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Alternative splicing of interleukin-33 and type 2 inflammation in asthma

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Type 2 inflammation occurs in a large subgroup of asthmatics, and novel cytokine-directed therapies are being developed to treat this population. In mouse models, interleukin-33 (IL-33) activates lung resident innate lymphoid type 2 cells (ILC2s) to initiate airway type 2 inflammation. In human asthma, which is chronic and difficult to model, the role of IL-33 and the target cells responsible for persistent type 2 inflammation remain undefined. Full-length IL-33 is a nuclear protein and may function as an "alarmin" during cell death, a process that is uncommon in chronic stable asthma. We demonstrate a previously unidentified mechanism of IL-33 activity that involves alternative transcript splicing, which may operate in stable asthma. In human airway epithelial cells, alternative splicing of the IL-33 transcript is consistently present, and the deletion of exons 3 and 4 (Δ exon 3,4) confers cytoplasmic localization and facilitates extracellular secretion, while retaining signaling capacity. In nonexacerbating asthmatics, the expression of Δ exon 3,4 is strongly associated with airway type 2 inflammation, whereas full-length IL-33 is not. To further define the extracellular role of IL-33 in stable asthma, we sought to determine the cellular targets of its activity. Comprehensive flow cytometry and RNA sequencing of sputum cells suggest basophils and mast cells, not ILC2s, are the cellular sources of type 2 cytokines in chronic asthma. We conclude that IL-33 isoforms activate basophils and mast cells to drive type 2 inflammation in chronic stable asthma, and novel IL-33 inhibitors will need to block all biologically active isoforms.

interleukin-33 | alternative splicing | type 2 inflammation | asthma | basophils

Type 2 inflammation is a dominant mechanism of asthma (1), and interleukin 33 (IL-33), IL-25, and thymic stromal lymphopoietin (TSLP) are epithelial cell cytokines that regulate type 2 responses (2). IL-33 signals through the ST2 receptor (ST2L) (3, 4) and is inhibited by a soluble decoy variant of the receptor (sST2) (5, 6). Multiple cell types secrete type 2 cytokines in response to IL-33, including $CD4^+$ Th2 cells, basophils, mast cells, and innate lymphocyte type 2 (ILC2) cells (7–12). Type 2 airway inflammation occurs in many, but not all, asthmatics (1, 13) and persists chronically in patients with the Th2-high or type 2-high asthma endotype (14).

In mouse models of asthma, activation of ILC2s by IL-33 initiates type 2 airway inflammation (15–17). Much less is known about how IL-33 might regulate persistent type 2 inflammation in chronic stable type 2-high asthma. IL-33 is a nuclear protein without a signal sequence, and the mechanism of its extracellular release in the airway is poorly understood. In some settings, IL-33 is released from cell nuclei during necrosis and necroptosis (18), a process that might feature prominently during virus-induced asthma exacerbations (19). This mechanism is unlikely to operate in the airways of stable asthmatics. Another possibility is that alternative splicing of *IL33* mRNA produces proteins that localize to the cytoplasm and are capable of release from living cells (20, 21). In support of this idea, studies have demonstrated that extracellular IL-33 release is dependent on increases in intracellular calcium concentrations, which can be induced by exogenous ATP administration (22–24). Moreover, it is not yet clear which cell type responds to IL-33 to sustain persistent type 2 inflammation in the airway. Like ILC2s and Th2 cells, mast cells and basophils also express ST2L and secrete type 2 cytokines when activated by IL-33 (9, 25). Some studies have found an increase in ILC2s in human asthma (26, 27), whereas others have shown prominent gene signatures for mast cells and basophils in airway biospecimens from type 2-high asthmatics (13, 28, 29).

Here we explore the mechanisms of persistent airway type 2 inflammation in type 2-high asthma with a focus on the IL-33/ST2 axis. We examine alternative splicing of *IL33* in airway epithelial cells and explore which IL-33–responsive cells promote persistent type 2 inflammation in asthma.

Methods

Analysis of Banked Biospecimens in the University of California, San Francisco Airway Tissue Bank. To examine the cellular localization of IL-33 and to quantify *IL33* transcripts in the airway, we studied histological sections from airway mucosal biopsies and epithelial brushing RNA from healthy and asthmatic subjects in the University of California, San Francisco (UCSF) Airway Tissue Bank (ATB) (*SI Appendix*, Table S1). One-step PCR and molecular cloning and sequencing were used to identify alternatively spliced variants of *IL33*. RNase H-dependent

Significance

Type 2 inflammation occurs in a large subgroup of asthmatics and is the target of multiple novel therapies for asthma; however, the mechanisms that drive type 2 inflammation in chronic asthma are poorly understood. In this study, we identify a previously unidentified mechanism of IL-33 activity involving alternative RNA transcript splicing and provide evidence that mast cells and basophils are major cellular targets of IL-33 activity driving type 2 cytokine production in stable asthma. These data advance our understanding of the mechanisms of type 2-high asthma and guide the selection of asthmatics likely to benefit from IL-33 inhibitor therapies.

