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Redox-dependent activation of Src kinase mediates epithelial IL-33 production and signaling during acute airway allergen challenge

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Abstract

The respiratory epithelium forms the first line of defense against inhaled pathogens, and acts as an important source of innate cytokine responses to environmental insults. One critical mediator of these responses is the IL-1 family cytokine, IL-33, which is rapidly secreted upon acute epithelial injury as an alarmin and induces type 2 immune responses. Our recent work highlighted the importance of the NADPH oxidase dual oxidase 1 (DUOX1) in acute airway epithelial IL-33 secretion by various airborne allergens, associated with H_2O_2 production and redox-dependent activation of Src kinases and epidermal growth factor receptor (EGFR) signaling. Here, we show that IL-33 secretion in response to acute airway challenge with house dust mite (HDM) allergen critically depends on the activation of Src by a DUOX1-dependent oxidative mechanism. Intriguingly, HDM-induced epithelial IL-33 secretion was dramatically attenuated by siRNA- or antibody-based approaches to block IL-33 signaling through its receptor IL1RL1(ST2), indicating that HDM-induced IL-33 secretion includes a positive feed-forward mechanism involving ST2-dependent IL-33 signaling. Moreover, activation of type 2 cytokine responses by direct airway IL-33 administration was associated with ST2-dependent activation of DUOX1-mediated H_2O_2 production and redox-based activation of Src and EGFR, and was attenuated in *Duox1*$^{-/-}$ and *Src*$^{+/-}$ mice, indicating that IL-33-induced epithelial signaling and subsequent airway responses involve DUOX1/Src-dependent pathways. Collectively, our findings suggest an intricate relationship between DUOX1, Src and IL-33 signaling in the activation of

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innate type 2 immune responses to allergens, involving DUOX1-dependent epithelial Src/EGFR activation in initial IL-33 secretion and in subsequent IL-33 signaling through ST2 activation.

Keywords

NADPH Oxidase; DUOX1; Cysteine; Sulfenic acid; EGFR; Innate immune response; Type 2 inflammation

Introduction

The airway epithelium plays an essential role in respiratory function as a first-line defense against inhaled pathogens (1, 2). Additionally, the respiratory epithelium plays a crucial role in signaling processes and mediator production to aid in tissue regenerative processes following injury (2, 3). Among the key initial features of these innate epithelial responses is the rapid production of IL-1 family cytokines, particularly the alarmin cytokine IL-33 (4) which critically contributes to the activation of type 2 immune responses that are important for tissue regeneration due to e.g. parasitic infections or other forms of injury (5, 6) but have also been strongly associated with development of allergic diseases such as asthma (7, 8).

IL-33 is a member of the IL-1 cytokine family that is constitutively expressed in epithelial cells in barrier tissues as a chromatin-bound nuclear factor, and additionally functions as an extracellular cytokine (4, 9–11) as the only known ligand for the heterodimeric IL1RL1 (ST2) receptor expressed on many effector cells (12). Following damage to the epithelium, IL-33 can be released either passively due to epithelial necrosis or actively through secretion pathways that are incompletely understood (13). Released IL-33 then binds the ST2 receptor on various target cells, forming an intracellular signaling complex (involving e.g. MyD88 and TRAF6), inducing type 2 immune responses and other various inflammatory processes (14, 15). Activation of IL-33 cytokine function also involves its proteolytic processing to a more potent, truncated 18 kD form (16), by exogenous proteases released from inflammatory cells or proteases associated with various allergens (17, 18). Extracellular IL-33 activity can also be regulated by its binding to a soluble truncated form of ST2 that acts as a decoy receptor for IL-33 (19), or through cysteine oxidation within IL-33 to an intracellular disulfide which reduces its cytokine function (20). We and others have recently shown that IL-33 secretion from the respiratory epithelium in response to various airborne allergens is mediated by an active signaling mechanism initiated by early damage signals such as ATP, activation of Ca^{2+}-dependent signaling, and H_2O_2 production by the NADPH oxidase family member dual oxidase 1 (DUOX1), which subsequently results in redox-dependent activation of cell signaling pathways, including epidermal growth factor receptor (EGFR) signaling (21–23). Activation of EGFR signaling is well-known to contribute to reparative and secretory processes within the airway epithelium (24, 25), and has also been implicated in allergic asthma (26). Recent studies have shown that redox-dependent regulation of EGFR involves increased kinase activity and autophosphorylation due to oxidation of a cysteine in its ATP-binding region to a sulfenic acid (27, 28). However, DUOX1-mediated redox regulation of EGFR appears to occur largely at the level of EGFR transactivation by initial activation of G-protein coupled receptors such as P2YR2 and

intermediate non-receptor kinases such as the Src kinase, a proto-oncogene involved in wound repair and cancer (25, 29). Indeed, we recently observed that DUOX1 also mediates allergen-induced activation of Src within the airway epithelium (21), but the importance of Src in allergen-induced IL-33 secretion or related responses has not yet been established.

Src is an extensively studied non-receptor tyrosine kinase that is involved in many critical cellular processes, such as cell migration, proliferation, and survival (30, 31). As a non-receptor kinase, Src activity is regulated by allosteric mechanisms and conformational changes resulting in the “unclamping” and activation of its tyrosine kinase domain, which is further enhanced by autophosphorylation at Tyr 416 within the kinase activation loop (32–35). Emerging findings also suggest that Src function is regulated by oxidation of one or more of its cysteines (36), and our recent studies have shown that Src activity can be enhanced by direct oxidation of two of its cysteines, Cys185 in its SH2 domain and Cys277 near the ATP-binding region of the kinase domain (37). The relevance for these oxidative events for allergen-induced IL-33 secretion and activation of type 2 immune responses is, however, still unclear.

The present studies were designed to specifically address the importance of DUOX1-dependent Src activation in acute IL-33 secretion and activation of downstream type 2 responses induced by the asthma-relevant allergen, house dust mite (HDM). In the process of these studies, we uncovered an unanticipated autoamplification mechanism in which IL-33 appears to promote its own secretion by activating ST2-dependent signaling within the epithelium. Following this, we now demonstrate that DUOX1-dependent oxidative Src activation represents an important component of IL-33-dependent signaling and activation of type 2 cytokine responses.

Materials and Methods

Reagents

All reagents, unless otherwise noted, were purchased from Thermo Fisher Scientific (Waltham, MA) or Sigma-Aldrich (St. Louis, MO) at the appropriate quality grade available and/or at grades appropriate for cell culture/*in vivo* use, depending on the application.

Mouse strains and experiments

C57BL/6J mice aged 8–12 weeks were purchased from Charles River Laboratories (Wilmington, MA) and were allowed to acclimate for at least 1 week prior to experimentation. *Src*-deficient mice (B6;129S7-*Src*^{tm1Sor}/J) were obtained from Jackson Laboratories (Bar Harbor, ME), and were bred to obtain *Src*$^{+/-}$ mice and littermate *Src*$^{+/+}$ WT controls (38). Animal genotypes were confirmed via supplier’s genotyping protocols using a universal reverse primer (GAG TTG AAG CCT CCG AAG AG) and the forward primers (TCC TAA GGT GCC AGC AAT TC) for WT and (CGC TTC CTC GTG CTT TAC GGT AT) for KO. *Duox1*$^{-/-}$ mice were originally provided by Miklós Geiszt (39) and were backcrossed onto C57BL/6NJ background (Jackson Laboratories, Bar Harbor, Maine), for at least 5 generations.

Mice were anaesthetized with isoflurane briefly before all instillation procedures. Src Inhibitor AZD0530 (Saracatinib; APExBIO, 677 ng/kg in saline prepared from a stock solution in DMSO) and α-ST2/IgG (R&D Systems, 20 μg/mouse) were instilled intranasally (50 μL/mouse) 1–2h prior to allergen challenge. House dust mite extract (*Dermatophagoides pteronyssinus*, HDM, 50 μg/mouse in PBS, Lot #269206, Greer Laboratories) or recombinant mouse IL-33 (BioLegend, 1.0 μg/mouse in PBS) were instilled oropharyngeally in a volume of 50 μL/mouse either once or on 4 consecutive days prior to sacrifice and harvest. Following sacrifice, bronchoalveolar lavage fluid (BALF, collected by 500 uL injection of PBS three times) and lung tissues were collected for analysis. Both male and female mice were used in experiments as evenly as possible. All procedures were reviewed and approved by the University of Vermont Institutional Animal Care and Use Committee prior to experiments (protocol #: PROTO202000078).

