

Targeting ST2L during CpG Therapy Enhances IL-12 Generation by DCs from Asthmatic Mice

The effect on IL-12 generation by targeting ST2L alone or during CpG therapy in DCs isolated from asthmatic mice was explored in a final series of experiments (Figure 10). In the first experiment, the direct effect of anti-ST2L mAb on DCs cultured with and without CpG is shown in Figure 10A. The addition of either 1 or 10 µg/mL of anti-ST2L mAb *in vitro* did not alter IL-12 generation by asthmatic DCs derived from asthmatic mice on day 28 after conidia. The addition of 2 nmol/L CpG to asthmatic DCs promoted a threefold increase in IL-12 levels. However, the inclusion of anti-ST2L mAb significantly enhanced, in a dose-dependent manner, the activating effect of CpG in asthmatic DCs (Figure 10A). Asthmatic DCs isolated from mice treated with IgG or anti-ST2L mAb (both at 100 µg per dose) generated similar amounts of IL-12 regardless of the *in vitro* condition (Figure 10B). However, IL-12 generation was more dramatically affected by the combined *in vivo* treatment of CpG and anti-ST2L mAb compared with control mice treated with CpG and IgG (Figure 10C). Thus, ST2L appeared to impair the generation of IL-12 by CpG-activated asthmatic DCs.

Discussion

The discovery that IL-33, through its membrane-associated receptor ST2L, is a potent activator of Th2-type inflammation led to investigation of their role in both experimental and clinical asthma.^{33,40} Although controversy exists about whether caspase-mediated cleavage of IL-33 produces an active or inactive form of the protein,^{16,41,42} IL-33-mediated activation of various lymphoid, myeloid, and epithelial cells via ST2L leads to

Figure 8. CpG and anti-ST2L mAb combination therapy reduced airway inflammation, mucus metaplasia, and peribronchial fibrosis; and promoted fungal clearance during fungal asthma. Whole lung tissue sections from *Aspergillus*-sensitized mice treated i.p. with 10 μ g of IgG (**A**, **F**, **K**, and **P**), 10 μ g of anti-ST2L mAb (**B**, **G**, **L**, and **Q**), and 5 μ g of CpG (**C**, **H**, **M**, and **R**), CpG plus IgG (**D**, **I**, **N**, and **S**), or CpG plus anti-ST2L mAb (**E**, **J**, **O**, and **T**) on alternate days from day 14 to day 28 after conidia challenge. Tissues were stained with H&E (**A–E**), Masson trichrome (**F–J**), PAS (**K–O**), or Gomori methenamine silver (**P–T**). Original magnification, $\times 200$ (all photomicrographs).

profound changes in the lung, including airway hyperresponsiveness and goblet cell metaplasia.²⁰ Various targeting strategies directed at either IL-33²⁵ or ST2L^{18–22} have proved successful in the treatment of experimental allergic asthma, largely through the reduction in Th2-type inflammation. In the present study, we observed that both ST2L and IL-33 were induced after the challenge of *A. fumigatus*-sensitized mice with live conidia from the same fungus. Targeting ST2L alone with an mAb beginning 2 weeks after the conidia challenge attenuated all features of airway disease in this model, thereby showing that ST2L activation contributed to the maintenance of fungal asthma. The finding that ST2L also functioned as a negative regulator of TLR9 activation in this model was unique to our study because blockade of ST2L revealed

the therapeutic effects of systemically administered CpG. The blockade of ST2L significantly enhanced the generation of IL-12p70 by CpG-activated DCs, thereby enhancing levels of a cytokine known to potently inhibit allergic asthma in mice.⁴³ Thus, ST2L activation contributes to experimental allergic asthma via two distinct mechanisms: the promotion of Th2-type responses and the negative regulation of TLR9 signaling.

