Soluble ST2 Blocks Interleukin-33 Signaling in Allergic Airway Inflammation*

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The ST2 gene produces a soluble secreted form and a transmembrane form, referred to as soluble ST2 and ST2L, respectively. A recent study has reported that interleukin (IL)-33 is a specific ligand of ST2L and induces production of T helper type 2 (Th2) cytokines. Although soluble ST2 is highly produced in sera of asthmatic patients and plays a critical role for production of Th2 cytokines, the function of soluble ST2 in relation to IL-33 signaling remains unclear. Here we show antagonistic effects of soluble ST2 on IL-33 signaling using a murine thymoma EL-4 cells stably expressing ST2L and a murine model of asthma. Soluble ST2 directly bound to IL-33 and suppressed activation of NF-κB in EL-4 cells stably expressing ST2L, suggesting that the complex of soluble ST2 and IL-33 fails to bind to ST2L. In a murine model of asthma, pretreatment with soluble ST2 reduced production of IL-4, IL-5, and IL-13 from IL-33-stimulated splenocytes. These results indicate that soluble ST2 acts as a negative regulator of Th2 cytokine production by the IL-33 signaling. Our study provides a molecular mechanism wherein soluble ST2 modulates the biological activity of IL-33 in allergic airway inflammation.

The interleukin (IL)-1 receptor family plays important roles in inflammatory and immunological responses. The ST2 gene is a member of the IL-1 receptor family, producing a soluble secreted form and a transmembrane form, soluble ST2 and ST2L, respectively (1–3). These proteins are generated by alternative splicing of pre-mRNA. The structure of ST2L is similar to that of IL-1 receptor type 1 (IL-1RI), consisting of three extracellular immunoglobulin domains and an intracellular Toll-interleukin-1 receptor domain. Although the extracellular domain is common to soluble ST2 and ST2L, soluble ST2 lacks the transmembrane and intracellular Toll-interleukin-1 receptor domains. The ST2 gene is expressed in several cells including fibroblasts and mast cells (1, 4). In particular, ST2L is preferentially expressed in murine and human Th2 cells and can be utilized as a specific marker of Th2 cells in in vitro experiments (5–8). Therefore, the function of ST2L has been suggested to correlate with Th2 cell-mediated immunological responses. However, ST2L has been an orphan receptor ever since it was first reported (5). Late in 2005, IL-33, a newly discovered member of the IL-1 cytokine family, was finally reported as a specific ligand for ST2L (9).

The IL-33 gene, also described as a nuclear factor expressed in high endothelial venules (NF-HEV) (10), codes a 31-kDa protein that does not contain a signal sequence for secretion, similar to the IL-1α, IL-1β, and IL-18 genes (11, 12). Previous study has demonstrated the processing and function of the IL-33 protein (9). The precursor 31-kDa protein (pre-IL-33) was cleaved by caspase-1 into a mature 18-kDa protein (IL-33) in vitro experiments using a recombinant protein. Functional analysis has shown that IL-33 bound to murine mast cells expressing ST2L and stimulated the intracellular signaling pathway, leading to the activation of NF-κB and mitogen-activated protein kinases. In addition, the production of Th2 cytokines and severe pathological changes in mucosal organs were induced by administration of IL-33 to mice. Previous studies before the discovery of IL-33 had already shown that ST2L is associated with the production of Th2 cytokines. Levels of Th2 cytokines were decreased in asthmatic mice by administration of the antibody that blocks ST2L and in a pulmonary granuloma model using mice lacking the ST2 gene (7, 13). Therefore, these results suggest that IL-33 signaling via ST2L plays important roles in Th2 cell-mediated immunological responses including the production of Th2 cytokines.

On the other hand, previous studies in human patients and animal models have shown that the level of soluble ST2 in sera was elevated in asthmatic disease (14, 15). Therefore, it has been suggested that soluble ST2 may also play a critical role in Th2 cell-mediated diseases. In fact, administration of a recombinant soluble ST2-Fc fusion protein or a soluble ST2 expression vector to asthmatic mice effectively attenuated inflammatory responses and production of Th2 cytokines (7, 15). These results of therapeutic experiments indicate that soluble ST2 negatively regulates the Th2 cell-mediated immunological responses, in opposition to ST2L. However, the molecular mechanism of negative regulation by soluble ST2 remains unclear. In addition, it has not been addressed whether soluble ST2 is associated with IL-33 signaling.

In this study, using a murine thymoma cell line, EL-4, stably expressing ST2L and a murine model of asthma, we demonstrated that soluble ST2 had a negative function in IL-33 signal-
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The gels were stained with ethidium bromide. The intensity of DNA bands was quantified using the public domain NIH Image program (developed at the United States National Institutes of Health). The size of PCR products was as follows: ST2 (754 bp), ST2L (739 bp), IL-33 (801 bp), and β-actin (576 bp).

Construction of Plasmids—Constructions of pET-21-mIL-33 and pET-21-mIL-1β proceeded as follows. The coding regions of mature IL-33 and IL-1β proteins were obtained from cDNA derived from spleens of BALB/c mice by PCR amplification. The nucleotide sequences of primers containing an EcoRI or an XhoI site were as follows: IL-33, forward 5'-GAATTCACATGTAGCATCGAACAGGAAAC-3' and reverse 5'-CTCGAGGATTTTGAGACTTACAA-3'; IL-1β, forward 5'-GAATTCGGTCCCATAGAAGCTGCA-3' and reverse 5'-CTCGAGGATCCCATAGAAGCTGCA-3'. The production of Th2 cytokines through IL-33 signaling were suppressed in the presence of soluble ST2. Our data suggest that soluble ST2 negatively modulates the activation of NF-κB and the production of Th2 cytokines in the IL-33 signaling were suppressed in the presence of soluble ST2. Our data suggest that soluble ST2 negatively modulates the production of Th2 cytokines through IL-33 signaling in allergic airway inflammation.