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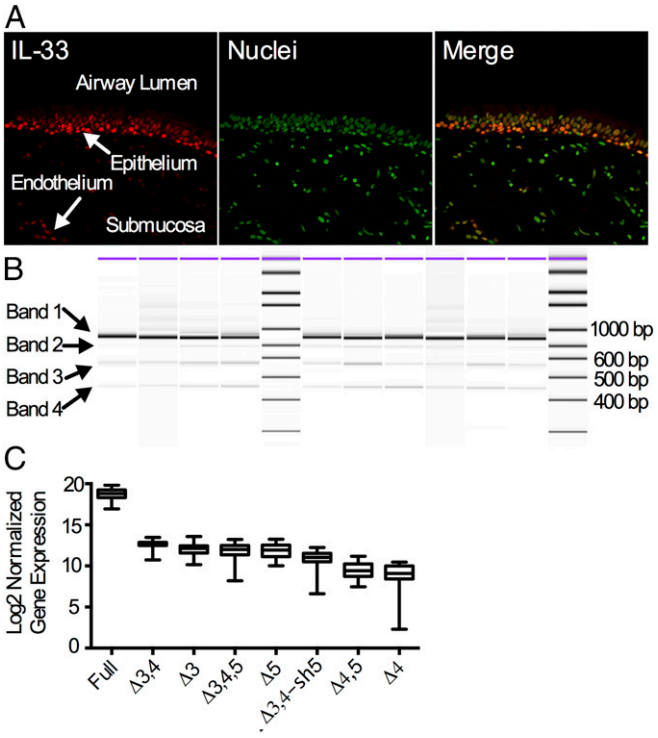


Fig. 1. Multiple *IL33* splice variants are expressed in human airway epithelial brushings. (*A*) IL-33 immunolocalizes to epithelial and endothelial cells in the submucosa. The image is from tissue sections of endobronchial biopsies taken from a representative healthy subject. (*B*) Multiple alternatively spliced transcripts of *IL33* are detectable in airway epithelial RNA from healthy subjects ($n = 10$). Exons 2–8 of *IL33* were reverse-transcribed, PCR-amplified, and analyzed on an Agilent 2100 Bioanalyzer. Splicing out of some or all of exons 3, 4, and 5 are revealed by cloning and sequencing of cDNA generated from airway epithelial cell RNA from human subjects (*SI Appendix*, Fig. S2). (*C*) By using RNase H-dependent primers, full-length *IL33* is the most abundant transcript in airway epithelial brushings, but multiple *IL33* isoforms are also highly expressed. The data are from airway epithelial brushings from 39 healthy controls and are corrected for primer efficiency (median ± interquartile range).

quantitative PCR (qPCR) was used to quantitatively measure *IL33* spliced transcripts (30). Details about subject characterization, immunostaining, and PCR are provided in *SI Appendix*.

In Vitro Characterization of IL-33 Isoforms. To assess the function of IL-33 isoforms, full-length and alternatively spliced *IL33* cDNA were cloned into CMV promoter-driven mammalian expression vectors with and without fluorescent protein tags or EF1α promoter-driven lentiviral mammalian expression vectors with a fluorescent protein tag. Tagged and untagged CMV promoter-driven plasmids were transfected into the cultured human primary epithelial cells to determine cellular localization. IL-33 isoforms were translated in vitro by using CMV promoter-driven plasmids or expressed in *Escherichia coli* and used to determine cytokine bioactivity. EF1α promoter-driven mammalian lentiviral expression vectors were used to create stable Beas2B-overexpressing epithelial cell lines to study IL-33 secretion. Details of the cell culture methods, immune assays, and the IL-33 bioactivity assay are provided in *SI Appendix*.

Analysis of Cells in Induced Sputum. To characterize ST2-bearing cells in the airway, we applied FACS and qPCR to prospectively collected samples of induced sputum from asthmatic subjects and healthy controls (*SI Appendix*, Table S2). We also stimulated sputum cells with cytokines in vitro and analyzed the response using RNA sequencing. Protocol visit details are described in *SI Appendix*, as are methods of sputum cell FACS, PCR, and stimulation.

Study Approval. All subjects provided written informed consent for the study in which they originally participated and also provided written informed consent for their biospecimens to be placed in the UCSF ATB for studies in addition to the original protocol. All studies and UCSF ATB procedures were reviewed and approved by the UCSF Committee on Human Research.

Statistical Analysis. Two group comparisons were made with the paired Student *t* test, and three or more group differences were assessed by using ANOVA with Bonferroni corrections for multiple comparisons. qPCR gene expression data were normalized and log2 transformed. Linear regression models were used to assess the relationship between different biologic splice variants of IL-33 and airway type 2 inflammation. Models were constructed with adjustment for age, gender, and inhaled corticosteroid use. Regression models were performed by using robust SEs. Simple correlations were made with Spearman's rank order correlation. Flow-cytometric data were analyzed by nonparametric *t* test because of the nonnormal distribution, and data are displayed as median ± interquartile range. All statistical analyses were performed in either STATA/SE (Version 11.0) or GraphPad Prism (Version 5.0d).