Cell culture and treatments

Primary mouse tracheal epithelial cells (MTEC) were isolated from WT or *Duox1*$^{-/-}$ C57BL/6NJ mice, and grown as previously described (40), NCI-H292 human pulmonary mucoepidermoid cells were purchased from ATCC (Manassas, VA) and were grown on plastic in RPMI-1640 (Gibco) supplemented with 10% fetal bovine serum and 1% Pen/Strep (Gibco). Upon reaching confluence, plated cells were starved (serum-free medium for H292, or medium without EGF for MTEC) overnight prior to experimentation. Media was refreshed 2h prior to treatment, and cells were pretreated for 30 min with inhibitors (1 μM AZD0530, 1 μM AG1478) or mouse α-ST2 blocking mAb/IgG (2 μg/mL; R&D Systems). Cells were then treated with HDM (50 μg/mL, Lot #213051), *A. alternata* extract (ALT, Greer Laboratories, 50 μg/mL), ATP (Sigma, 100 μM), or with human/mouse recombinant IL-33 (Peprotech/BioLegend, respectively, 100 ng/mL) and media was collected at appropriate times for analysis of cytokines/growth factors or ATP release. Control studies showed that neither HDM, AZD0530, or AG1478 caused significant loss of viability at the doses used, assessed using CellTiter-Glo Viability Assay (Promega, Madison, WI). Following treatments, cells were lysed utilizing Western Solubilization Buffer (WSB, 50 mM HEPES, 250 mM NaCl, 1.5 mM $MgCl_2$, 1% Triton-X100, 10% glycerol, 1 mM EDTA, 1 mM phenylmethylsulfonyl fluoride, 2 mM Na_3VO_4, 10 mg/ml aprotinin, and 10 mg/ml leupeptin (pH 7.4)) or GeneJet RNA extraction kit lysis buffer (Invitrogen) supplemented with 2% β-mercaptoethanol, for analysis of protein or RNA, respectively.

siRNA silencing

Silencing of gene expression in H292 cells or MTEC was carried out when cells were 60–70% confluent using targeted siRNAs and the DharmaFECT reagent (Dharmacon), according to manufacturer protocol. Briefly, cells were treated with siRNA mixed with Dharmafect reagent and serum-free media at a concentration of 100 nM 72h prior to experimentation. After 24h, media was removed and replaced with full growth media for 24h, followed by starvation media 24h prior to treatment. Specific RNA used were On-Target Plus Smartpool NS siRNA, targeting either human or mouse Src, or mouse *Il1rl1* (ST2), (Dharmacon). DUOX1 was silenced using siRNA reagents (GCUAUGCAGAUGGCGUGUAtt; antisense, UACACGCCAUCUGCAUAGCtg; sense) and control siRNA (Invitrogen) as previously described (41).

ELISA analyses

BALF harvested from mice or cell supernatants were analyzed for IL-33, IL-5, IL-13, and amphiregulin (Areg) using DuoSet ELISA kits (R&D systems) according to manufacturer protocol and were read on a BioTek Synergy HT plate reader.

H_2O_2 detection assay

Production of extracellular H_2O_2 from H292 cells was analyzed using peroxidase-catalyzed tyrosine crosslinking, as previously described (42). Reaction mixtures were analyzed by high performance liquid chromatography and fluorescence detection of dityrosine and compared to exogenous standards generated using H_2O_2.

Analysis of protein sulfenylation

Protein sulfenylation was measured as previously described (29, 43). Briefly, following 10-min stimulation of either H292 cells or MTEC, cells were lysed in WSB containing 1 mM DCP-Bio 1, 200 U/mL Catalase, and 10 mM N-ethylmaleimide for 1h on ice. Equal amounts of protein (~100–500 μg), measured by BCA assay, were then loaded on to Amicon Ultra-0.5 filtration units and washed with 6 changes of 20 mM Tris-HCl. Washed proteins were then added to prewashed NeutrAvidin beads (Pierce) and rotated overnight at 4°C. Following multiple washes, Biotin-tagged proteins were eluted from the beads with a 50 mM Tris, 2% SDS, 1 mM EDTA buffer pH 7.4 and boiling for 10 min, followed by mixing the supernatant solution with 6x Laemmli buffer for analysis by SDS-PAGE and western blot.

Western blotting

Cell culture lysates were analyzed with BCA Assay (Pierce) and mixed with Laemmli sample buffer and briefly boiled. Equal amounts of protein from each sample were separated using Criterion 10% SDS-PAGE (Bio-Rad), and transferred to nitrocellulose membranes. Membranes were then blocked with either 5% BSA or non-fat milk and probed with antibodies against pY1068 EGFR (1:1000, 3777S), pY845 EGFR (1:1000, 2231S), EGFR (1:1000, 2646S), p-Src Tyr 416 (1:1000; 2101S), Src (L4A1; 1:1000; 2110S) (Cell Signaling Technologies) and β-Actin (1:5000, A5441, Sigma), in manufacturer recommended diluent and supplemented with 0.05% Sodium Azide overnight at 4°C. Membranes were then probed with horseradish peroxidase (HRP)- conjugated secondary antibodies and detected using Pico or Femto chemiluminescence substrates (Fisher and GeneTex, respectively) and an AI600 Chemiluminescent Imager (GE Lifesciences).

RT-PCR

Tissue RNA was isolated from mouse inferior lobes as previously described (22). RNA from cultured cells was isolated using GeneJet RNA extraction kit lysis buffer (Invitrogen) supplemented with 2% β-mercaptoethanol. RNA from both tissues and cultured cells was then purified using GeneJet RNA purification kits (Invitrogen) according to manufacturer protocol. cDNA was synthesized using the iScript cDNA synthesis kit (Bio-Rad) and/or applied biosystems High-Capacity cDNA Reverse Transcription Kit (Applied Biosystems) according to manufacturer protocol. Quantitative RT-PCR was performed using Sybr Green

PCR supermix (Bio-Rad) 0.5 μL cDNA and 0.5 μM primer mix as previously described (23). Primer sets used included: *Src* (F: GAC CGA GCT CAC CAC TAA GG, R: CTG TGG CTC AGC GAA CGT AA), *Il33* (F: GAT GGG AAG AAG GTG ATG GGT G, R: TTG TGA AGG ACG AAG AAG GC), *Il13* (F: CCA CGG CCC CTT CTA ATG A, R: GCC TCT CCC CAG CAA AGT CT), *Il5* (F: ATG GAG ATT CCC ATG AGC AC, R: CCC ACG GAC AGT TTG ATT CT), *Areg* (F: AAC GGT GTG GAG AAA AAT CC, R: TTG TCC TCA GCT AGG CAA TG), *Duox1* (F: GAC CCC AGT ATC TCC CCA GA, R: ATG ACT GGG AAT CCC CTG GA), *Il1rl1 (ST2)* (F: GTG ACA CCT TAC AAA ACC CG, R: TCA AGA ACG TCG GGC AGA G), *Muc5ac* (F: AGT CTC TCT CCG CTC CTC TCA AT, R: CAG CCG AGA GGA GGG TTT GAT CT).

Immunohistochemistry

Immunohistochemistry analyses were performed as previously described (22). Tissue slides were quenched of endogenous peroxidase activity with 3% H_2O_2 and incubated with antibodies against Src pY416 (Millipore pSrc Tyr 418 07–909, 1:600) and EGFR pY1068 (CST 3777S, 1:125) overnight. Finally, samples were conjugated with biotinylated secondary antibodies and streptavidin-HRP (strep-HRP) using the Vectastain ABC kit (Vector Laboratories) according to the manufacturer's protocol. Antibodies were visualized by reacting strep-HRP with 3,3'-diaminobenzidine and monitored until color was sufficiently developed for analysis. Control stainings were performed by omitting primary antibodies or by using isotype IgG1 controls to assure staining specificity. Quantification of positive staining was calculated as a percentage against total counterstained airway using MetaMorph imaging software (Molecular Devices).

Analysis of lung single-cell suspensions

Mouse lungs were dissociated into single-cell suspensions using enzymatic digestion (Miltenyi Biotec Lung dissociation Kit 130–095-927) and a GentleMACS Dissociator (Miltenyi Biotec Inc.) following the manufacturer's protocol. Hematopoietic cell types were isolated with CD45 Microbeads (Miltenyi Biotec, mouse CD45 Microbeads, #130–052-301) using LS Columns (Miltenyi Biotec, #130–042-401) and QuadroMACS Separator (Miltenyi Biotec), according to manufacturer's recommendations, and CD326(EpCAM)-positive (epithelial) cells were enriched from the $CD45^-$ fraction using Miltenyi Biotec CD326 EpCAM Microbeads (#130–105-958). $CD45^-/CD326^+$, $CD45^-/CD326^-$ and $CD45^+$ cell fractions were pooled from 2 mice for RNA extraction using QIAGEN RNeasy Micro kit and RT-PCR analysis.