EXPERIMENTAL PROCEDURES

Animals—Male and female BALB/c mice, 7–8 weeks of age, were purchased from Japan SLC, Inc. (Shizuoka, Japan). All of the mice were housed in an animal research facility of the Jichi Medical University under pathogen-free conditions. All of the experimental procedures were approved by the Animal Research Ethics Board of Jichi Medical University.

Sensitization and Aeroallergen Challenge—The mice were sensitized by intraperitoneal injection with 100 μg of ovalbumin (OVA) (Sigma-Aldrich) and 20 mg of aluminum potassium sulfate (Sigma-Aldrich) in saline or 20 mg of aluminum potassium sulfate alone in saline on days 0 and 7. On days 14 and 15, the mice were challenged twice daily at intervals of 4 h with 1% (w/v) OVA in saline or saline alone for 30 min using an ultrasonic nebulizer (Omron Corp., Tokyo, Japan) (15). The mice were sacrificed 3–48 h after the last allergen challenge, and sera and tissues were obtained for further analyses. Briefly, blood was drawn from the caudal vena cava and left for 30 min, and then the serum was separated by centrifugation. The sera were stored at −80°C until assay.

Cell Culture—Human embryonic kidney HEK293T cells were cultured in Dulbecco’s modified Eagle’s medium (Sigma-Aldrich) supplemented with 10% fetal bovine serum (Thermo Electron, Melbourne, Australia). Murine thymoma EL-4 cells were cultured in RPMI 1640 medium supplemented with 5% fetal bovine serum (Sigma-Aldrich) and 50 μM 2-mercaptoethanol (RPMI 1640 growth medium).

Reverse Transcription-PCR Analysis—Total RNAs were isolated from murine tissues using TRI reagent (Sigma-Aldrich). The total RNA was treated with RNase-free DNase I; then first-strand cDNA was synthesized as described previously (16). PCR amplification was performed using 0.5 units of AmpliTaq Gold DNA polymerase (Applied Biosystems, Foster, CA), 0.5 μM each of the forward and reverse primers, and the first-strand cDNAs derived from 0.25 μg of DNase I-treated RNA. After cDNAs were treated at 94 °C for 10 min, PCR was carried out for 25 (β-actin), 28 (IL-33), or 33 (ST2 and ST2L) cycles at 94 °C for 1 min, 60 °C for 1 min, and 72 °C for 1.5 min, followed by treatment at 72 °C for 10 min. The nucleotide sequences of primers used were as follows: ST2, forward 5'-TGGCATGAT-TAAGAGAACCATAAGGCT-3' and reverse 5'-GTTTAAACTAGTCTGCTAAGTTGAAG-3'; β-actin, forward 5'-ATGAG-ACCTAAGAAGGTATTTCCA-3' and reverse 5'-TACTCCTGCTGTCTATACACA-3'; and β-actin, forward 5'-ATGAG-ACCTAAGAAGGTATTTCCA-3' and reverse 5'-TACTCCTGCTGTCTATACACA-3'. Seven microliters of PCR products were developed by electrophoresis on 2% agarose gels, and then the gels were stained with ethidium bromide. The intensity of DNA bands was quantified using the public domain NIH Image program (developed at the United States National Institutes of Health). The size of PCR products was as follows: ST2 (754 bp), ST2L (739 bp), IL-33 (801 bp), and β-actin (576 bp).
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Purification of Recombinant IL-33 and IL-1β Proteins—Recombinant murine IL-33 and IL-1β proteins containing a T7 tag at the N terminus and a His tag at the C terminus (rIL-33 and rIL-1β) were produced in bacteria. BL-21 Codon-Plus (DE3)-RIL (Stratagene, La Jolla, CA) was transformed with pET-21-mIL-33 or pET-21-mIL-1β. The bacteria were cultured at 37 °C until the A500 reached 0.6; then expression of recombinant protein was induced by the addition of isopropyl β-D-thiogalactopyranoside to a final concentration of 1 mm. Three hours after culture at 25 °C, the bacteria were harvested, and the pellets were resuspended in lysis buffer (50 mM Na2HPO4, 300 mM NaCl, 10 mM imidazole). After sonication, the soluble cytoplasmic fraction was isolated by centrifugation. The fraction was loaded onto a nickel-nitrilotriacetic acid-agarose (Qiagen) column. The proteins were eluted with elution buffer (50 mM Na2HPO4, 300 mM NaCl, 250 mM imidazole). After dialysis against T7 tag binding buffer (4.3 mM Na2HPO4, 1.5 mM KH2PO4, 2.7 mM KCl, 137 mM NaCl, 0.1% (v/v) Tween 20, pH 7.3), the proteins were purified using a T7 tag affinity purification kit (Novagen). The proteins were eluted from the column with 0.1M citric acid (pH 2.2) and neutralized with 2 M Tris base (pH 10.4). After desalting and concentrating the protein using Centricon YM-3 (Millipore, Bedford, MA), the purified proteins were dialyzed against PBS (8 mM Na2HPO4, 1.5 mM KH2PO4, 2.7 mM KCl, 137 mM NaCl). The protein concentration was determined by the Bradford method using protein assay dye reagent (Bio-Rad) with calibration using bovine serum albumin (Sigma-Aldrich). The protein purity was evaluated using a silver staining kit (Daichi Pure Chemicals, Tokyo, Japan).