Results

IL-33 Localizes to Epithelial Cells and Endothelial Cells in the Airway. We examined protein expression of IL-33 using immunofluorescence in sections of airway biopsies collected during research bronchoscopy. We found that IL-33 localizes predominantly to epithelial and endothelial cells within the airway (Fig. 1*A* and *SI Appendix*, Fig. S1*A*).

IL-33 Splice Variants Are Expressed in Human Airway Epithelial Cells. Previous studies in cell lines, primary human keratinocytes, fibroblasts, and endothelial cells indicated that exons 3, 4, and 5 can be alternatively spliced out of the *IL33* transcript (20). To evaluate *IL33* splicing patterns in human airway epithelial cells, we generated cDNA amplicons spanning exons 2–8 using RNA extracted from epithelial brushings from human subjects. Four distinct PCR product bands were expressed in all samples (Fig. 1*B*). We cloned and sequenced species from each band and found 10 splice variants corresponding to alternative splicing of some or all of exons 3, 4, or 5 (*SI Appendix*, Fig. S2). The largest band (~861 bp) contained full-length *IL33* and amplicons using an alternative splice site between exons 4 and 5, resulting in exclusion of the first 18 nucleotides of exon 5 (Δshort5). The second band (~698 bp) included species with exon 3, exon 4, exon 5, or exon 3 and the first 18 nucleotides of exon 5 spliced out. The amplicons are of similar size because exons 3, 4, and 5 are each 126 bp. The third band (~575 bp) included amplicons with exons 3 and 4, exons 4 and 5, or exon 3 and 4 and the first 18 nucleotides of exon 5 spliced out. The smallest band (~460 bp) contained an amplicon with exons 3, 4, and 5 spliced out. We quantified the expression of eight variants in airway epithelial brushings from 39 healthy subjects using RNase H-dependent primers overlying splice junctions (Fig. 1*C* and *SI Appendix*, Fig. S3*A*). Each assay was specific for and linearly amplified its target (*SI Appendix*, Figs. S3*B* and S4). After correcting for primer efficiency, we found that full-length *IL33* mRNA was the most highly expressed variant, followed by Δexon 3,4; Δexon 3; Δ exon 3,4,5; and Δ exon 5 (Fig. 1*C*).

Splicing of Exons 3 and 4 Results in Cytoplasmic Localization of IL-33. To determine whether IL-33 splice variant proteins localize to different cellular compartments, we overexpressed the 10 variants in primary human airway epithelial cells using constructs tagged with a fluorescent protein. Full-length IL-33 localized exclusively to the cell nucleus (Fig. 2*A*). In contrast, Δ exon 3,4 localized to the nucleus and the cytoplasm (Fig. 2*A*). Deletion of either exon 3 or 4 alone resulted in localization predominantly in the nucleus (Fig. 2*A*). The Δ exon 3,4,5 colocalized with an autophagosome marker (LC3B) (Fig. 2*A*). The cellular localization of untagged proteins was similar to that of the tagged proteins (*SI Appendix*, Fig. S5).