The expression of ST2L, sST2, and IL-33 was enhanced both in structural cells, such as lung epithelial cells, and in immune cells, such as DCs, after the induction of fungal asthma. ST2L was prominently expressed in both types of cells, and its expression was regulated in a time- and TLR-dependent manner. Human lung alveolar epithelial cells generate sST2,⁴⁴ and elevated sST2 has been reported in asthmatic patients (regardless of disease severity) compared with healthy volunteers. However, the greatest differences in sST2 were observed between asthmatic patients who had experienced a short-term exacerbation and those who had not experienced an exacerbation.³² The present study demonstrated that sST2 was increased after the conidia challenge and that TLR-activated asthmatic DCs were a prominent source of this soluble receptor, but serum levels of this receptor did not change during the course of the model. The highest levels of sST2 were detected in samples from patients with moderate asthma. Increased IL-33 expression in the airway epithelium and airway smooth muscle cells during fungal asthma was consistent with that observed in lung biopsy specimens and

Figure 9. Quantitative PCR analysis of *Aspergillus* conidia in whole lung samples from asthmatic mice treated with IgG (10 μ g per dose), anti-ST2L mAb (10 μ g per dose), CpG (5 μ g per dose), CpG plus IgG, or CpG plus anti-ST2L mAb. Conidia content in lung samples was determined using primers for 18S ribosomal DNA specific for *A. fumigatus* conidia. Data are expressed as the mean $\pm$ SEM ($n = 5$ per group per time point). *** $P \leq 0.001$.

Figure 10. The combination of CpG plus anti-ST2L mAb enhanced CpG-induced IL-12 generation by cultured DCs. **A:** Results from DCs obtained from untreated asthmatic mice on day 28 after conidia challenge; these cells were cultured for 24 hours with the indicated reagents. **B:** Results from DCs obtained on day 28 after conidia from asthmatic mice that received 100 µg per dose of IgG or anti-ST2L mAb on alternate days from day 14 to day 28 after conidia challenge. **C:** Results from DCs obtained on day 28 after conidia from asthmatic mice that received CpG, IgG, anti-ST2L mAb, CpG plus IgG, or CpG plus anti-ST2L mAb on alternate days from day 14 to day 28 after conidia challenge. In all experiments, DCs were cultured either in medium alone or with CpG (2 nmol/L) for 24 hours (**A–C**). ELISA was used to detect IL-12p70 in cell-free supernatants. Data are expressed as the mean $\pm$ SEM ($n = 5$ per group). *** $P \leq 0.001$ versus the appropriate control group.

bronchoalveolar lavage samples from asthmatic patients.^{34,35} Whole lung levels of IL-33 peaked on day 7 after conidia challenge, but circulating levels of IL-33 were less than the limits of detection in the fungal asthma model. However, IL-33 normally resides in the cell nucleus^{45,46} and inflammatory events leading to cell necrosis allow its release.³³ Anaphylactic shock appears to be one human condition that is characterized by the release of major amounts of IL-33 into the circulatory system.⁴⁷ In contrast, a few human plasma samples contained detectable levels of IL-33, although the amount of IL-33 detected in plasma samples from patients with moderate asthma was significantly greater than levels in control and other diseased samples. TLR ligand activation of DCs induced ST2L, sST2, and IL-33 transcript expression in our experimental model, but levels of IL-33 protein produced by these cells were less than the limits of detection of the ELISA used. Together, these data demonstrate that ST2 and IL-33 expression are increased during both experimental and clinical asthma.

DCs are among the many types of myeloid-derived innate allergic cells that express ST2L, and the increased expression of this receptor renders DCs refractory to proinflammatory maturation-inducing stimuli.⁴⁸ IL-33 recently directly promoted the differentiation of DCs via its triggering of granulocyte-monocyte colony-stimulating factor production by these cells.³⁶ IL-33-activated DCs induce what has been described as an atypical Th2-type response characterized by IL-5 and IL-13, with no IL-4.³⁷ The addition of recombinant IL-33 to cultured asthmatic DCs in the present study dose dependently induced chemokine ligands 17 and 22, but not IL-12p70, generation by these cells (H.R. and C.M.H., unpublished data). Together, these findings are consistent with the Th2-inducing effects of IL-33 and further highlight the manner in which ST2L-positive DCs might contribute to allergic inflammation.