Purification of Recombinant Soluble ST2 Proteins—Recombinant soluble ST2 proteins containing V5-His or FLAG-His tags at the C terminus (ST2-V5 and ST2-FLAG) were purified as described previously (18). Briefly, HEK293T cells were transiently transfected with pEF6-mST2-V5-His or pEF6-mST2-FLAG-His using the calcium-phosphate method. Sixteen hours after transfection, the cells were cultured in serum-free Dulbecco’s modified Eagle’s medium for 48 h. The secreted recombinant proteins in the culture supernatant were purified by affinity chromatography using nickel-nitrilotriacetic acid-agarose (Qiagen). The proteins were eluted with 50 mM sodium phosphate buffer (pH 8.0) containing 300 mM NaCl and 250 mM imidazole. Desalting and concentrating the proteins were performed using Centricon YM-30 (Millipore). Finally, the purified proteins were dialyzed against PBS. The method for measuring the protein concentration is described under “Measurement of Soluble ST2 and Cytokines.” Deglycosylation with N-glycosidase F (Roche Applied Science) was performed as described previously (17). The protein purity was evaluated using a silver staining kit.

Establishment of Stable Cell Lines—Empty vector (pEF6-V5-His) and expression vectors (pEF6-mST2L-FLAG and pEF6-mIL-1R1-FLAG) were linearized with FspI. EL-4 cells (1 × 107 cells) were mixed with 50 μg of linearized plasmid DNA in serum-free RPMI 1640 medium, and the mixtures were left for 10 min on ice. Electroporation was carried out using a Gene Pulser (Bio-Rad) at 270 V and 960 microfarads, and then the cells were left on ice for 10 min. The transfected cells were returned to the RPMI 1640 growth medium and were incubated at 37 °C in 5% CO2. Forty-eight hours after transfection, the transfected cells were selected with G418 (Invitrogen). Stable clones were cultured in RPMI 1640 growth medium containing 6 μg/ml G418.

Flow Cytometry—Splenocytes (1 × 106 cells) and stably transfected EL-4 cells (5 × 105 cells) were used for flow cytometric analysis. Preparation of splenocytes proceeded as follows. The spleen was homogenized into a single-cell suspension in PBS by filtration through nylon mesh (70 μm). After depletion of erythrocytes by osmotic lysis, the splenocytes were washed with PBS and resuspended in PBS containing 5% fetal bovine serum. Subsequently, anti-mouse CD16/CD32 antibody (BD Biosciences PharMingen, San Diego, CA) was mixed with the splenocytes to block the Fc receptor for 5 min on ice. Binding analysis of IL-33 and IL-1β on the cell surface was performed as follows. The cells were mixed with 100 or 500 ng of rIL-33 or rIL-1β for 1 h on ice, followed by staining with biotinylated anti-T7 tag antibody (Novagen) for 1 h on ice. Then the cells were stained with R-phycoerythrin (RPE)-conjugated streptavidin (DakoCytomation, Glostrup, Denmark) for 30 min on ice. In case of binding analysis in the presence of soluble ST2, 1 μg of ST2-V5 was added to the cells at 1 h before, at 1 h after, or at the same time as the addition of rIL-33 or rIL-1β. Binding of ST2-V5 was detected with fluorescein isothiocyanate (FITC)-conjugated anti-V5 antibody (Invitrogen) for 1 h on ice. ST2L or IL-1RI was stained with FITC-conjugated anti-mouse T1/T2/ST2 antibody (BD Biosciences, Zürich, Switzerland), RPE-conjugated anti-mouse IL-1RI antibody (BD Biosciences PharMingen), or each isotype control antibody for 1 h on ice. After the stained cells were washed twice with PBS containing 5% fetal bovine serum, the cells were resuspended in PBS and filtered through nylon mesh (35 μm). Analysis was performed on Becton Dickinson LSR using Cell Quest software (BD Biosciences).

Immunoprecipitation—Five hundred ng of ST2-V5 was mixed with 2 μg of rIL-33 or rIL-1β in 500 μl of RIPA buffer (50 mM Tris-HCl, pH 8.0, 150 mM NaCl, 1% (v/v) Nonidet P-40, 0.5% (w/v) deoxycholate, 0.1% (w/v) SDS), and the mixture was agitated overnight at 4 °C. The protein complexes were immunoprecipitated with 40 μl of 50% (v/v) slurry of anti-T7 tag antibody-conjugated agarose (Novagen). After the protein-bound agarose was washed three times with RIPA buffer, binding protein complexes were eluted with 0.1 M citric acid (pH 2.2) and neutralized with the addition of 2 M Tris (pH 10.4). The eluted proteins were subjected to Western blotting.