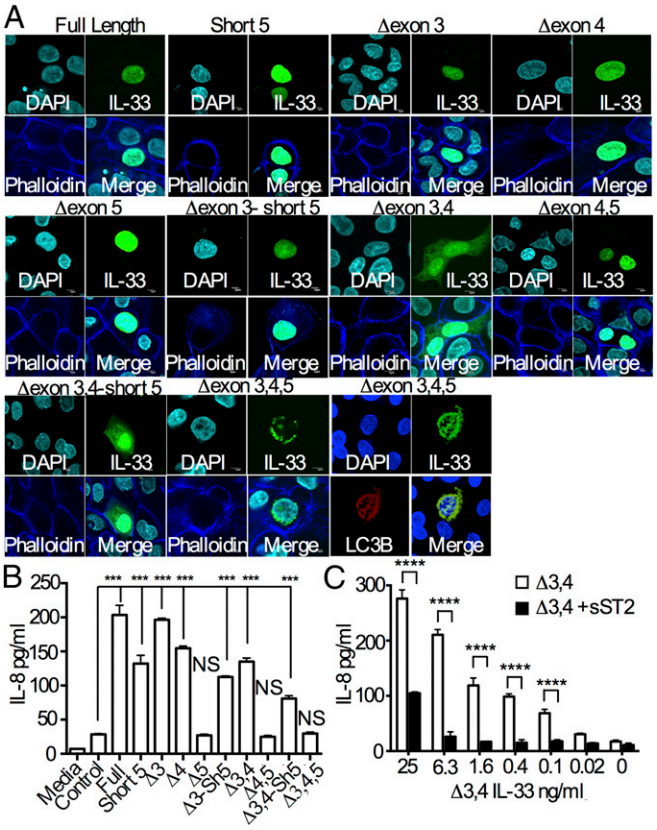


Fig. 2. Deletion of exons 3 and 4 confers cytoplasmic localization of IL-33 in primary airway epithelial cells, and exon 5 is required for cytokine activity. (*A*) Full-length and alternatively spliced *IL33* cDNA were cloned into CMV-driven mammalian expression vectors tagged with venus fluorescent protein, and plasmids were transfected into primary human airway epithelial cells. Full-length, short 5, Δ exon 3, Δ exon 4, Δ exon 5, Δ exon 3–short 5, and Δ exon 4,5 isoforms localized to the nucleus, whereas the Δ exon 3,4 and Δ exon 3,4–short 5 isoforms localized to both the nucleus and the cytoplasm. The Δ exon 3,4,5 isoform demonstrates a punctate, perinuclear staining, which colocalized with the autophagosome marker LC3B. (*B*) In vitro-translated IL-33 protein isoforms show differences in signaling activity in HMC-1 cells. Whereas isoforms containing exon 5 cause release of IL-8, proteins with a deletion in exon 5 do not. (*C*) Recombinant purified Δ exon 3,4 IL-33 protein demonstrates cytokine bioactivity in HMC-1 cells (EC_{50}: 0.8–1.1 ng/mL). The activity of Δ exon 3,4 IL-33 in HMC-1 cells is inhibited by sST2. Data in *B* and *C* represent mean ± SD for three replicates for each experimental condition. ***$P < 0.001$; ****$P < 0.0001$.

Exon 5 Is Required for IL-33 Cytokine Activity. To determine whether IL-33 splice variants retain their ability to signal via the ST2 receptor, we generated proteins using in vitro transcription and translation and examined signaling in a mast cell line (HMC-1). All IL-33 isoforms produced proteins (*SI Appendix*, Fig. S2*B*). Full-length IL-33 protein demonstrated cytokine activity, as did Δ exon 3, Δ exon 4, and Δ exon 3,4 splice variants, but IL-33 variants missing exon 5 did not (Fig. 2*B*). This result is consistent with reports that exon 5 encodes amino acid residues that are critical for binding of IL-33 to ST2 (31). We purified Δ exon 3,4 protein (*SI Appendix*, Fig. S6) and found that it signals in a dose-dependent manner and is inhibited by recombinant sST2 (Fig. 2*C*). We demonstrate that Δ exon 3,4 protein binds avidly to recombinant ST2-Fc receptor with a K_D of 0.25 nM (*SI Appendix*, Fig. S7*A*), and stimulation of primary human basophils with Δ exon 3,4 in combination with IL-3 results in an increase of IL-13 and -5 protein production (*SI Appendix*, Fig. S7 *B* and *C*).

The Δ Exon 3,4 Splice Variant Correlates with Airway Type 2 Inflammation. To determine whether biologically active forms of IL-33 are related to airway type 2 inflammation, we measured *IL33* splice variant transcripts in epithelial brush RNA from 85 mild to moderate asthmatics with stable disease (*SI Appendix*, Table S1). These subjects previously had gene expression measured for *periostin*, *CLCA1*, and *serpinB2*, which are markers of airway epithelial cell activation by IL-13 (28, 32) that can be combined into a single "three-gene-mean" (TGM) metric (32). Among the biologically active IL-33 splice variants, we found that Δ exon 3,4 was the only variant significantly associated with the TGM (Fig. 3 and *SI Appendix*, Table S3). Because the TGM is decreased by inhaled corticosteroids (32), we adjusted for corticosteroid use and found that the relationship remained significant (Fig. 3 and *SI Appendix*, Table S3). Full-length *IL33* was not associated with the TGM. We also measured IL-33 protein in the bronchoalveolar lavage fluid from a subset of these subjects ($n = 51$) and found that IL-33 levels were below the limit of detection (1.7 pg/mL) in the majority of the samples.

The Δ Exon 3,4 Splice Variant Is Actively Secreted from an Airway Epithelial Cell Line. To further explore the role of IL-33 as an extracellular cytokine, we used lentiviral plasmids to stably overexpress full-length IL-33 and Δ exon 3,4 fused with GFP in Beas2B cells (a transformed, nontumorigenic human bronchial epithelial cell line). We found that Beas2B cells expressed very little IL-33 transcript or protein under baseline conditions and that stable overexpression resulted in increased full-length and Δ exon 3,4 RNA transcripts (Fig. 4*A*). Notably, full-length IL-33 protein was more abundant than Δ exon 3,4 protein in the intracellular lysates (Fig. 4*B*). Although we were unable to detect IL-33 peptides in our cell culture-conditioned medium, we did detect the GFP fusion tag in the conditioned medium of both the full-length IL-33–GFP- and Δ exon 3,4–GFP-overexpressing cells (Fig. 4*C*) without evidence of cellular necrosis (Fig. 4*D*). In both unstimulated cells and ionomycin-stimulated cells, we found that the concentration of extracellular GFP in the conditioned medium of Δ exon 3,4-GFP cells was significantly higher than in full-length IL-33-GFP cells (Fig. 4*E*).