For analysis by flow cytometry, 10^6 cells per sample were incubated in Live/Dead Fix Blue (ThermoFisher # L23105) for 20 min at 4°C and then washed with BioLegend Cell Staining Buffer (BioLegend #420201). Cells were incubated with BioLegend TrueStain FcX anti-mouse CD16/32 block (BioLegend 1#01320) for 10 min at room temperature and appropriate antibodies were added for 30 min at 4°C (CD45 conjugated to Alexa Fluor 700, BioLegend #103128; CD326 conjugated to Brilliant Violet 510, BioLegend #118231; and ST2 conjugated to PE, ThermoFisher #12–9335-82). Cells were then washed twice with BioLegend Cell Staining Buffer and analyzed on BD LSRII flow cytometer and FlowJo

v10.7 software. Cells of interest were gated on single live cells that were $CD45^{-}$ and $CD326^{+}$.

Measurement of airway hyperresponsiveness

Following 4-day IL-33 challenge, mice were anesthetized on day 5 and their tracheas were cannulated. Following paralysis with pancuronium bromide (0.8 μg/kg), mice were connected to a flexiVent ventilator apparatus (SIREQ) and exposed to aerosolized PBS or increasing doses of methacholine (12.5, 25, and 50 mg/mL) after stabilizing. Newtonian resistance (R_n), tissue damping (G), and tissue elastance (H) we measured by forced oscillation analysis using a constant phase model of impedance (44, 45).

BALF cytology—BALF were briefly centrifuged at 150 x *g* and the supernatant was collected for analysis. Pelleted cells were resuspended in PBS and cells were counted using a hemocytometer prior to loading into an EZ Cytofunnel (Thermo Scientific) and centrifuged at 600 RPM for 10 minutes. Slides were fixed and stained using the Hema 3 kit (Thermo Scientific) and cell differentials were counted based on at least 200 cells per slide.

Statistical Analyses

All data are represented as means and SEMs. Statistical significance was evaluated using 2-way ANOVA, unless otherwise noted, through the GraphPad Prism Software (Version 7.0, GraphPad Software, La Jolla, CA). All values were considered significant if $P<0.05$.

Results

Src mediates airway epithelial IL-33 secretion in response to acute airway HDM challenge

We previously demonstrated that acute challenge of the airway epithelium with allergens results in secretion of IL-33, which was associated with redox-dependent activation of Src and EGFR (21, 22). To address the role of Src in such acute airway epithelial responses, mice were pretreated with the Src family kinase (SFK) inhibitor AZD0530 (Saracatinib) (46), prior to acute airway challenge with HDM, after which BAL fluids and lung tissues were harvested (Fig. 1A, left). Consistent with previous findings (25, 29), IHC analysis showed increased Src and EGFR activation following HDM administration at 1 and 6h, which was in both cases diminished with AZD0530 treatment (Fig. 1B and Fig. S1A,B). This suggests that EGFR activation was mediated by Src-mediated transactivation (25), since AZD0530 is a relatively weak direct inhibitor of EGFR (47). As expected (21), HDM challenge caused significant IL-33 secretion into the BALF after 1h, which returned to baseline levels at 6h. HDM-induced IL-33 secretion was significantly attenuated following SFK inhibition, suggesting that Src activity is involved in IL-33 secretion following HDM exposure (Fig. 1C). HDM challenge also induced airway secretion of IL-13 and IL-5 at 6h, consistent with their induction by IL-33 secretion and signaling, and these responses were also inhibited following SFK inhibition by AZD0530 (Fig. 1C). Finally, we observed corresponding increases in mRNA expression of these cytokines, which were similarly suppressed following AZD0530 treatment, indicating that Src also regulates transcriptional regulation of *Il33*, as well as *Il5* and *Il13*, after HDM challenge (Fig. S1C).

While clinically relevant, AZD0530 does not discriminate among the different SFK members (47) and therefore does not provide insight into the relative contribution of different SFKs (e.g. Fyn, Yes, etc.). To address this concern, we utilized heterozygous *Src* knockout mice in similar HDM challenge studies (Fig. 1A, right). We observed a trend (p=0.066) towards decreased HDM-induced IL-33 secretion into the BALF of $Src^{+/-}$ mice compared to WT mice at 1h, and significant suppression of IL-13 and IL-5 secretion at 6h (Fig. 1D). In summary, these findings demonstrate that HDM-induced secretion and expression of IL-33, and IL-33-induced type 2 cytokines, are dependent on Src activation and signaling.

To assess the specific impact of Src in epithelial responses, we performed similar analyses of in vitro HDM-stimulated MTEC, after siRNA silencing of endogenous *Src* expression (Fig. S1D). We treated MTEC with HDM for 1 or 6 hrs and analyzed cytokines secreted into the media. Consistent with our in vivo studies, we observed increased IL-33 secretion at 1h which returned to baseline at 6h (Fig. 1E). Consistent with previous studies indicating the ability of lung epithelial cells to generate type 2 cytokines upon appropriate stimulation (48–50), MTEC were also capable of producing IL-5 and IL-13 upon HDM challenge, which were increased at 6h likely due to their induction by initially secreted IL-33. In both cases, these responses were significantly diminished after *Src* silencing (Fig. 1E). Interestingly, we did not see any significant change to *Il33* gene expression in these cells in response to HDM treatment (Fig. S1E). Overall, these findings demonstrate that Src is involved in IL-33 secretion from epithelial cells following HDM challenge.

Oxidation of Src C185 and C277 are involved in Src-dependent IL-33 secretion

We previously showed that HDM-induced epithelial IL-33 secretion is dependent on initial production of H_2O_2 by the NADPH oxidase DUOX1 and redox-dependent activation of Src and EGFR (21). Extending these previous findings, we found that oxidation and activation of Src and EGFR in response to HDM treatment were markedly attenuated after siRNA-dependent silencing of *Src* (Fig. S1D), indicating that HDM-induced EGFR oxidation and activation were dependent on Src-mediated transactivation. We recently reported that H_2O_2 can directly enhance Src activation by oxidizing Cys185 and Cys277 within the Src protein (37). To address the importance of Src C185 or C277 residues in HDM-mediated secretion of IL-33, we transfected H292 cells with FLAG-tagged WT Src as well as C185A and C277A mutant constructs. HDM-induced IL-33 secretion was increased after transfection with WT Src, compared to empty vector control, but cells transfected with either C185A or C277A mutants displayed lower HDM-induced IL-33 secretion compared to cells transfected with WT Src cells or empty vector (Fig. 1G). These findings are consistent with the proposed importance of these Cys residues in Src activation and also suggest that oxidant-resistant Src may act in a dominant-negative fashion, since Src activation may involve the formation of Src dimers (51). Overall, these results indicate that HDM-induced IL-33 secretion involves redox-dependent activation of Src and is dependent on two critical Cys residues within Src that influence oxidant-mediated kinase activation.

HDM-induced IL-33 secretion involves an autoamplification mechanism involving IL-33 signaling

In an attempt to verify whether HDM-induced production of type 2 cytokines, such as IL-13 or IL-5, are indeed due to initial IL-33 secretion and signaling through its receptor, IL1RL1/ST2, we used an ST2-targeted monoclonal blocking antibody (mAb) to prevent IL-33 signaling. To our surprise, we observed that ST2 receptor blockade also markedly suppressed HDM-induced secretion of IL-33 itself into the BALF (Fig. 2A,B). Follow-up studies of HDM-challenged MTEC treated with the ST2 mAb (Fig. 2C) or with ST2-targeted siRNA (Fig. 2D and Fig. S2E) showed similar findings, as both approaches markedly attenuated HDM-induced IL-33 secretion compared to corresponding controls. Similar findings were obtained with alternative insults such as *A. alternata* extract (a fungal allergen) or exogenous ATP (as a direct mimic of damage response) (Fig. 2E). Collectively, these findings indicate that IL-33 secretion induced by HDM or other injurious insults appears to largely depend on a feed-forward mechanism in which initially-released IL-33 further enhances its own secretion, by signaling through ST2 receptors expressed on epithelial cells (50, 52). We performed flow cytometry analysis of lung single cell suspensions to validate the expression of IL1RL1/ST2 on epithelial cells, which indicated that a fraction of about 6% of $CD45^{-}/CD326^{+}$ epithelial cells also displayed positive IL33R/ST2 staining (Fig. 2F and Fig. S3F), in agreement with earlier studies (52).