Western Blotting—The protein samples were separated by electrophoresis on SDS-polyacrylamide gels. The proteins were transferred to a polyvinylidene difluoride membrane (Millipore) and were probed with mouse monoclonal anti-T7 tag (Novagen), rabbit polyclonal anti-IL-33 (Adipogen Inc., Seoul, South Korea), goat polyclonal anti-IL-1β (Santa Cruz Biotechnology, Santa Cruz, CA), mouse monoclonal anti-V5 (Invitrogen), mouse monoclonal anti-His (C Term) (Invitrogen), rabbit polyclonal anti-IκBα (Santa Cruz), or mouse monoclonal anticycraldehyde-3-phosphate dehydrogenase (Santa Cruz) antibody as the primary antibody. The proteins were detected with

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horseradish peroxidase-conjugated goat anti-mouse Ig (Bio-Rad), horseradish peroxidase-conjugated horse anti-goat Ig (Vector, CA), or horseradish peroxidase-conjugated donkey anti-rabbit Ig (Amersham Biosciences) as the secondary antibody. The proteins were visualized using Immobilon Western detection reagents (Millipore), and the membranes were exposed to x-ray films (RX-U; Fuji Photo Film Co., Tokyo, Japan).

Preparation of Cytoplasmic and Nuclear Extracts—Preparation of cytoplasmic and nuclear extracts from stably transfected EL-4 cells was performed as described previously (16). Briefly, the cells were harvested and lysed with PBS. The cell pellets were resuspended in 5 volumes of a buffer solution (10 mM Hepes-KOH, pH 8.0, 10 mM KCl, 1.5 mM MgCl₂, 1 mM dithiothreitol). After addition of Nonidet P-40 to a final concentration of 0.1% (v/v), cytoplasmic extracts were separated by centrifugation. The nuclei were resuspended in 2.5 volumes of a buffer solution (20 mM Hepes-KOH, pH 8.0, 420 mM KCl, 1.5 mM MgCl₂, 0.2 mM EDTA, 25% (v/v) glycerol, 1 mM dithiothreitol, 0.5 mM phenylmethylsulfonyl fluoride) and mixed by agitation. The debris was removed by centrifugation, and the supernatants were dialyzed against a buffer solution (20 mM Hepes, pH 8.0, 100 mM KCl, 0.2 mM EDTA, 20% (v/v) glycerol, 1 mM dithiothreitol, 0.5 mM phenylmethylsulfonyl fluoride). After centrifugation, the supernatants were harvested as nuclear extracts. The protein concentration was determined by the Bradford method. Cytoplasmic and nuclear extracts were stored at −80 °C until assay.

Electrophoretic Mobility Shift Assay (EMSA)—EMSA was performed as described previously, with some modifications (16, 19). Binding reactions were performed at 30 °C for 30 min in a total volume of 15 μl containing nuclear extracts (5 μg of protein) and 20,000 cpm of 32P-labeled oligonucleotide probe. Supershift assay was carried out using antibodies against p50, p52, p65, and c-Rel (Santa Cruz). The protein-DNA complexes were separated on 4% nondenaturing polyacrylamide gels at 80 °C until assay.

Luciferase Assay—The luciferase assay was performed as described previously (16). EL-4 cells stably expressing ST2L or IL-1RI in EL-4 cells, IL-1RI was further expressed in IL-1RI/EL-4 cells to a final concentration of 500 ng/ml for 3 h. Then the cells were stimulated with 10 ng/ml rIL-33 or rIL-1β for 48 h. The culture supernatant was harvested and stored at −80 °C until assay.

Measurement of Soluble ST2 and Cytokines—The concentrations of soluble ST2 in sera and purified recombinant proteins were measured by a sandwich enzyme-linked immunosorbent assay (ELISA) as described previously (15). The concentrations of IL-4, IL-5, IL-13, and IFN-γ in culture supernatants were measured using ELISA kits (BIOSOURCE International Inc., Camarillo, CA).

Statistical Analysis—The data are represented as the means ± S.E. The data were analyzed by the Turkey-Kramer test. A value of p < 0.05 was considered to be significant.

RESULTS

Specific Binding of IL-33 to ST2L-positive Cells—To study the binding and function of IL-33, we developed systems for the expression and purification of mature IL-33 as a recombinant protein (designated as rIL-33) (Fig. 1A). In this study, recombinant mature IL-1β (designated as rIL-1β) was also used for control experiments, because IL-1β has binding activity for IL-1R1, but not for ST2L. Recombinant IL-33 and IL-1β containing T7 and His tags were expressed in bacteria and subjected to affinity purification. The purities of rIL-33 and rIL-1β proteins were examined by silver staining and Western blotting (Fig. 1A, a and b). Although rIL-33 was purified as a single band, the purification product of rIL-1β contained a cleaved product. Next, to establish a clear analysis system for the binding of IL-33, we generated cell lines stably expressing ST2L or IL-1R1 using EL-4 cells (Fig. 1B). Although ST2L was hardly detected in cells stably transfected with an empty vector (EV/EL-4) or an IL-1R1 expression vector (IL-1R1/EL-4), remarkable expression of ST2L was observed in ST2L expression vector-transfected cells (ST2L/EL-4). In addition to a constitutive expression of IL-1R1 in EL-4 cells, IL-1R1 was further expressed in IL-1R1/EL-4 cells. We examined the binding activity of IL-33 using these stably transfected cell lines (Fig. 1C). The ST2L/EL-4 cells clearly shifted according to the increasing concentration of rIL-33 (Fig. 1C, middle panel). Conversely, EV/EL-4 and IL-1R1/EL-4 cells showed little change corresponding to the expression of ST2L. These results demonstrate that IL-33 specifically binds to ST2L.