Expression of the ST2 Receptor (ST2L) Correlates with Expression of Type 2 Cytokines and Mast Cell/Basophil Genes in Sputum. IL-33 activates ST2 receptor-bearing cells to stimulate the production of type 2 cytokines, but the specific cells driving the production in type 2-high asthma are unknown. We previously found that gene expression for type 2 cytokines is increased in asthmatic sputum cells (29). Here we measured gene expression for the ST2 receptor

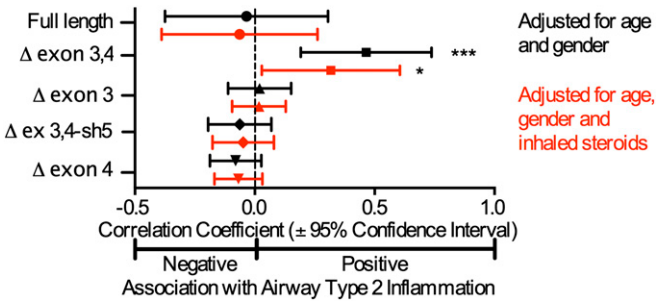


Fig. 3. The Δ exon 3,4 *IL-33* transcript is associated with type 2 airway inflammation. A forest plot shows the odds ratios (*x* axis) for the association between the expression of 5 different IL-33 isoforms (*y* axis) in airway epithelial cells and airway type 2 inflammation in 85 stable asthmatic subjects. The type 2 inflammation outcome is based on a composite metric of three IL-13–responsive genes in airway epithelial cells (*periostin*, *CLCA1*, and *serpinB2*). The plot shows that only the Δ exon 3,4 *IL33* isoform is significantly and positively associated with airway type 2 inflammation. Data presented in black are adjusted for age and gender, and data presented in red are adjusted for age, gender, and use of inhaled steroids. Δ ex 3,4–sh5 refers to Δ exon 3,4–short 5. *$P < 0.05$; ***$P < 0.001$.

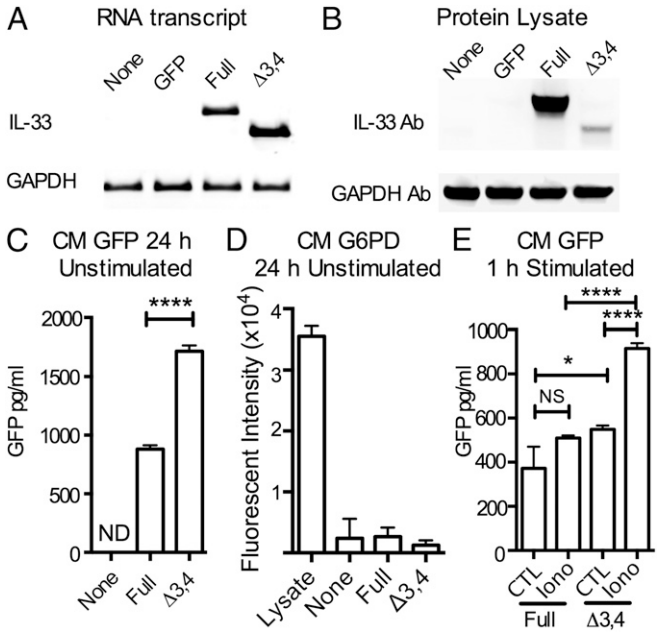


Fig. 4. The Δ exon 3,4 IL-33 splice variant is secreted from an airway epithelial cell line. (*A*) Stable overexpression of full-length IL-33 fused with GFP (full), or Δ exon 3,4 IL-33 fused with GFP (Δ3,4) in Beas2B cells using lentiviral transduction results in increased full-length and Δ exon 3,4 RNA transcripts. None, untransduced Beas2B cells; GFP, overexpression of GFP alone. (*B*) Despite similar levels of transcript overproduction, full-length IL-33 is more abundant in the cellular lysate than Δ exon 3,4 IL-33. (*C*) At 24 h, GFP is present in the conditioned medium (CM) of the cells expressing full-length IL-33–GFP, but it is more robust in the cells expressing Δ exon 3,4 IL-33–GFP. (*D*) There is no evidence of cellular necrosis in untransduced cells, full-length IL-33–GFP, and Δ exon 3,4 IL-33–GFP cell lines as measured with glucose-6-phosphate dehydrogenase (G6PD) release assay. Beas2B total protein lysate (60 μg) demonstrates a positive G6PD signal. (*E*) After 1-h stimulation with 5 μM ionomycin (Iono), there was an increase in extracellular GFP in cells expressing Δ exon 3,4 IL-33–GFP, but not those expressing full-length IL-33–GFP. $*P < 0.05$; $****P < 0.0001$.