Targeting ST2L via an antibody approach^{18,21} or enhancing levels of sST2²⁴ have both proved effective in attenuating ovalbumin-induced allergic asthma responses in Th2-

biased BALB/c mice. Our targeting of ST2L alone using a novel mAb in the fungal asthma model confirmed, and also extended, these previous studies. As others have shown, targeting ST2L during fungal asthma resolved all features of allergic airway disease (ie, airway hyperresponsiveness and remodeling), concomitant with a significant reduction in Th2-mediated allergic inflammation (ie, peribronchial eosinophilia). However, our findings contrasted with those obtained from an ovalbumin-induced disease model in the C57BL/6 mouse that showed little disease, normalized IL-33 levels, and few ST2-positive T cells at 1 week after challenge.²¹ Indeed, elevated levels of ST2L and, to a lesser extent, IL-33 were maintained during the 1-month course of fungal asthma followed in our model. The maintenance or persistence of disease allowed for a therapeutic assessment of anti-ST2L mAb treatment in established fungal asthma, beginning at 2 weeks after induction with conidia challenge. Thus, targeting ST2L during experimental fungal asthma is consistent with the hypothesis that IL-33 promotes or amplifies established Th2 responses.³³

Aside from its role in directing Th2-type responses, ST2L also has a well-characterized inhibitory effect on MyD88-dependent TLR activation.^{17,49,50} Although TLR activation can promote Th2-type allergic inflammation,⁵¹ targeting TLR9 with CpG has proved to be a highly effective therapeutic approach in experimental asthma.⁵² The containment of *Aspergillus* in the allergic host requires TLR9 expression,³⁰ and exogenous CpG reverses fungal asthma when administered at a dose of 50 µg for 2 weeks by intranasal, but not i.p., injection.⁸ CpG given in this manner has caused tumor necrosis factor- α -dependent toxicity in rodents.⁵³ In the present study, CpG therapy was optimized (ie, systemic administration of a nontoxic dose of 5 µg) with the addition of subtherapeutic amounts of anti-ST2L mAb. All features of allergic asthma were markedly attenuated by this combination therapeutic approach, and prominent induction of IL-12p70 and MIG/CXCL9 was observed in whole lung and isolated asthmatic DCs. IL-12 has potent therapeutic effects in

allergic asthma,⁴³ including attenuation of asthmatic responses driven by both fungus³⁷ and fungal proteases.⁵⁴ The role of MIG/CXCL9 in asthma is less clear, but this CXC chemokine is strongly induced by IL-12.⁵⁵ Asthmatic DCs produced significant amounts of IL-12p70 after CpG activation, but it was evident from the present study that the ST2L had an inhibitory effect on CpG-mediated generation of this cytokine. Because IL-33 protein was not detected in culture supernatants of DCs, the increased presence of ST2L alone on these cells might have been sufficient to sequester the adaptor protein MyD88¹⁷ or inhibit I κ B α degradation,⁵⁶ thereby impairing TLR signaling. These findings are consistent with the observations of Espinassous et al,⁵⁷ who showed that the exogenous addition of IL-33 enhanced lipopolysaccharide activation in macrophages, findings that mirror those we obtained from cultured asthmatic DCs (H.R. and C.M.H., unpublished data). The persistence of ST2L, not IL-33, expression during fungal asthma might indicate that the negative regulatory role of ST2L becomes more prominent during later stages of this model. Thus, IL-12 generation by CpG-activated DCs is impaired by the presence of ST2L. The data from this study suggest that targeting ST2L with an mAb abrogates this inhibitory effect, and further studies will address whether this mAb-ST2L interaction alters the conformation of ST2L, thereby preventing it from sequestering MyD88.