Inhibition of IL-33 Binding Activity by Soluble ST2—The amino acid sequence of soluble ST2 except for 9 amino acids in the C terminus is the same as that of the extracellular domain of ST2L (1, 3). Therefore, it seemed possible that soluble ST2 might also bind to IL-33. To investigate this possibility, we generated recombinant soluble ST2 containing either V5 or FLAG and His tags in the C terminus (designated as ST2-V5 or ST2-
of input was performed using the anti-His antibody because both recombinant proteins were His-tagged. These experiments clearly revealed that ST2-V5 specifically bound to rIL-33 but not to rIL-1β (Fig. 2B, compare lanes 3–5).

To further study the interaction between soluble ST2 and IL-33, we analyzed the binding of rIL-33 to ST2L-EL-4 cells or rIL-1β to IL-1RI/EL-4 cells in the presence of ST2-V5 (Fig. 2C). Stably transfected EL-4 cells were either left untreated or treated with ST2-V5 and then added with either rIL-33 or rIL-1β. ST2-V5 inhibited the binding of rIL-33 to ST2L-EL-4 cells (Fig. 2C, upper panel). In contrast, rIL-1β binding activity for IL-1RI/EL-4 cells was not affected in the presence of ST2-V5 (Fig. 2C, lower panel). Next, we examined whether the additive order of soluble ST2 influences the binding activity of IL-33 (Fig. 2D). ST2-V5 was added to ST2L/EL-4 cells before, after, or at the same time as the addition of rIL-33. When rIL-33 was added prior to ST2-V5, the binding of IL-33 was hardly influenced by the treatment with ST2-V5 (Fig. 2D, compare panels c and d). On the other hand, the binding of IL-33 was inhibited by the treatment with ST2-V5 at the same time or before the addition of rIL-33 (Fig. 2D, compare panels e, f, and j). In addition, the binding of ST2-V5 to ST2L/EL-4 cells was not observed regardless of the binding of IL-33 in this system. These results indicate that soluble ST2 specifically binds to free IL-33 and inhibits the binding activity of IL-33 for ST2L and that the

Recombinant soluble ST2, expressed in HEK293T cells and secreted into the culture supernatant, was affinity-purified. The purity was confirmed by silver staining (Fig. 2A). SDS-PAGE analysis showed that purified recombinant soluble ST2 was detected as a single broad band of 55–65 kDa because of N-linked glycosylation. After deglycosylation with N-glycosidase F, the molecular mass shifted to 37 kDa, corresponding to an unmodified form. We tested whether soluble ST2 directly interacted with IL-33 in vitro (Fig. 2B). ST2-V5 was mixed with either rIL-33 or rIL-1β, and protein complexes were immunoprecipitated with anti-T7 tag antibody-conjugated agarose and then eluted from agarose. The eluates were analyzed by Western blotting using anti-V5 and anti-T7 tag antibodies. Analysis of input was performed using the anti-His antibody because both recombinant proteins were His-tagged. These experiments clearly revealed that ST2-V5 specifically bound to rIL-33 but not to rIL-1β (Fig. 2B, compare lanes 3–5).

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induced the activation of NF-κB (Fig. 3A, panel a, lanes 4, 7, and 10) and the degradation of IκBα (Fig. 3A, panel b, lanes 3, 6, and 9) in all cell lines, using constitutively expressed IL-1RI. Next, we investigated components of activated NF-κB using specific antibodies against NF-κB. Supershift assay showed that the p50 and p65 subunits were contained in the IL-33-induced DNA/NF-κB complex (Fig. 3B, lanes 3 and 5), as well as in the IL-1β-induced DNA/NF-κB complex (Fig. 3B, lanes 8 and 10).

To further investigate the effect of soluble ST2 on IL-33 signaling, we examined the DNA binding activity of NF-κB and the degradation of IκBα in the presence of soluble ST2 (Fig. 3C and supplemental Fig. S2). Stably transfected EL-4 cells were either left untreated or treated with ST2-V5 and then were left unstimulated or stimulated with rIL-33 or rIL-1β. In IL-33-stimulated ST2L/EL-4 cells, the DNA/NF-κB complex was gradually decreased as the concentration of ST2-V5 increased (Fig. 3C, panel a, lanes 5–7). In contrast, pretreatment with ST2-V5 led to the repression of IκBα degradation (Fig. 3C, panel b, lanes 4–6). On the other hand, ST2-V5 did not affect the NF-κB activation and IκBα degradation in the IL-1β signaling (Fig. 3C, panel a, lanes 11–13, and panel b, lanes 10–12). In addition, we examined NF-κB-dependent luciferase activity in IL-33- or IL-1β-stimulated EL-4 cells (Fig. 3D). Stimulation with IL-33 effectively induced NF-κB-dependent luciferase activity in ST2L/EL-4 cells. The IL-33-induced luciferase activity also slightly increased in IL-1RI/EL-4 cells coincident with low expression levels of ST2L. Furthermore, pretreatment with ST2-V5 reduced the IL-33-induced luciferase activities in both cell lines. On the other hand, IL-1β-induced luciferase activities in both cell lines were not affected by the addition of ST2-V5. These results demonstrate that soluble ST2 specifically suppresses the activation of NF-κB by IL-33 signaling via ST2L.