(*ST2L*) in induced sputum cells from asthmatics and healthy controls (*SI Appendix*, Table S2) and found that *ST2L* expression is markedly increased in asthmatics (*SI Appendix*, Fig. S8) and is tightly correlated with a composite metric of *IL4*, *IL5*, and *IL13* gene expression (Fig. 5*A*). Notably, carboxypeptidase A3 (*CPA3*), a human mast cell and basophil protease, and tryptase, a mast cell protease, are also very strongly correlated with *ST2L* expression in sputum cells (Fig. 5*A*), suggesting that mast cells and basophils, expressing the ST2 receptor, are the cells in the asthmatic airway that produce type 2 cytokines.

Basophils Are Increased in Induced Sputum from Asthmatics. We used flow cytometry to examine innate and adaptive immune cells known to express ST2 in induced sputum cells. We defined populations of $CD3^+CD4^-$ and $CD3^+CD4^+$ T cells, basophils ($Lin^-FcεR1^+$), and ILC2s ($Lin^-CD3^-CD4^-$ $FcεR1^-ST2^+CD25^+$) in peripheral blood mononuclear cells (PBMCs) collected from asthmatic and healthy subjects (*SI Appendix*, Table S2 and Fig. S9*A*). We used fluorescence-minus-one controls to identify positive ST2 staining (Fig. 5*B*). ST2 was observed on all of our defined cell populations, but was strongest in basophils ($Lin^-FcεR1^+$) (Fig. 5*B*), which also expressed CD123 (*SI Appendix*, Fig. S10). We performed flow cytometry on concomitantly collected sputum samples and used the gating strategy defined in PBMCs to identify immune cell subsets in sputum (*SI Appendix*, Fig. S9*B*). We found that ILC2s are rare and not increased in number in asthmatic subjects, whereas basophils are more abundant in asthmatic than in healthy subjects (Fig. 5*C*). Although our gating strategy did not allow us to identify mast cells, we performed additional analysis of sputum cells in a smaller group of asthmatic subjects to confirm the presence of mast cells. We found Lin^+cKit^+ cells in sputum that strongly express ST2, consistent with mast cells (*SI Appendix*, Fig. S10 *B* and *C*), but these mast cells are much less abundant than basophils (*SI Appendix*, Fig. S10 *D* and *E*).

Transcriptomic Analysis Reveals Basophils and Mast Cells as the Target of IL-33 Activity in Asthma. If persistent type 2 inflammation depends on basophils and mast cells, then sputum cells that show type 2 responses after IL-33 stimulation should be enriched in basophils and mast cells. We activated sputum cells from 12 asthmatic donors using a combination of IL-33 (signaling peptide 112–270 amino acids) and TSLP (Fig. 6*A*), because these cytokines synergize in stimulating type 2 cytokine secretion from $ST2^+$ cells (33). Sputum cells from 6 of the 12 asthmatics showed

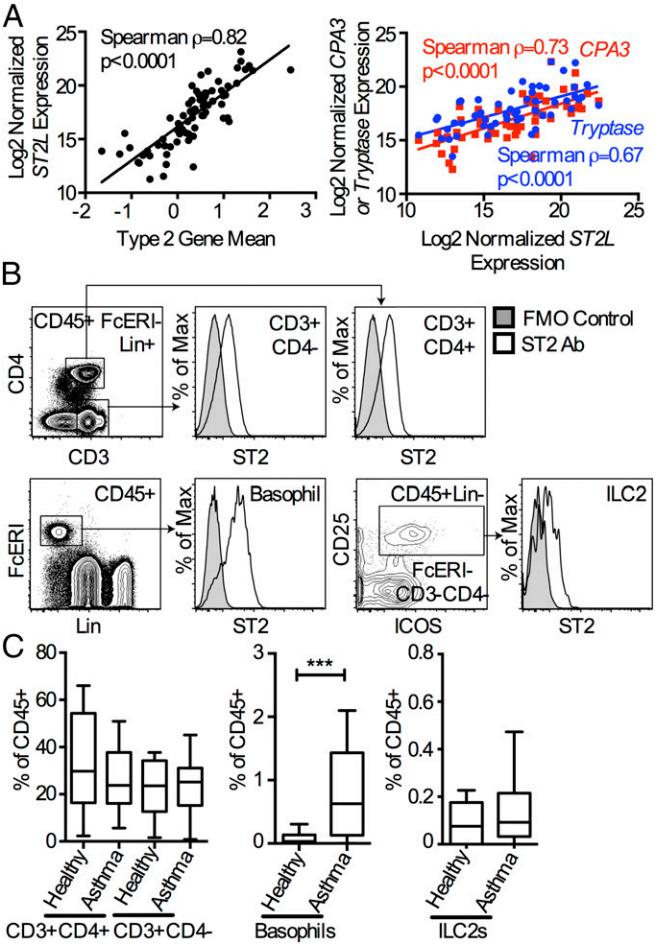
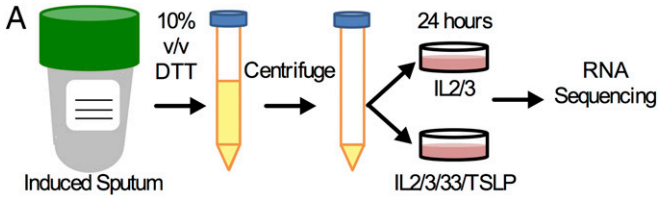
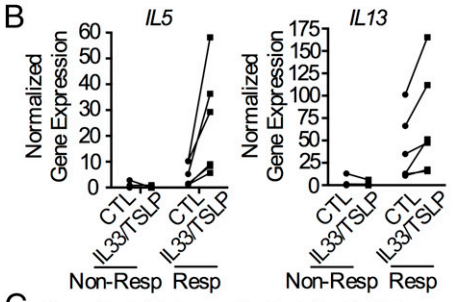