In summary, targeting ST2L alone or in the presence of subtherapeutic amounts of CpG promoted the resolution of established fungal asthma. The therapeutic effects of targeting ST2L were consistent with its direct role in Th2-type responses,³³ but the present study also revealed that ST2L negatively regulates the therapeutic effects of CpG during fungal asthma. Given the difficulty in reproducing the therapeutic effects of CpG in clinical asthma,⁹ it is conceivable that the implementation of strategies that limit the activity of ST2L might both impair Th2-type allergic inflammation and enhance therapeutic TLR activation.

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References

- Galli SJ, Tsai M, Piliponsky AM: The development of allergic inflammation. *Nature* 2008, 454:445–454
- Kuipers H, Lambrecht BN: The interplay of dendritic cells: Th2 cells and regulatory T cells in asthma. *Curr Opin Immunol* 2004, 16:702–708
- Duez C, Gosset P, Tonnel AB: Dendritic cells and toll-like receptors in allergy and asthma. *Eur J Dermatol* 2006, 16:12–16
- Sur S, Wild JS, Choudhury BK, Sur N, Alam R, Klinman DM: Long term prevention of allergic lung inflammation in a mouse model of asthma by CpG oligodeoxynucleotides. *J Immunol* 1999, 162:6284–6293
- Serebrisky D, Teper AA, Huang CK, Lee SY, Zhang TF, Schofield BH, Kattan M, Sampson HA, Li XM: CpG oligodeoxynucleotides can reverse Th2-associated allergic airway responses and alter the B7.1/B7.2 expression in a murine model of asthma. *J Immunol* 2000, 165:5906–5912
- Kline JN, Kitagaki K, Businga TR, Jain VV: Treatment of established asthma in a murine model using CpG oligodeoxynucleotides. *Am J Physiol Lung Cell Mol Physiol* 2002, 283:L170–L179
- Banerjee B, Kelly KJ, Fink JN, Henderson JD Jr, Bansal NK, Kurup VP: Modulation of airway inflammation by immunostimulatory CpG oligodeoxynucleotides in a murine model of allergic aspergillosis. *Infect Immun* 2004, 72:6087–6094
- Ramaprakash H, Hogaboam CM: Intranasal CpG therapy attenuated experimental fungal asthma in a TLR9-dependent and -independent manner. *Int Arch Allergy Immunol* 2010, 152:98–112
- Gauvreau GM, Hessel EM, Boulet LP, Coffman RL, O'Byrne PM: Immunostimulatory sequences regulate interferon-inducible genes but not allergic airway responses. *Am J Respir Crit Care Med* 2006, 174:15–20
- Mutwiri G, van Drunen Littel-van den Hurk S, Babiuk LA: Approaches to enhancing immune responses stimulated by CpG oligodeoxynucleotides. *Adv Drug Deliv Rev* 2009, 61:226–232
- Liew FY, Xu D, Brint EK, O'Neill LA: Negative regulation of toll-like receptor-mediated immune responses. *Nat Rev Immunol* 2005, 5:446–458
- O'Neill LA: When signaling pathways collide: positive and negative regulation of toll-like receptor signal transduction. *Immunity* 2008, 29:12–20