Src and EGFR contribute to IL-33-induced type 2 cytokine production

We next sought to explore whether IL-33 signaling can directly activate Src and/or EGFR within the epithelium and thereby contribute to type 2 cytokine secretion. To this end, we first challenged WT or $Src^{+/-}$ mice with recombinant IL-33 (Fig. 3A), and indeed observed that IL-33-induced production of both IL-5 and IL-13 within the BALF were significantly reduced in $Src^{+/-}$ mice compared to WT controls (Fig. 3B). We also observed significant upregulation of *Il33*, *Il13*, and *Il5* mRNA at 6h following IL-33 challenge, but this was not significantly attenuated in $Src^{+/-}$ mice (Fig. S2A). Similarly, siRNA silencing of *Src* in MTEC also significantly attenuated IL-13 secretion induced by IL-33 (Fig. 3C), and pharmacological inhibition of either Src (AZD0530) or EGFR (AG1478) similarly suppressed IL-33-induced IL-13 secretion in H292 cells (Figs. 3D,E). Consistent with these findings, we showed that IL-33 stimulation of either H292 cells or MTEC increased activation of both Src and EGFR, as measured by Src autophosphorylation at Y416 and EGFR phosphorylation at Y845 (a Src target) and Y1068 (autophosphorylation) (Fig. S3D and Fig. 4F).

Src has been implicated in EGFR transactivation, but may also be involved in the activation of EGFR ligands (25). One such EGFR ligand that is strongly involved in type 2 immune responses is amphiregulin (AREG), which contributes to epithelial regeneration and remodeling (53). Indeed, we observed rapid *Areg* mRNA expression and AREG secretion in response to airway HDM challenge, which was inhibited by the Src inhibitor AZD0530 (Fig. S2B). Moreover, AREG secretion from MTECs in response to HDM was also Src-dependent (Fig. S2C). Finally, ST2 blockade indicated that HDM-induced AREG secretion critically depends on IL-33 signaling (Fig. S2D,E), and AREG was secreted into the BALF of mice upon in vivo IL-33 treatment in a Src-dependent manner (Fig. S2F). Collectively,

although Src and EGFR are not typically implicated in canonical IL-33-dependent signaling through ST2, our findings demonstrate that activation of Src and EGFR are both important for epithelial type 2 cytokine production in response to IL-33, and may also involve IL-33-dependent activation of AREG.

IL-33-induced signaling in airway epithelial cells is DUOX1-dependent

Our findings implicating Src and EGFR in IL-33 signaling prompted us to explore the potential contribution of DUOX1 to IL-33-induced epithelial responses given our previous findings demonstrating DUOX1-dependent regulation of Src and EGFR (21, 22). Indeed, *Duox1*$^{-/-}$ mice challenged with IL-33 displayed diminished IL-5 and IL-13 secretion into the BALF compared to WT mice (Fig. 4A and 4B), suggesting that DUOX1 is involved in downstream cytokine secretion in response to IL-33 signaling, in addition to its previously reported role in initial secretion by allergen challenge (21). Since HDM challenge generates additional innate epithelial cytokines such as IL-1a and IL-25 in a DUOX1-dependent fashion (21), we explored whether direct IL-33 challenge may also contribute to the production of these additional cytokines. Indeed, we observed that IL-33 challenge resulted in rapid production of IL-1α and more delayed production of IL-25 into the BAL, which appeared to be dependent on both Src and DUOX1 (Fig. S2G and Fig. S3A). To address the cellular origin of IL-33-induced production of IL-5 and IL-13, we prepared single cell suspensions from lung tissues following acute IL-33 challenge, and separated epithelial (EpCAM/CD326^{+}) and hematopoietic (CD45^{+}) cells from other lung cell types and assessed *Il5* and *Il13* mRNA expression in these cell fractions. As shown, IL-33 airway challenge markedly enhanced *Il5* and *Il13* mRNA in CD45^{+} cells, as expected (4), but also in epithelial (CD326^{+}) cells (Fig. 4C), even though we can't completely rule out a contribution of CD45^{+} cells as a potential impurity in this latter cell fraction. Consistent with our in vivo findings, IL-33-induced production of IL-13 (and to a lesser extent IL-5) was also suppressed in MTECs from *Duox1*$^{-/-}$ mice or in H292 cells in which *Duox1* was silenced (Fig. 4D and Fig. S3B,C). To demonstrate whether IL-33 signaling through ST2 is in fact capable of activating DUOX1, we assessed the ability of IL-33 to stimulate epithelial H_2O_2 production. Indeed, we observed an increase in extracellular H_2O_2 in H292 cells following IL-33 stimulation, which was attenuated when *Duox1* was silenced using siRNA (Fig. 4E). Additionally, we examined whether IL-33 induces oxidative activation of Src and/or EGFR. Indeed, IL-33-dependent activation of Src and EGFR in either H292 cells or MTECs (Fig. S3D and Fig. 4F) was in both cases DUOX1-dependent and corresponded with DUOX1-mediated cysteine oxidation within both Src and EGFR (Fig. 4F and Fig. S3D).

Repeated IL-33 exposure induces DUOX1- and Src-dependent type 2 inflammation.—Our results so far demonstrate that IL-33 can induce its own secretion from the airway epithelium and that DUOX1-mediated activation of Src is critical for such IL-33 autoamplification and activation of EGFR and type 2 cytokine responses. Based on a recent study demonstrating that development of eosinophilic inflammation and airway hyperresponsiveness in response to chronic airway challenge with exogenous IL-33 is largely dependent on endogenous IL-33 (54), we wondered whether DUOX1 and/or Src could similarly contribute to such outcomes. Therefore, we exposed *Duox1*$^{-/-}$ or *Src*$^{+/-}$

mice or their corresponding littermate controls to repeated airway IL-33 challenge on 4 consecutive days, after which we assessed type 2 cytokine responses, inflammation, airway remodeling, and airway hyperresponsiveness by flexiVent analysis (Fig. 5A). Consistent with our previous findings in the context of acute challenge, we observed significant increases in BAL IL-33, IL-13, and IL-5 levels in response to chronic IL-33 challenge, which was in each case markedly attenuated in both *Duox1*$^{-/-}$ or *Src*$^{+/-}$ mice (Figs. 5B,C). Similar findings were observed with respect to AREG secretion under these conditions, as well as production of other type 2 cytokines such as IL-4 and IL-9 (Figs. S4A,B). As expected, chronic IL-33 challenge also significantly increased lung tissue mRNA levels of *Il33*, *Duox1, Il5, Il13, and Il1rl1*, but not *Areg* (Figs S4C,D), however, neither response was significantly altered in *Duox1*- or *Src*-deficient mice, suggesting that DUOX1 and Src primarily affect these cytokine responses at the level of secretion. Intriguingly, repeated IL-33 challenge resulted in decreased lung tissue *Src* mRNA expression (Figs. S4C,D), which may potentially reflect a negative feedback mechanism in chronic conditions of IL-33-driven inflammation. Conversely, Src-deficiency appeared to enhance overall IL-33 expression (Fig. S4C), further supporting such an antagonistic relationship.

As expected (54), chronic IL-33 challenge resulted in increased airway inflammation, illustrated by marked increases in total BALF cell counts, which were largely represented by increased eosinophils, and to a lesser extent neutrophils and lymphocytes. However, neither were significantly altered in *Duox1*$^{-/-}$ mice or *Src*$^{+/-}$ mice, with the exception of a slight increase in neutrophils in IL-33 challenged *Duox1*$^{-/-}$ mice (Figs. 5D,E). Additionally, we observed an increase in the mucus metaplasia marker *Muc5ac* following IL-33 challenge, but this was not altered in *Duox1*$^{-/-}$ or *Src*$^{+/-}$ mice (Figs. S4C,D). Similarly, repeated IL-33 challenge enhanced expression of remodeling/fibrosis markers *Col1a1* and *Col3a1*, this was similarly not different between the various mouse groups (Figs. S4C,D). Finally, we observed that chronic IL-33 challenge increased airway hyperresponsiveness (AHR), indicated by increases in Newtonian resistance, tissue damping, as well as tissue elastance, in response to increasing methacholine dosage (Figs. 5F,G). Interestingly, all three AHR parameters were attenuated in *Duox1*$^{-/-}$ mice (Fig. 5F), consistent with recent findings indicating that increases in AHR due to exogenous IL-33 challenge largely depend on endogenously secreted IL-33 (54). However, no significant decrease was observed in IL-33-induced AHR in *Src*$^{+/-}$ mice compared to corresponding WT mice, and instead there was an apparent increase in Newtonian resistance and tissue elastance (Fig. 5G). Moreover, *Src*$^{+/-}$ mice also appeared to display decreased tissue damping and elastance in PBS treatment groups. Overall, these results suggest that DUOX1 and to a lesser extent Src contribute to type 2 cytokine responses and associated airway hyperresponsiveness in mice following repeated IL-33 challenge.