Fig. 5. Sputum cell analysis links basophils and mast cells to type 2 inflammation. (*A*) The expression of the IL-33 receptor (*ST2L*) in sputum cells from asthmatics ($n = 55$), is tightly correlated with a combined metric, type-2-gene-mean, that represents the expression of *IL4*, *IL5*, and *IL13*. *ST2L* expression in sputum cells is strongly correlated with expression of mast cell and basophil proteases, carboxypeptidase A3 (*CPA3*), and *tryptase* among asthmatics. (*B*) ST2 is highly expressed on basophils in PBMCs from healthy and asthmatic subjects (one representative plot from healthy subject is shown). White histogram indicates surface ST2 staining, whereas gray indicates fluorescence-minus-one FMO control. (*C*) Basophil numbers are significantly higher in induced sputum cells from asthmatic subjects ($n = 28$) than in healthy controls ($n = 16$); the numbers of $CD3^+CD4^-$, $CD3^+CD4^+$, and ILC2 cells are not significantly different between groups. Data are presented as Tukey boxplots with median ± IQR. $***P < 0.001$.





C Gene Set Enrichment Analysis by Cell Type

Immune Cell	Resp P-value	Non-resp P-value	Set Size
Mast cell/Basophil	0.0055	0.9945	95
Eosinophil	0.0787	0.9213	38
B Cell	0.0005	0.9995	38
Macrophage	0.9999	0.0001	142
Neutrophil	0.9962	0.0038	137
NK cell	0.0948	0.9052	12

Fig. 6. Transcriptomic analysis reveals basophils and mast cells as the target of IL-33 activity in asthma. (*A*) We performed in vitro stimulation of sputum cells with IL-2 and -3 (CTL) or IL-2, -3, -33, and TSLP (IL33/TSLP) from 12 asthmatic subjects. After 24 h, cells were collected, RNA was extracted, and RNA sequencing was performed. (*B*) Subjects were classified as responders (Resp) ($n = 6$) if the gene expression of *IL5* and *IL13* increased after stimulation. All others were classified as nonresponders (Non-Resp). (*C*) Gene set enrichment analysis reveals that the sputum cell transcriptomes of IL-33 responders are enriched in mast cell/basophil and B-cell gene transcripts, whereas IL-33 nonresponders are enriched in macrophage and neutrophil gene transcripts.

increased *IL5* and *IL13* gene expression after IL-33/TSLP activation ("responders"), whereas 6 donors showed no increase ("nonresponders") (Fig. 6B). *IL4* expression was undetectable in most samples. Comparing the gene expression profiles of the responder and nonresponder sputum cells under control conditions, we found that mast cell and basophil genes (*CPA3*, *GATA2*, *HDC*, and *KIT*) were prominent among the 94 differentially expressed genes (Dataset S1), which separate the two groups in cluster analysis (*SI Appendix*, Fig. S11). Using the Human Immune Cell Transcriptome dataset (National Center for Biotechnology Information Gene Expression Omnibus), we compared the transcriptomes of six human immune cell types to the transcriptome of our responders or nonresponders in the control condition. Using gene set enrichment analysis, we found that the IL-33 responders were enriched in transcripts found in mast cells/basophils as well as B cells (Fig. 6*C* and *SI Appendix*, Fig. S12*A*). The IL-33 nonresponders were enriched in transcripts found in macrophages and neutrophils (Fig. 6*C* and *SI Appendix*, Fig. S12*B*).

Discussion

In this study, we explored the mechanisms underlying persistent type 2 inflammation in asthma with a focus on the IL-33/ST2 axis. We show that a splice variant of IL-33 missing exons 3 and 4 localizes to the cytoplasm of airway epithelial cells, retains signaling capacity, is actively secreted from epithelial cells, and is strongly associated in asthma with an airway gene expression signature of type 2 cytokine activity. Among ST2-bearing sputum cells, we found that basophils are increased in asthma, and sputum cells that have a type 2 cytokine response to IL-33 in vitro have a gene profile that is enriched in genes typical of basophils and mast cells. We propose a model in which persistent type 2 inflammation in asthma results from activation of basophils and mast cells by alternatively spliced variants of IL-33 in addition to other epithelial cytokines (TSLP and IL-25).