- Lang T, Mansell A: The negative regulation of Toll-like receptor and associated pathways. *Immunol Cell Biol* 2007, 85:425–434
- Lohning M, Grogan JL, Coyle AJ, Yazdanbakhsh M, Meisel C, Gutierrez-Ramos JC, Radbruch A, Kamradt T: T1/ST2 expression is enhanced on CD4+ T cells from schistosome egg-induced granulomas: analysis of Th cell cytokine coexpression ex vivo. *J Immunol* 1999, 162:3882–3889
- Lambrecht BN, De Veerman M, Coyle AJ, Gutierrez-Ramos JC, Thielemans K, Pauwels RA: Myeloid dendritic cells induce Th2 responses to inhaled antigen, leading to eosinophilic airway inflammation. *J Clin Invest* 2000, 106:551–559
- Schmitz J, Owyang A, Oldham E, Song Y, Murphy E, McClanahan TK, Zurawski G, Moshrefi M, Qin J, Li X, Gorman DM, Bazan JF, Kastelein RA: IL-33, an interleukin-1-like cytokine that signals via the IL-1 receptor-related protein ST2 and induces T helper type 2-associated cytokines. *Immunity* 2005, 23:479–490
- Brint EK, Xu D, Liu H, Dunne A, McKenzie AN, O'Neill LA, Liew FY: ST2 is an inhibitor of interleukin 1 receptor and Toll-like receptor 4 signaling and maintains endotoxin tolerance. *Nat Immunol* 2004, 5:373–379
- Coyle AJ, Lloyd C, Tian J, Nguyen T, Eriksson C, Wang L, Ottoson P, Persson P, Delaney T, Lehar S, Lin S, Poisson L, Meisel C, Kamradt T, Bjerke T, Levinson D, Gutierrez-Ramos JC: Crucial role of the interleukin 1 receptor family member T1/ST2 in T helper cell type 2-mediated lung mucosal immune responses. *J Exp Med* 1999, 190:895–902
- Townsend MJ, Fallon PG, Matthews DJ, Jolin HE, McKenzie AN: T1/ST2-deficient mice demonstrate the importance of T1/ST2 in developing primary T helper cell type 2 responses. *J Exp Med* 2000, 191:1069–1076
- Kondo Y, Yoshimoto T, Yasuda K, Futatsugi-Yumikura S, Morimoto M, Hayashi N, Hoshino T, Fujimoto J, Nakanishi K: Administration of IL-33 induces airway hyperresponsiveness and goblet cell hyperplasia in the lungs in the absence of adaptive immune system. *Int Immunol* 2008, 20:791–800
- Kearley J, Buckland KF, Mathie SA, Lloyd CM: Resolution of allergic inflammation and airway hyperactivity is dependent upon disruption of the T1/ST2-IL-33 pathway. *Am J Respir Crit Care Med* 2009, 179:772–781
- Kurowska-Stolarska M, Stolarski B, Kewin P, Murphy G, Corrigan CJ, Ying S, Pitman N, Mirchandani A, Rana B, van Rooijen N, Shepherd M, McSharry C, McInnes IB, Xu D, Liew FY: IL-33 amplifies the polarization of alternatively activated macrophages that contribute to airway inflammation. *J Immunol* 2009, 183:6469–6477
- Oshikawa K, Yanagisawa K, Tominaga S, Sugiyama Y: Expression and function of the ST2 gene in a murine model of allergic airway inflammation. *Clin Exp Allergy* 2002, 32:1520–1526
- Hayakawa H, Hayakawa M, Kume A, Tominaga S: Soluble ST2 blocks interleukin-33 signaling in allergic airway inflammation. *J Biol Chem* 2007, 282:26369–26380