Discussion

Type 2 immune signaling and wound responses are critical regenerative processes of various tissues in response to injury due to e.g. parasitic infections, toxins, etc. (55). The importance of SFKs in wound healing has been recognized for some time, illustrated by a critical role for Fyn signaling in tail fin regeneration in zebrafish (56), or previous findings implicating Src in airway epithelial wound responses (25). Building on this, the

present findings highlight the specific involvement of Src in the secretion of the alarmin IL-33 from airway epithelial cells in response to airway challenge with HDM, a common asthma-inducing allergen, and in subsequent production of type 2 cytokines such as IL-13 and IL-5 by various lung cell types, including the respiratory epithelium itself. Therefore, epithelial Src may be important for innate host defense against acute non-microbial triggers and allergen challenge, which largely rely on type 2 immune activation, but could also contribute to dysregulated type 2 inflammation during e.g. allergen inflammation, similar to previously demonstrated role(s) of EGFR in the context of chronic allergen challenge that more closely resembles diseases such as allergic asthma (22, 26). Indeed, Src has been implicated in various aspects of asthma (57, 58), based on its involvement in e.g. smooth muscle proliferation (59) or TNF-α-induced disruption of epithelial barrier integrity (60). Our present findings implicating Src in chronic type 2 inflammation may further justify selective Src targeting approaches as a potential therapeutic strategy for allergic airways diseases associated with increased type 2 inflammation.

Our findings also further highlight the importance of redox-based regulation of Src in the context of IL-33-mediated type 2 inflammation. Consistent with our previous findings implicating the importance of specific cysteines within Src in regulating its activity (37), we show that allergen-induced IL-33 secretion is dependent on the oxidation of specific Cys residues, namely C185 and C277. Moreover, we demonstrate that such redox-dependent Src activation is mediated by the epithelial NADPH oxidase DUOX1, which was previously implicated in airway epithelial injury responses (21, 22, 29). The Cys residues implicated in these responses are largely unique to Src within the SFK family (37) and C277 has been exploited as a target for covalent Src inhibitor development (61). However, our observations of unique redox-based regulation of Src may have implications for such covalent inhibitor approaches, and may warrant alternative redox-based inhibitor strategies that selectively target Src and avoid off-target effects related to inhibition of other SFKs or related kinases.

An intriguing aspect of our present findings is the observation of an apparent feed-forward autoamplification mechanism with respect to epithelial IL-33 secretion in the context of acute allergen challenge. It has been well-recognized that airway epithelial expression of IL-33 and its receptor ST2 are subject to positive feedback regulation by paracrine mechanisms, e.g. by IL-13 generated by ILC2 (62). More recently, a similar paracrine amplification of epithelial IL-33 expression was attributed to mast cells that shift from the submucosa to the epithelium during allergic asthma, and in fact involve mast cell-derived IL-33 (63). Moreover, recent studies indicate that inflammatory responses induced by repeated airway administration of IL-33 are largely mediated by endogenous IL-33 production and activation (54). Our findings reveal an alternative epithelial-specific feed-forward mechanism, in which IL-33 induces further secretion of IL-33 from epithelial cells in the context of *acute* allergen challenge. This feed-forward mechanism depends on ST2 receptor signaling but appears to be independent of transcriptional IL-33 regulation, as it was not associated with increased epithelial IL-33 mRNA. Furthermore, our present findings demonstrate that the previously reported involvement of DUOX1 and redox-dependent Src/EGFR activation in acute IL-33 secretion in response to protease allergens such as HDM (21) may in fact largely be due to their role(s) in IL-33-dependent signaling in this feed-forward mechanism. Since epithelial IL-33 secretion by injurious triggers may involve

diverse mechanisms (13), including both passive IL-33 release from necrotic cells as well as cell signaling mechanisms initiated by early damage signals (e.g. ATP) through receptor-mediated signaling, we speculate that the feed-forward IL-33 secretion mechanism observed in our present study may reflect initial effects of early passive IL-33 secretion amplified by subsequent IL-33 secretion via IL-33 signaling and DUOX1/Src/EGFR activation.

We do not fully understand how IL-33 signaling through ST2 leads to the activation of DUOX1. DUOX1 activation in e.g. wound responses typically requires the activation of Ca^{2+} signaling, initiated by activation of ionotropic or metabotropic receptors, such as purinergic receptors (activated by initial ATP release as a damage signal) (25), histaminergic receptors (64), or TRP channels such as TRPV1 (23). IL-33 signaling typically involves the formation of a heterodimeric receptor complex between ST2 and IL-1RAcP, which then can complex with multiple signaling proteins (e.g. MyD88, TRAF6, various IRAKs), inducing MAP kinase signaling and other downstream kinase responses (4, 65), but emerging findings indicate that IL-33 can also induce Ca^{2+} mobilization, e.g. in neurons (66) or in IgE-sensitized mast cells (67), although the mechanisms are yet unestablished. However, the overlap of canonical IL-33 signaling, or the potential for an alternative signaling pathway, with the known DUOX1 activation pathway should be addressed.

Another intriguing aspect of our findings is the apparent close association between IL-33 and the EGFR ligand AREG, which are both produced acutely upon HDM challenge. It has recently been shown that EGFR and ST2 are closely associated in type 2 T helper cells (Th2 cells), and that the association of IL-33 and AREG with their receptors is not only required for this association, but necessary for the production of IL-13 by these cells (68). Also, IL-33 signaling and ST2 expression are closely linked with EGFR/AREG signaling during e.g. injury responses within the intestinal and airway epithelia (69). We recently reported that IL-33 is capable of inducing DUOX1-dependent AREG secretion in epithelial cells (22), and our present findings indicate the importance of IL-33 signaling in epithelial AREG secretion in response to HDM. Both IL-33 and AREG are known to be important in the context of epithelial injury and wound responses (4, 53), and our current findings offer further insight into the potential relationship between these mediators, and highlight the potential importance of DUOX1 and Src in such interrelations.

In summary, our current findings reveal the central importance of Src in HDM-induced airway epithelial type 2 responses, specifically in responses involving IL-33 secretion and signaling. Additionally, we have identified a novel autoamplification mechanism with respect to acute IL-33 secretion mechanisms, which is mediated by redox-dependent activation of Src and EGFR, and further demonstrate the intricate relationship between IL-33, AREG, and EGFR signaling in the context of innate injury responses and in chronic airway diseases characterized by increased type 2 inflammation. With respect to the physiological significance of our findings, DUOX1-mediated type 2 cytokine responses were associated with increased AHR in the context of chronic IL-33 challenge, as was previously observed in the context of HDM-mediated allergic airways disease (22), but this did not appear to apply to Src as IL-33-mediated AHR was not attenuated and perhaps even enhanced in *Src*$^{+/-}$ mice. This latter finding could potentially be due to the use of heterozygous mice rather than *Src*-null mice or to the fact that Src is ubiquitously present

in all cell types, and that non-selective Src deletion in other lung cell types may have unanticipated consequences impacting on overall lung function. Epithelial-specific targeting of Src may therefore have greater benefit. Lastly, since DUOX1 (and possibly Src) impaired IL-33 driven type 2 cytokine production and also AHR could also suggest that IL-33 (or related type 2 cytokines), via DUOX1 and/or Src, may activate sensory nerves in the airways as an important mechanism in promoting AHR (66, 70–72). Future studies that go beyond the scope of the present work would be required to test this possibility.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Key points:

- Epithelial IL-33 secretion involves IL-33 signaling via its ST2 receptor
- IL-33-dependent signaling activates Src kinase and EGFR
- Epithelial IL-33 signaling involves activation of the NADPH oxidase DUOX1