Full-length IL-33 contains both nuclear localization signals and a chromatin-binding motif that explain its prominent nuclear localization (34, 35). Extracellular release of full-length IL-33 may occur when cells undergo necrosis and necroptosis (18), but excessive cellular necrosis is unlikely in the airways of stable asthmatics (13, 28, 29). We considered the possibility that release of IL-33 in type 2-high asthma might involve alternatively spliced *IL33* transcripts that produce proteins stored in the cytoplasm of epithelial cells. It is known that alternative splicing of *IL33* mRNA produces proteins that localize to the cytoplasm (20), but the biological activity of these splice variants and their regulation in airway disease have not been demonstrated. We found that deletion of exon 5 rendered IL-33 isoforms incapable of signaling via the ST2 receptor. Moreover, we found that deletion of exons 3 and 4 is required to confer cytoplasmic localization in primary airway epithelial cells. Among the five IL-33 isoforms that we found to be capable of cytokine signaling, only Δ exon 3,4 was strongly associated with a gene expression signature of type 2 cytokine activity in the airway in asthma. Finally, we show that, although both full-length and Δ exon 3,4 protein variants were secreted from the Beas2B cell line in the absence of cell death, the Δ exon 3,4 variant was secreted more abundantly, especially in ionomycin-stimulated cells. Literature has shown that the secretion of IL-33 from living primary airway epithelial cells is calcium-dependent (22–24). Our finding linking cytoplasmic localization and secretion of IL-33 protein to airway type 2 cytokine activity is supported by in vitro and in vivo mouse studies. Deletion of the N terminus of IL-33 results in cytoplasmic localization in HEK-293T cells (36), and a similar N-terminal deletion causes high systemic IL-33 levels and widespread eosinophilia in mice (37). Mechanisms that confer cytoplasmic localization, such as alternative splicing, are likely to be critical for IL-33 extracellular release. It is notable that we were unable to detect extracellular IL-33 peptides in either cell culture medium or lavage fluid, although we were able to detect extracellular GFP when we fused this tag to the IL-33 protein. Many factors may contribute to the inability to detect extracellular IL-33, including binding of IL-33 by soluble ST2, IL-33 alterations such as oxidation (38), or proteolytic degradation (39). These factors likely function to inactivate IL-33 signaling and limit the effects of this potent cytokine. Together, our findings that Δ exon 3,4 variant is localized to the cytoplasm and actively secreted into the extracellular space, has cytokine activity, and is associated with type 2 inflammation in asthma suggest that this variant acts as a secreted cytokine in living cells to drive type 2 inflammation. The association between airway expression of the Δ exon 3,4 variant and type 2 inflammation remained significant, despite controlling for the use of inhaled steroids. Previous reports suggest that lung IL-33 expression and secretion may be resistant to steroid treatment (40, 41), a finding that may relate directly to Δ exon 3,4 variant expression. An important implication of our findings is that asthma treatments that aim to directly inhibit IL-33 cytokine activity will need to be designed to block this Δ exon 3,4 variant. Such therapeutics may be particularly beneficial in a population of steroid-resistant asthmatics.

We provide several lines of evidence that basophils and mast cells produce type 2 cytokines in chronic type 2-high asthma. In qPCR studies, we showed that the expression of type 2 cytokine genes in sputum cells from asthmatics is tightly correlated with the expression of *ST2L* and that *ST2L* expression is very strongly correlated with the expression of tryptase and CPA3 (proteases whose expression is restricted to basophils and mast cells). In

FACS studies, we showed that basophils (but not $CD4^+$ T cells or ILC2 cells) are increased in sputum in asthma. Our FACS strategy did not allow us to comprehensively evaluate the relative abundance of mast cells in asthmatic compared with healthy subjects; however, we documented that mast cells are present, but rare, in the sputum of asthmatics. Consistent with this finding, we previously identified mast cells in airway biopsies using immunohistochemistry and found that they interdigitate within the epithelium and are more abundant in patients with the Th2-high endotype (42). Finally, we show that asthmatics whose sputum cells have a type 2 cytokine response to IL-33 in vitro also have a gene transcript profile enriched in the expression of genes found in basophils, mast cells, and B cells. This basophil and mast cell gene profile includes expression of proteases (tryptase and CPA3), transcription factors (GATA-2), and surface receptors (ST2L, CRLF2/TSLPR, and MS4A2). Although these markers cannot distinguish between mast cells and basophils, they provide support for the conclusion that mast cells and basophils are the key ST2-bearing cells that produce type 2 cytokines in response to IL-33. The enrichment of B cells in this group of asthmatics may reflect the overlapping role of IgE-dependent immune responses (43, 44). Finally, the presence of macrophage and neutrophil transcripts in the airways of asthmatics who do not demonstrate type 2 responses to IL-33 stimulation is notable. Although subgroups of asthmatics with neutrophilic airway inflammation have been described (45, 46), it will be important to understand whether these or other associated cell types may be suppressing type 2 responses and promoting alternative inflammatory pathways in asthma. Nevertheless, our study in chronic human asthma implicates alternative splicing of IL-33 as well as basophils and mast cells in the maintenance of airway type 2 inflammation. Therapeutics targeting the IL-33 and the type 2 axis will likely benefit subjects with an accumulation of these cell types.

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