25. Liu X, Li M, Wu Y, Zhou Y, Zeng L, Huang T: Anti-IL-33 antibody treatment inhibits airway inflammation in a murine model of allergic asthma. *Biochem Biophys Res Commun* 2009, 386:181–185
26. Chupp GL, Lee CG, Jarjour N, Shim YM, Holm CT, He S, Dziura JD, Reed J, Coyle AJ, Kiener P, Cullen M, Grandsaigne M, Dombret MC, Aubier M, Pretolani M, Elias JA: A chitinase-like protein in the lung and circulation of patients with severe asthma. *N Engl J Med* 2007, 357:2016–2027
27. American Thoracic Society. Proceedings of the ATS workshop on refractory asthma: current understanding, recommendations, and unanswered questions. *Am J Respir Crit Care Med* 2000, 162:2341–2351
28. Hogaboam CM, Blease K, Mehrad B, Steinhauser ML, Standiford TJ, Kunkel SL, Lukacs NW: Chronic airway hyperreactivity, goblet cell hyperplasia, and peribronchial fibrosis during allergic airway disease induced by *Aspergillus fumigatus*. *Am J Pathol* 2000, 156:723–732
29. Fursov N, Johnston E, Duffy K, Cotty A, Petley T, Fisher J, Jiang H, Ryczyn MA, Giles-Komar J, Powers G: Generation and characterization of rat anti-mouse ST2L monoclonal antibodies. *Hybridoma (Larchmt)* 2011, 30:153–162
30. Ramaprakash H, Ito T, Standiford TJ, Kunkel SL, Hogaboam CM: Toll-like receptor 9 modulates immune responses to *Aspergillus fumigatus* conidia in immunodeficient and allergic mice. *Infect Immun* 2009, 77:108–119
31. Paterson PJ, Seaton S, McLaughlin J, Kibbler CC: Development of molecular methods for the identification of *aspergillus* and emerging moulds in paraffin wax embedded tissue sections. *Mol Pathol* 2003, 56:368–370
32. Oshikawa K, Kuroiwa K, Tago K, Iwahana H, Yanagisawa K, Ohno S, Tominaga SI, Sugiyama Y: Elevated soluble ST2 protein levels in sera of patients with asthma with an acute exacerbation. *Am J Respir Crit Care Med* 2001, 164:277–281
33. Smith DE: IL-33: a tissue derived cytokine pathway involved in allergic inflammation and asthma. *Clin Exp Allergy* 2010, 40:200–208
34. Prefontaine D, Lajoie-Kadoch S, Foley S, Audusseau S, Olivenstein R, Halayko AJ, Lemiere C, Martin JG, Hamid Q: Increased expression of IL-33 in severe asthma: evidence of expression by airway smooth muscle cells. *J Immunol* 2009, 183:5094–5103
35. Prefontaine D, Nadigel J, Chouaili F, Audusseau S, Semlali A, Chakir J, Martin JG, Hamid Q: Increased IL-33 expression by epithelial cells in bronchial asthma. *J Allergy Clin Immunol* 2010, 125:752–754
36. Mayuzumi N, Matsushima H, Takashima A: IL-33 promotes DC development in BM culture by triggering GM-CSF production. *Eur J Immunol* 2009, 39:3331–3342
37. Rank MA, Kobayashi T, Kozaki H, Bartemes KR, Squillace DL, Kita H: IL-33-activated dendritic cells induce an atypical TH2-type response. *J Allergy Clin Immunol* 2009, 123:1047–1054
38. Kool M, van Nimwegen M, Willart MA, Muskens F, Boon L, Smit JJ, Coyle A, Clausen BE, Hoogsteden HC, Lambrecht BN, Hammad H: An anti-inflammatory role for plasmacytoid dendritic cells in allergic airway inflammation. *J Immunol* 2009, 183:1074–1082
39. Ashino S, Wakita D, Zhang Y, Chamoto K, Kitamura H, Nishimura T: CpG-ODN inhibits airway inflammation at effector phase through down-regulation of antigen-specific Th2-cell migration into lung. *Int Immunol* 2008, 20:259–266
40. Kakkar R, Lee RT: The IL-33/ST2 pathway: therapeutic target and novel biomarker. *Nat Rev Drug Discov* 2008, 7:827–840
41. Luthi AU, Cullen SP, McNeela EA, Duriez PJ, Afonina IS, Sheridan C, Brumatti G, Taylor RC, Kersse K, Vandenabeele P, Lavelle EC, Martin SJ: Suppression of interleukin-33 bioactivity through proteolysis by apoptotic caspases. *Immunity* 2009, 31:84–98