Expression of Soluble ST2 and ST2L in a Murine Model of Asthma—Previous studies have shown that expressions of soluble ST2 and ST2L increased in asthma. To investigate the expressions of soluble ST2 and ST2L...
FIGURE 3. Suppression of IL-33-induced NF-κB activation by soluble ST2. A, analysis of intracellular responses in the IL-33 and IL-1β signalings. Stably transfected EL-4 cells (2 × 10^7 cells) were either left unstimulated or stimulated with rIL-33 or rIL-1β (10 ng/ml) for 30 min, followed by preparation of cytoplasmic and nuclear extracts. Panel a, EMSA using nuclear extracts with a 32P-labeled oligonucleotide probe containing an NF-κB-binding site. The DNA-protein complexes were separated on a 4% nondenaturing polyacrylamide gel. Panel b, detection of IκBα and glyceraldehyde-3-phosphate dehydrogenase (GAPDH) in cytoplasmic extracts. Cytoplasmic extracts were separated on SDS-12.5% polyacrylamide gels, followed by Western blotting with anti-IκBα and anti-glyceraldehyde-3-phosphate dehydrogenase antibodies. Panel c, supershift assay using anti-NF-κB antibodies. Nuclear extracts were kept on ice for 1 h without antibody (lanes 2 and 7) or with a series of anti-NF-κB antibodies (lanes 3–6 and 8–11), and then the 32P-labeled oligonucleotide probe was admixed. The mixture was kept at 30 °C for 30 min and then subjected to EMSA. Panel d, suppression of DNA binding activity of NF-κB by the addition of ST2-V5. Stably transfected EL-4 cells were either left untreated or treated with ST2-V5 (10 and 100 ng) for 3 h and then left unstimulated or stimulated with rIL-33 or rIL-1β for 30 min. After stimulation, cytoplasmic and nuclear extracts were prepared. Panel a, DNA binding activity of NF-κB was analyzed by EMSA. Panel b, degradation of IκBα was analyzed by Western blotting. Panel c, and D (panel a) contained the 32P-labeled oligonucleotide probe alone. The DNA/NF-κB and supershifted complexes are indicated by white, gray (supershifted by αp50), and black (supershifted by αp65) arrowheads, respectively. D, transcriptional activity of rIL-33- or rIL-1β-induced NF-κB. Stably transfected EL-4 cells (1 × 10^7 cells) were transiently transfected with pNF-κB-Luc (40 μg) and pRL-TK (4 μg). The transfected cells were either left untreated or treated with ST2-V5 (500 ng/ml) for 3 h and then either left unstimulated or stimulated with rIL-33 or rIL-1β (10 ng/ml) for 24 h. The cells were harvested and subjected to luciferase assay. Firefly luciferase activity was normalized with Renilla luciferase activity, and the luciferase activity of the untreated and unstimulated cells was given a reference value of 1. The data are shown as the means ± S.E. from four independent experiments. **, p < 0.01, IL-33 alone versus ST2 plus IL-33). The data of clone numbers 1-1-A-3 (EV/EL-4 cells), 1-2-G-12 (ST2L/EL-4 cells), and 3-2-A-8 (IL-1RI/EL-4 cells) are represented in each panel.
Suppression of IL-33 Signaling by Soluble ST2

Proteins in vivo, we utilized a murine model of asthma caused by OVA. BALB/c mice were sensitized with saline (SAL) or OVA and then challenged with an aerosol of SAL or OVA. Twenty-four hours after the last aeroallergen challenge, the concentration of soluble ST2 in sera was measured by sandwich ELISA, and the expression of ST2L in splenocytes was detected by flow cytometry. The level of soluble ST2 was predominantly elevated in OVA-sensitized and -challenged (OVA/OVA) mice (Fig. 4A). In contrast, the production of soluble ST2 in other groups was low and showed no significant difference. However, soluble ST2 was below the detectable level in bronchoalveolar lavage fluids of any groups in our sandwich ELISA system (data not shown). Moreover, the expression of ST2L in splenocytes was apparently induced in OVA/OVA mice. On the other hand, the expression of ST2L in SAL/OVA mice was as low as that in untreated mice (Fig. 4B and data not shown). These results indicate that the expressions of soluble ST2 and ST2L were specifically induced in asthmatic mice by the combination of sensitization and challenge with OVA.

In addition, we also examined the protein expression of IL-33 in asthmatic mice. So far, a detection system such as ELISA for secreted murine IL-33 has not been developed. Therefore, we tried to detect IL-33 protein by Western blotting using commercially available anti-mouse IL-33 antibody. Cellular extracts were prepared from the thymus of asthmatic mice because the expression of IL-33 mRNA was highly induced in the thymus (Fig. 5A). However, we could detect neither the precursor nor the mature IL-33 protein in this experiment.

Expression of ST2, ST2L, and IL-33 mRNAs after the OVA Challenge—To investigate the expressions of the ST2 and IL-33 genes in various tissues of OVA/OVA mice, we performed a reverse transcription-PCR analysis (Fig. 5). Expression of the ST2 gene was induced in the thymus, lung, lymph node, spleen, and ovary after the last OVA challenge. However, the expression in the brain, heart, liver, kidney, and skeletal muscle was low or absent, as was the case for the testis (Fig. 5A and data not shown). Although the expression of the ST2 gene in the stomach was detected, the level of expression was not altered before and after the OVA challenge (data not shown). Interestingly, biphasic expression of the ST2 gene was observed in the thymus, lung, lymph node, and spleen. The expression of the ST2 gene was increased at 3 h, dropped at 6 h, and then increased again until 12 h or 24 h (Fig. 5). In addition, the expression profile was different in female and male mice. Expression of the ST2 gene was gradually induced in the ovary after the OVA challenge, but not in the testis. In the expression of the IL-33 gene, pronounced biphasic expression was observed in the thymus and lung (Fig. 5). IL-33 mRNA was expressed in the lymph node, ovary, and testis; however, the expression was hardly observed in the spleen. These results indicate that the expression of the ST2 gene is induced in immunological response-associated tissues after the OVA challenge and that the expression of the IL-33 gene is also induced in several tissues of asthmatic mice.