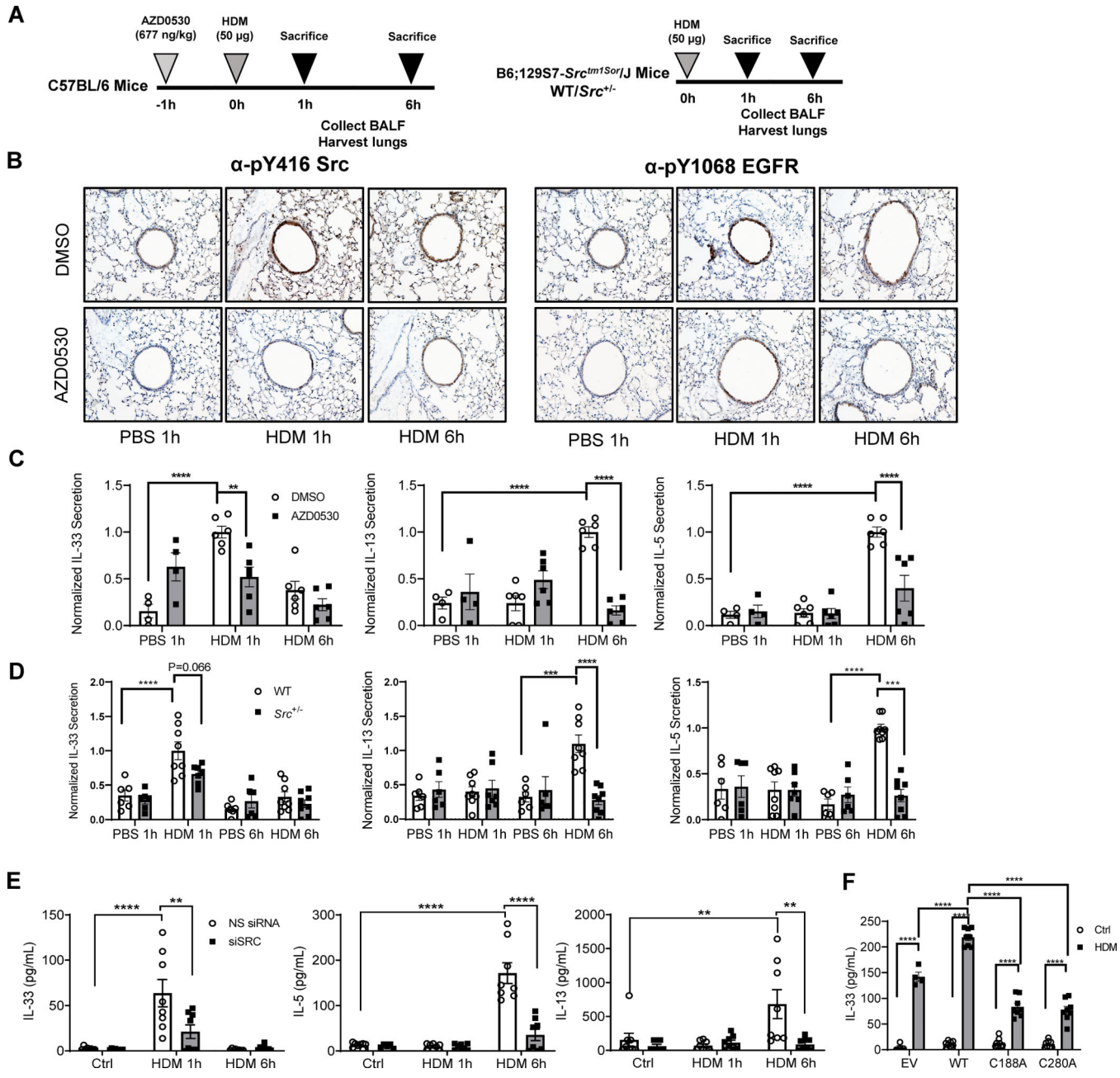

Figure 1: Src activity and oxidation are involved with airway epithelial type 2 responses.

(A) Experimental design for mouse experiments using Src inhibitor AZD0530 (Saracatinib) or *Src* WT/$Src^{+/-}$ mice. **(B)** Representative IHC analysis of lung sections from mice with α-pY416 Src and α-pY1068 EGFR antibodies. **(C)** ELISA analyses of IL-33, IL-13 and IL-5 in BALF from AZD0530-treated mice. **(D)** ELISA analyses of IL-33, IL-13 and IL-5 in BALF from WT or $Src^{+/-}$ mice. Data in C and D are normalized to average concentrations seen in WT HDM 1h (IL-33) or WT HDM 6h (IL-13 or IL-5). **(E)** MTEC were transfected with *Src*-targeted siRNA or non-silencing control (NS) and stimulated with 50 µg/mL HDM for 1 or 6 hours. Media was collected and analyzed by ELISA for IL-33, IL-5, or IL-13 secretion. **(F)** H292 cells were transfected with FLAG-WT, FLAG-C185A, FLAG-C277A or

empty vector control (EV), stimulated with HDM for 2h, and collected media was analyzed by ELISA for IL-33. Results are expressed as mean ± SEM from two separate experiments. AZD inhibitor studies were conducted with an n=4 for PBS control n=6 for HDM treated mice per group. WT/*Src*$^{+/-}$ experiments were conducted with an n=6 for PBS control and n=8 for HDM treated mice per group. Data were analyzed for significance using 2-way ANOVA where *P<0.05, **P<0.01, ***P<0.001, ****P<0.0001.

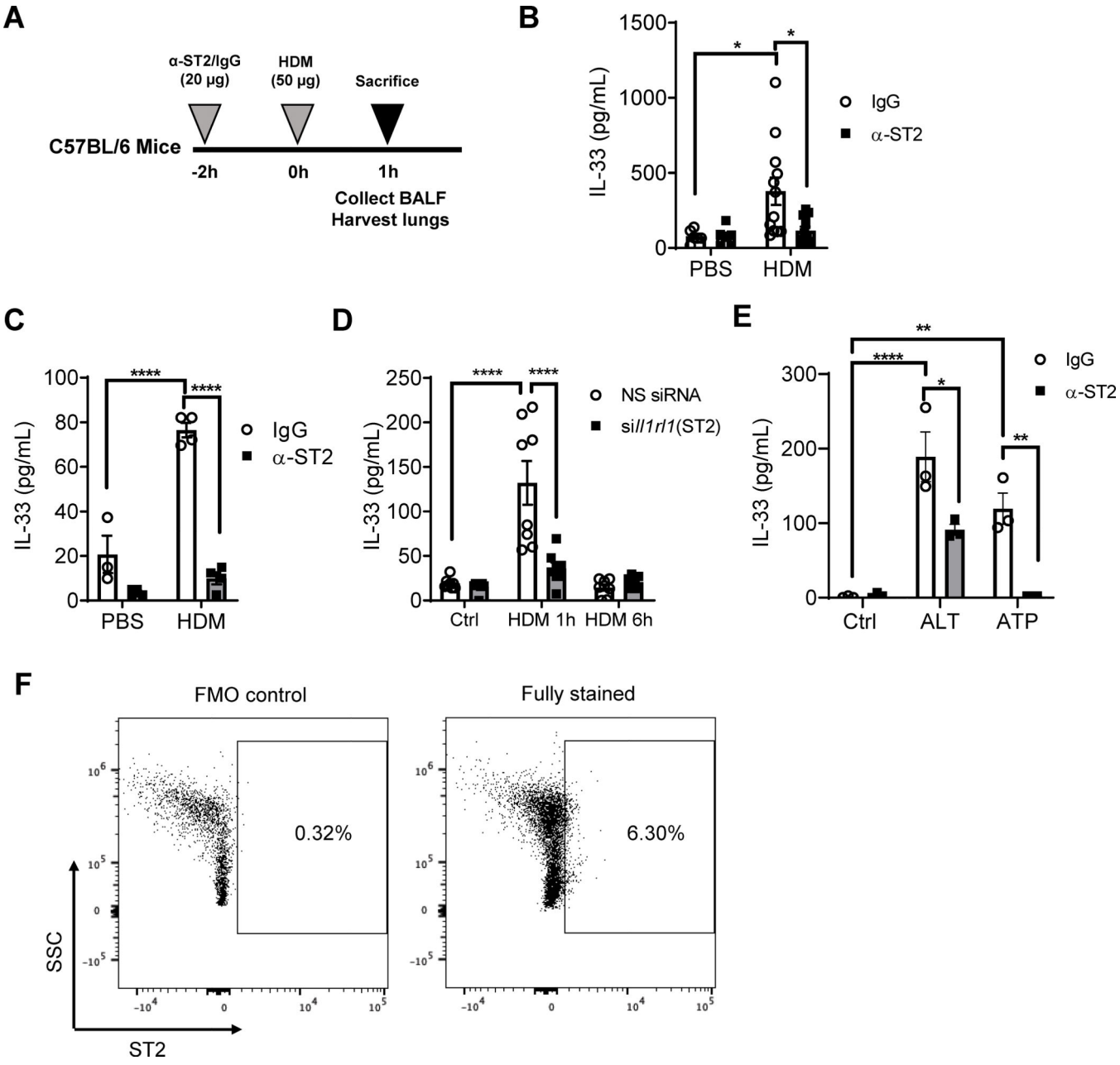

Figure 2: Airway epithelial IL-33 secretion is dependent on IL-33 signaling.