42. Talabot-Ayer D, Lamacchia C, Gabay C, Palmer G: Interleukin-33 is biologically active independently of caspase-1 cleavage. *J Biol Chem* 2009, 284:19420–19426
43. Gavett SH, O'Hearn DJ, Li X, Huang SK, Finkelman FD, Wills-Karp M: Interleukin 12 inhibits antigen-induced airway hyperresponsiveness, inflammation, and Th2 cytokine expression in mice. *J Exp Med* 1995, 182:1527–1536
44. Mildner M, Storka A, Lichtenauer M, Mlitz V, Ghannadan M, Hoetzelnecker G, Nickl S, Dome B, Tschachler E, Ankersmit HJ: Primary sources and immunological prerequisites for sST2 secretion in humans. *Cardiovasc Res* 2010, 87:769–777
45. Carriere V, Roussel L, Ortega N, Lacorre DA, Americh L, Aguilar L, Bouche G, Girard JP: IL-33, the IL-1-like cytokine ligand for ST2 receptor, is a chromatin-associated nuclear factor in vivo. *Proc Natl Acad Sci U S A* 2007, 104:282–287
46. Kuchler AM, Pollheimer J, Balogh J, Sponheim J, Manley L, Sorensen DR, De Angelis PM, Scott H, Haraldsen G: Nuclear interleukin-33 is generally expressed in resting endothelium but rapidly lost upon angiogenic or proinflammatory activation. *Am J Pathol* 2008, 173:1229–1242
47. Pushparaj PN, Tay HK, H'Ng SC, Pitman N, Xu D, McKenzie A, Liew FY, Melendez AJ: The cytokine interleukin-33 mediates anaphylactic shock. *Proc Natl Acad Sci U S A* 2009, 106:9773–9778
48. Turnquist HR, Sumpter TL, Tsung A, Zahorchak AF, Nakao A, Nau GJ, Liew FY, Geller DA, Thomson AW: IL-1 β -driven ST2L expression promotes maturation resistance in rapamycin-conditioned dendritic cells. *J Immunol* 2008, 181:62–72
49. Sweet MJ, Leung BP, Kang D, Sogaard M, Schulz K, Trajkovic V, Campbell CC, Xu D, Liew FY: A novel pathway regulating lipopolysaccharide-induced shock by ST2/T1 via inhibition of Toll-like receptor 4 expression. *J Immunol* 2001, 166:6633–6639
50. Liu J, Buckley JM, Redmond HP, Wang JH: ST2 negatively regulates TLR2 signaling, but is not required for bacterial lipoprotein-induced tolerance. *J Immunol* 2010, 184:5802–5808
51. Eisenbarth SC, Piggott DA, Huleatt JW, Visintin I, Herrick CA, Bottomly K: Lipopolysaccharide-enhanced, toll-like receptor 4-dependent T helper cell type 2 responses to inhaled antigen. *J Exp Med* 2002, 196:1645–1651
52. Hayashi T, Beck L, Rossetto C, Gong X, Takikawa O, Takabayashi K, Broide DH, Carson DA, Raz E: Inhibition of experimental asthma by indoleamine 2,3-dioxygenase. *J Clin Invest* 2004, 114:270–279
53. Campbell JD, Cho Y, Foster ML, Kanzler H, Kachura MA, Lum JA, Ratcliffe MJ, Sathe A, Leishman AJ, Bahl A, McHale M, Coffman RL, Hessel EM: CpG-containing immunostimulatory DNA sequences elicit TNF- α -dependent toxicity in rodents but not in humans. *J Clin Invest* 2009, 119:2564–2576
54. Lamhamedi-Cherradi SE, Martin RE, Ito T, Kheradmand F, Corry DB, Liu YJ, Moyle M: Fungal proteases induce Th2 polarization through limited dendritic cell maturation and reduced production of IL-12. *J Immunol* 2008, 180:6000–6009
55. Schuh JM, Blease K, Kunkel SL, Hogaboam CM: Chemokines and cytokines: axis and allies in asthma and allergy. *Cytokine Growth Factor Rev* 2003, 14:503–510
56. Takezako N, Hayakawa M, Hayakawa H, Aoki S, Yanagisawa K, Endo H, Tominaga S: ST2 suppresses IL-6 production via the inhibition of IkappaB degradation induced by the LPS signal in THP-1 cells. *Biochem Biophys Res Commun* 2006, 341:425–432
57. Espinassous Q, Garcia-de-Paco E, Garcia-Verdugo I, Synguelakis M, von Aulock S, Sallenave JM, McKenzie AN, Kanellopoulos J: IL-33 enhances lipopolysaccharide-induced inflammatory cytokine production from mouse macrophages by regulating lipopolysaccharide receptor complex. *J Immunol* 2009, 183:1446–1455