Suppression of the Production of Th2 Cytokines from IL-33-stimulated Splenocytes—A previous study showed that IL-33 induces the production of Th2 cytokines (9). To study the effects of soluble ST2 on the biological activity of IL-33, we analyzed the production of Th2 cytokines from splenocytes of asthmatic mice. We first examined whether rIL-33 binds to splenocytes using flow cytometry (Fig. 6A). Splenocytes were prepared from SAL/OVA and OVA/OVA mice at 24 h after the last OVA challenge. Recombinant IL-33 apparently bound to ST2L-positive splenocytes prepared from OVA/OVA mice. Next, we analyzed the production of Th1 and Th2 cytokines. Fig. 6B shows a scheme for the stimulation of splenocytes. The splenocytes were stimulated with OVA for activation of lymphocytes. The OVA-stimulated splenocytes were either untreated or treated with ST2-FLAG for 3 h. Subsequently, the splenocytes were left unstimulated or stimulated with rIL-33 for 48 h, followed by harvest of culture supernatants. Stimulation with rIL-33 specifically induced the productions of IL-4, IL-5, and IL-13 from splenocytes of OVA/OVA mice, whereas the production of these cytokines was reduced by pretreatment with ST2-FLAG (Fig. 6C). On the other hand, the production of IFN-γ was increased according to the reduction of Th2 cytokine production in splenocytes of OVA/OVA mice. Although the production of IFN-γ from splenocytes of SAL/OVA mice was also induced by the addition of IL-33 alone or ST2-FLAG plus IL-33, the reasons are presently unclear. Where the splenocytes of OVA/OVA mice were unstimulated with OVA, IL-33-induced production of Th2 cytokines was low (data not shown). These results suggest that IL-33 induces the production of Th2...
cytokines from activated splenocytes via ST2L. Taken together, soluble ST2 suppresses the IL-33-induced production of Th2 cytokines.

**DISCUSSION**

This study has examined the regulation of IL-33 signaling by the soluble secreted form of the ST2 gene products (soluble ST2). We found that soluble ST2 has antagonistic effects on IL-33 signaling in allergic airway inflammation.

IL-33 is a member of the IL-1 cytokine family; the intracellular pathway of IL-33 signaling is similar to that of IL-1 signaling (9). We have studied IL-33 signaling using EL-4 cells stably expressing ST2L or IL-1RI (Figs. 1 and 3). IL-33 specifically bound to ST2L/EL-4 cells, but not to IL-1RI/EL-4 cells. The binding of IL-33 to ST2L induced the degradation of IκBα and subsequent activation of DNA binding activity of NF-κB. IL-33-induced DNA/NF-κB complex contained the p50 and p65 subunits. Furthermore, we found that soluble ST2 also bound to

**FIGURE 5. Expression of ST2, ST2L, and IL-33 mRNAs after the last OVA challenge.** A, reverse transcription-PCR analysis of expression of ST2, ST2L, and IL-33 mRNAs in tissues of asthmatic mice. DNase I-treated total RNAs were prepared from tissues of untreated mice (control; lanes C) and OVA/OVA mice at the indicated time shown above each lane (3–48 h) after the last OVA challenge and then were subjected to reverse transcription-PCR analysis. β-Actin was detected as an internal control. PCR products were separated on 2% agarose gels. The data show one of three independent experiments. B, kinetic analysis of expression of ST2, ST2L, and IL-33 mRNAs. Densitometric analysis was performed using the public domain NIH Image program. Expression of ST2, ST2L, and IL-33 mRNAs was normalized with that of β-actin mRNA, and expression of control mice was given a reference value of 1. The data are shown as arbitrary units (means ± S.E.) from three independent experiments. Nd, no data for low level of gene expression.
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![Image](https://i.imgur.com/5.jpg)

**FIGURE 6. Suppression of Th2 cytokine production from IL-33-stimulated splenocytes of asthmatic mice.**

A, binding analysis of rIL-33 to splenocytes by flow cytometry. Splenocytes were prepared from SAL/OVA and OVA/OVA mice at 24 h after the last OVA challenge. The splenocytes (1 × 10^6 cells) were mixed with rIL-33 (1 μg) for 1 h. The splenocytes were stained with biotinylated anti-T7 tag antibody, RPE-conjugated streptavidin, and FITC-conjugated anti-mouse T1/ST2 antibody. Percentages of IL-33-bound ST2L-positive splenocytes are shown as the means ± S.E. (n = 8 mice in SAL/OVA, n = 9 mice in OVA/OVA; *, p < 0.05, OVA/OVA versus SAL/OVA). B, schematic diagram for stimulation of splenocytes. The splenocytes were prepared from SAL/OVA and OVA/OVA mice at 24 h after the last OVA challenge. Stimulation of splenocytes was performed as described under "Experimental Procedures." After the culture supernatants were harvested, the concentrations of IL-4, IL-5, IL-13, and IFN-γ were measured by ELISA. C, levels of Th1 and Th2 cytokines in culture supernatants. White and black bars indicate data obtained from splenocytes of SAL/OVA and OVA/OVA mice, respectively. The data are shown as the means ± S.E. (n = 8 mice in SAL/OVA, n = 9 mice in OVA/OVA; **, p < 0.01, IL-33 alone versus ST2 plus IL-33 in splenocytes of OVA/OVA mice). ND, not detected.