(A) Experimental setup for *in vivo* α-ST2 experiment. **(B)** Analysis of BALF from HDM-treated mice for IL-33 secretion using ELISA. **(C)** MTEC were treated with 2 μg/mL IgG or α-ST2 blocking mAb for 30 min prior to 50 μg/mL HDM challenge. Media was collected and analyzed for IL-33 secretion by ELISA. **(D)** MTECs were transfected with *Il1rl1* (ST2) siRNA and then treated with 50 μg/mL HDM. Media was collected and analyzed for IL-33 secretion with ELISA. **(E)** MTEC were treated with 2 μg/mL IgG or α-ST2 blocking mAb for 30 min prior to stimulation with 50 μg/mL ALT or 100 μM ATP for 2h. Media was collected and analyzed for IL-33 secretion by ELISA. Results are expressed as Mean ± SEM from 3–8 replicates. In vivo analyses were conducted with n=6

for PBS controls and n=12 for HDM-treated mice. **(F)** Analysis of mouse lung single cell suspensions by flow cytometry for ST2-positive populations of $CD326^{+}$ cells. Viable cells were gated on $CD45^{-}/CD326^{+}$ cell populations for analysis of ST2 expression, compared to the Fluorescence-Minus-One (FMO) control. Representative results from 3 separate analyses are shown. All data were analyzed for significance using 2-way ANOVA where *P<0.05, **P<0.01, ***P<0.001, ****P<0.0001.

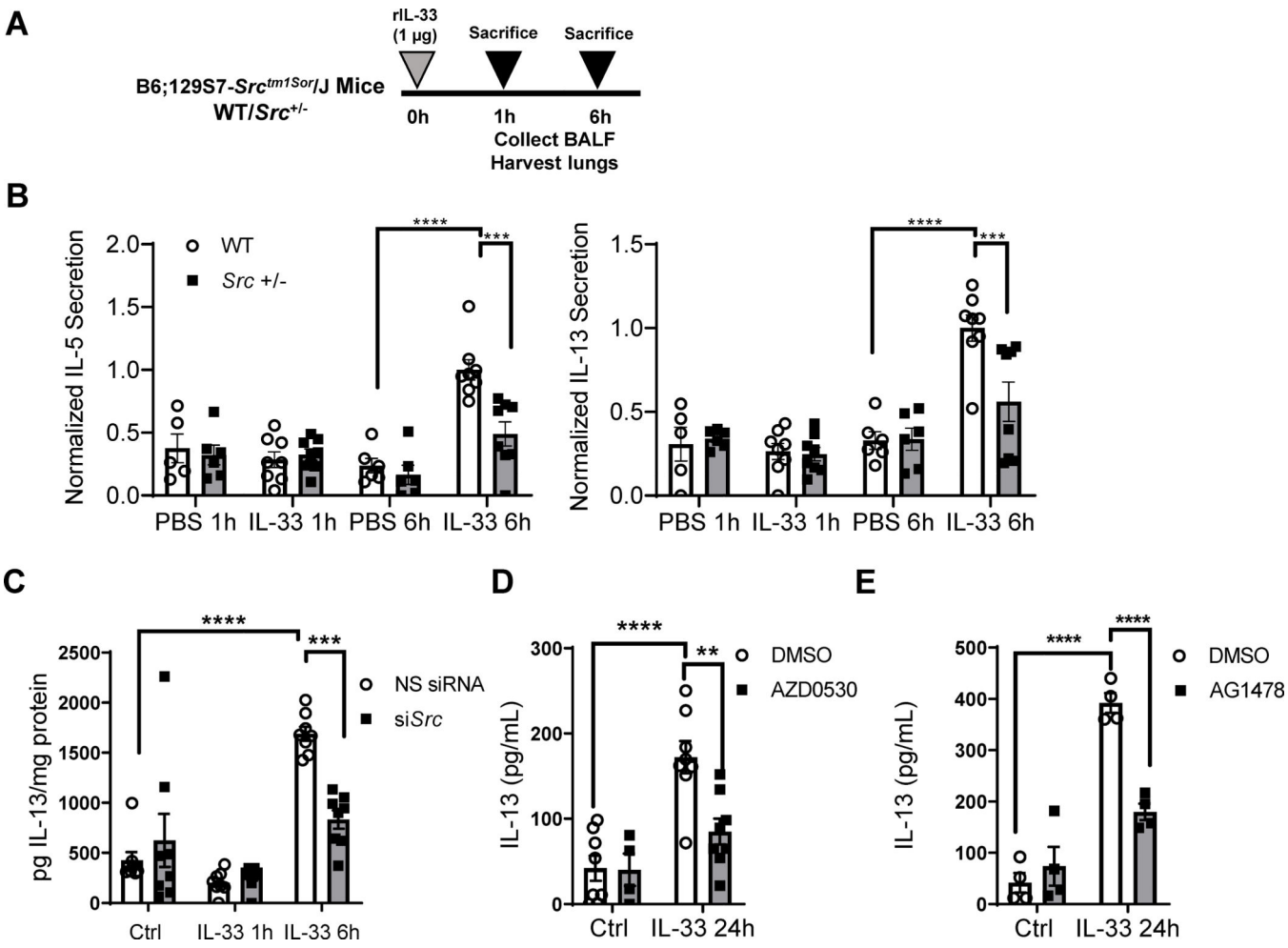

Figure 3: Epithelial IL-33 signaling involves Src and EGFR activation.

(A) Experimental design of in vivo IL-33 challenge studies. **(B)** Analysis of IL-5 and IL-13 secretion in BALF of mice treated with IL-33 or PBS by ELISA. **(C)** MTEC were transfected with Src siRNA for 72 hrs and treated with IL-33 for indicated times, after which media was collected and analyzed for IL-13 secretion by ELISA. **(D, E)** H292 cells were treated for 30 min with either a Src inhibitor (AZD0530, 1 µM; **D**) or the EGFR inhibitor AG1478 (1µM; **E**) followed by 24h stimulation with 100 ng/mL IL-33. Conditioned media was analyzed for IL-13 secretion by ELISA. Results are expressed as Mean ± SEM. *In vivo* analyses were conducted where n=5–6 for PBS control and n=8 for IL-33 treated mice per group. Data were analyzed for significance using 2-way ANOVA where *P<0.05, **P<0.01, ***P<0.001, ****P<0.0001.

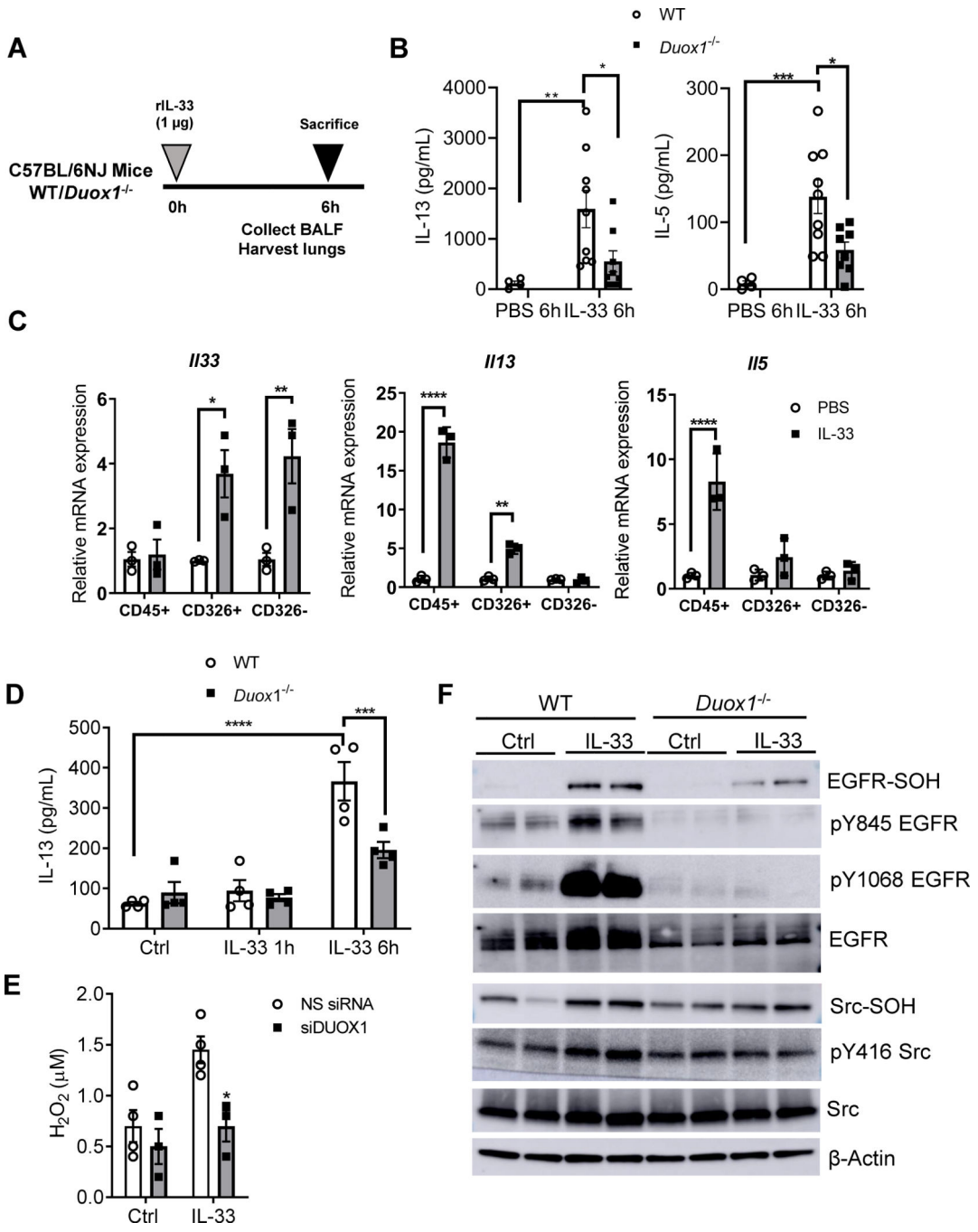

Figure 4: IL-33 signaling is dependent on DUOX1 activation in the airway epithelium.

(A) Experimental design of IL-33 challenge studies. **(B)** BALF IL-13 secretion from WT or *Duox1*$^{-/-}$ mice instilled with IL-33, analyzed by ELISA. (C) Lung single-cell suspensions from PBS- or IL-33-challenged mice were separated into CD45+ and CD45- fractions, and CD326+ cells were isolated from CD45- cell fractions. RNA was extracted from all 3 cell populations for mRNA analysis of *Il33*, *Il13* and *Il5* and was normalized to *Gapdh* as a house-keeping gene (n=3). **(D)** MTEC from WT or *Duox1*$^{-/-}$ mice were treated with 100 ng/mL IL-33 and IL-13 secretion into the media was analyzed by ELISA. **(E)** H292 cells

were transfected with NS or DUOX1 siRNA and stimulated with 100 ng/mL IL-33 for 10 min for analysis of H_2O_2 production by HPLC. **(F)** Western blot analysis of phosphorylated and sulfenylated (-SOH, measured by DCP-BIO1 alkylation and biotin affinity purification) forms of Src and EGFR from WT or *Duox1*$^{-/-}$ MTEC treated with 100 ng/mL IL-33 for 10 min. Results are expressed as Mean ± SEM. *In vivo* analyses were conducted where n=4 for PBS treated mice and n=8–9 for IL-33 treated mice per group. Mouse data were analyzed for statistical significance using a T-Test with Welch's Correction where *P<0.05, **P<0.01, ***P<0.001, ****P<0.0001. All other data were analyzed for significance using 2-way ANOVA where *P<0.05, **P<0.01, ***P<0.001, ****P<0.0001.

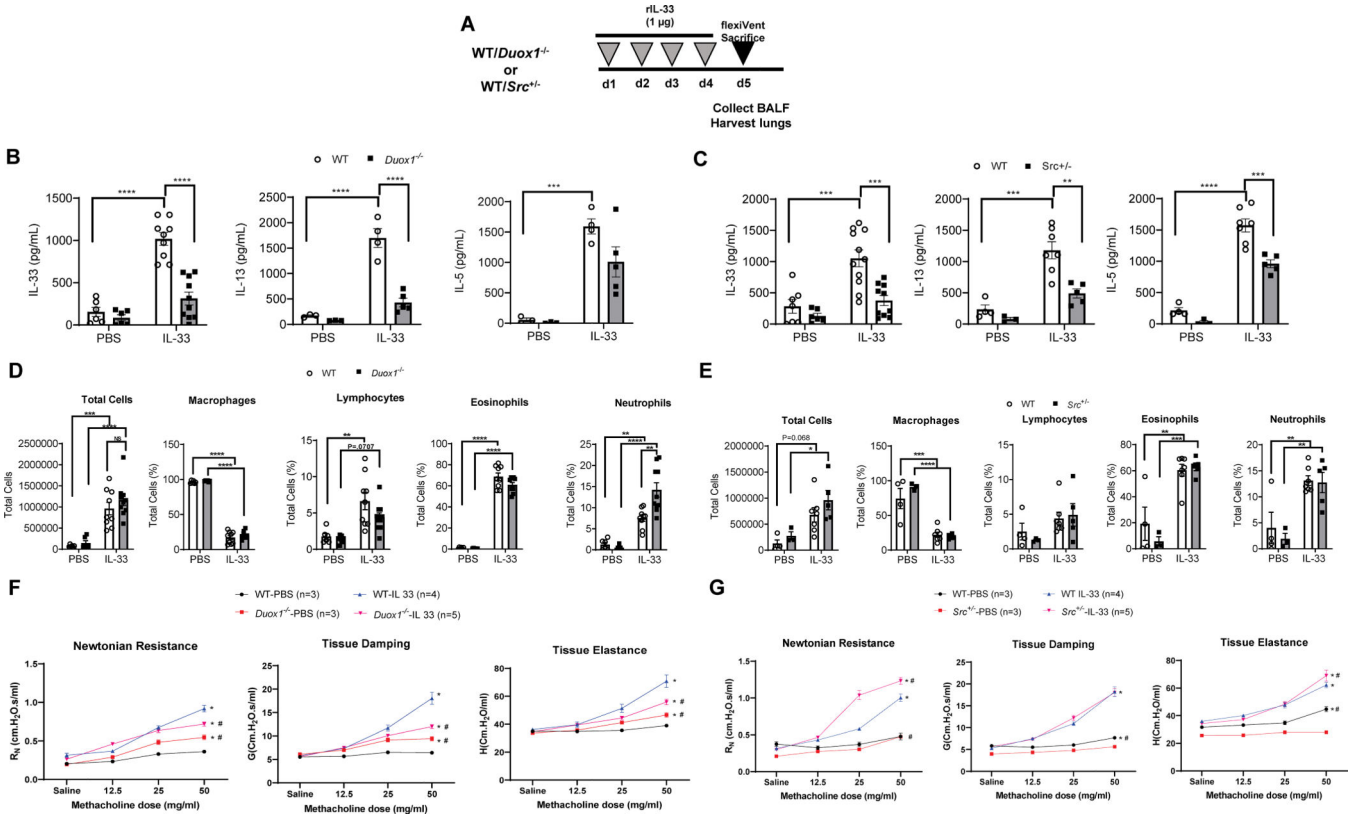

Figure 5: Role of DUOX1 and Src in type 2 inflammation and airways hyperresponsiveness upon repeated IL-33 challenge.

(A) Experimental design for repeated IL-33 challenge and analysis. **(B,C)** Cytokine secretion analyzed from BALF collected following 4-day IL-33 challenge in *Duox1*$^{-/-}$ **(B)** or *Src*$^{+/-}$ mice **(C). (D,E)** Measurement of total and cell differentials (expressed as percentage of total cell counts) in BALF from IL-33 exposed WT, *Duox1*$^{-/-}$ **(D)** or *Src*$^{+/-}$ mice **(E)**. Analysis of central airway (Newtonian) resistance, tissue damping, and tissue elastance in response to increasing methacholine dosage by flexiVent in WT, *Duox1*$^{-/-}$ **(F)** and *Src*$^{+/-}$ **(G)** mice. Results are expressed as Mean ± SEM. Experiments were conducted where n=3 for PBS control and n=4–5 for IL-33 treated mice per group. Data were analyzed for significance using 2-way ANOVA where *P<0.05, **P<0.01, ***P<0.001, ****P<0.0001. Flexivent results are expressed as Mean ± SEM. Experiments were conducted where n=3 for PBS control and n=4–5 for IL-33 treated mice per group. Data were analyzed for significance using 1-way ANOVA for 50 mg/mL methacholine dose where *P<0.05 vs WT PBS and $^{\#}$P<0.05 vs WT IL-33.