IL-33 and that pretreatment with soluble ST2 suppressed the IL-33-induced NF-κB activation in ST2L/EL-4 cells (Figs. 2 and 3). Thus, our results indicate that the binding activity of IL-33 for ST2L was inhibited by the formation of an ST2/IL-33 complex, leading to the suppression of IL-33 signaling.

Several studies have shown that soluble forms of cytokine receptors function as positive or negative regulators in the expression of cytokines and growth factors. The soluble form of IL-1 receptor type II (sIL-1RII), IL-4 receptor α-chain (sIL-4Rα), and IL-13 receptor α-chain 2 (sIL-13Rα2) have antagonistic effects on IL-1, IL-4, and IL-13 signalings, respectively (20–22). In contrast, the soluble IL-6 receptor α-chain (sIL-6Rα) has agonistic effects on IL-6 signaling and modulates the expression of chemokines (23, 24). Soluble forms of cytokine receptors are generated by several mechanisms including alternative splicing of pre-mRNA and proteolytic cleavage of receptors (25). In the case of the ST2 protein, soluble ST2 is generated by alternative splicing of pre-mRNA, and its amino acid sequence is mostly consistent with that of the extracellular domain of ST2L (1, 3). Therefore, it was a reasonable result that soluble ST2 possessed binding activity for IL-33.

Besides soluble ST2 and ST2L, ST2V in humans and ST2LV in chickens have been reported as variant forms of the ST2 gene products (26–28). These variants might also be related to the regulation of IL-33 signaling because of containing the extracellular domain.

In patients and model mice of allergic asthma, the level of soluble ST2 was elevated in sera (14, 15), and the number of CD4-positive T cells expressing ST2L was increased in the lung and lymph nodes (29). On the other hand, the expression of IL-33 in allergic asthma is not investigated yet. In this study, we demonstrated that the expression of IL-33 mRNA was induced in several tissues after the OVA challenge in a murine model of asthma (Fig. 5). In addition, IL-33 bound to ST2L-positive splenocytes of asthmatic mice and induced the productions of IL-4, IL-5, and IL-13 (Fig. 6). Although we could not clarify a level and a time course for protein expression of IL-33, these results suggest that IL-33 expresses and functions as a cytokine in allergic asthma. Furthermore, we showed a detailed expression profile of the ST2 gene in asthmatic mice. The ST2 gene was predominantly expressed in the thymus, lung, lymph nodes, and spleen (Fig. 5). The expression of the ST2 gene in these tissues was regulated by the distal promotor (data not shown), which functions in Th2 cell-mediated immunological responses (16, 30). The biphasic expression pattern of ST2 mRNA corresponds to the production pattern of soluble ST2 in sera after the OVA challenge (15), suggesting that these tissues might be sources of soluble ST2. In addition, the level of soluble ST2 in sera was dramatically elevated in comparison with that of ST2L on the cell surfaces of splenocytes after the OVA challenge (Fig. 4). This drastic increase of the soluble ST2 concentration may contribute to the suppression of IL-33 signaling in vivo. In fact, pretreatment with soluble ST2 was effective for the suppression of IL-4, IL-5, and IL-13 productions from IL-33-stimulated splenocytes in asthmatic mice (Fig. 6). This negative effect on Th2 cytokine production was consistent with the results of therapeutic experiments using a recombinant soluble ST2-Fc protein or a soluble ST2 expression vector (7, 15). Thus, our results strongly support the paradigm that soluble ST2 neg-
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NF-κB is a key regulator in the IL-33 signaling pathway, although it remains unclear how NF-κB regulates the expression of Th2 cytokine genes. Previous studies using animal models have provided evidence that NF-κB plays an essential role in Th2 cell-mediated immunological responses. The p50-deficient (p50−/−) and c-Rel-deficient (c-Rel−/−) mice do not develop allergic airway inflammation (34, 35). Furthermore, p50 is required for the expression of transcription factor GATA-3, which regulates the expression of Th2 cytokine genes (36). Transgenic mice expressing dominant negative GATA-3 exhibit the inhibition of allergic inflammation (37). Hereafter, analysis of the downstream regulation of NF-κB is required to advance understanding of IL-33 signaling. In addition, the processing mechanism of the IL-33 protein has not been elucidated in vivo. A recent study reported that pre-IL-33 also functioned as a transcriptional repressor in nucleus besides acting as a cytokine (38). Therefore, the regulation for secretion of native IL-33 protein should be studied for better understanding of the biological and pathological functions of IL-33.

In conclusion, we showed the biological function of soluble ST2 in vitro and in vivo. We demonstrated that soluble ST2 suppresses the activation of NF-κB and the production of Th2 cytokines in IL-33 signaling, suggesting that this suppression leads to attenuation of allergic inflammatory responses in asthma. Furthermore, our findings may serve to advance knowledge in relation to the biological functions of IL-33 and therapeutic effects of soluble ST2 in allergic airway inflammation.

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