

Airway epithelial dual oxidase 1 mediates allergen-induced IL-33 secretion and activation of type 2 immune responses

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Background: The IL-1 family member IL-33 plays a critical role in type 2 innate immune responses to allergens and is an important mediator of allergic asthma. The mechanisms by which allergens provoke epithelial IL-33 secretion are still poorly understood.

Objective: Based on previous findings indicating involvement of the NADPH oxidase dual oxidase 1 (DUOX1) in epithelial wound responses, we explored the potential involvement of DUOX1 in allergen-induced IL-33 secretion and potential alterations in airways of asthmatic patients.

Methods: Cultured human or murine airway epithelial cells or mice were subjected to acute challenge with *Alternaria alternata* or house dust mite, and secretion of IL-33 and activation of subsequent type 2 responses were determined. The role of DUOX1 was explored by using small interfering RNA approaches and DUOX1-deficient mice. Cultured nasal epithelial cells from healthy subjects or asthmatic patients were evaluated for DUOX1 expression and allergen-induced responses.

Results: *In vitro* or *in vivo* allergen challenge resulted in rapid airway epithelial IL-33 secretion, which depended critically on DUOX1-mediated activation of epithelial epidermal growth factor receptor and the protease calpain-2 through a redox-dependent mechanism involving cysteine oxidation within epidermal growth factor receptor and the tyrosine kinase Src. Primary nasal epithelial cells from patients with allergic asthma were found to express increased DUOX1 and IL-33 levels and demonstrated enhanced IL-33 secretion in response to allergen challenge compared with values seen in nasal epithelial cells from nonasthmatic subjects.

Conclusion: Our findings implicate epithelial DUOX1 as a pivotal mediator of IL-33-dependent activation of innate airway type 2 immune responses to common airborne allergens and indicate that enhanced DUOX1 expression and IL-33 secretion might present important contributing features of allergic asthma. (J Allergy Clin Immunol 2015;■■■■:■■■-■■■.)

Key words: Asthma, redox signaling, H₂O₂, Src, epidermal growth factor receptor, IL-13

The respiratory epithelium plays an integral role in innate and adaptive immune responses to diverse environmental pollutants, allergens, and pathogens beyond its commonly accepted role as a physical barrier.^{1,2} Mucosal barriers, such as the respiratory epithelium, are increasingly recognized for their critical contribution to type 2 immune responses as a mechanism of enhanced host tolerance and resistance against a wide variety of nonmicrobial stimuli, allergens, and parasites^{3,4} and also as an important contributing mechanism to the development of allergic diseases, such as asthma.^{2,5} Central to such type 2 responses is the epithelial production of various cytokines that promote T_H2-polarized immune responses, among which the IL-1 family member IL-33 (IL1F11) has garnered considerable recent interest.

IL-33 production contributes importantly to T_H2-mediated host defense against parasitic infections^{6,7} but is also increasingly recognized as an important mediator of allergic asthma based on observations of increased expression and secretion of IL-33 within the airways of asthmatic patients,⁸ identification of IL-33 and ST2/IL1RL1 (IL-33 receptor) genes as major asthma susceptibility loci in genome-wide association studies,⁹ and a rapidly growing number of studies in experimental models of asthma demonstrating the critical importance of IL-33 and ST2/IL1RL1 in the development of allergen-induced eosinophilic inflammation, mucus metaplasia, and airways hyperresponsiveness.^{10,11} In spite of this, mechanisms mediating IL-33 expression, secretion, or both in response to allergic stimuli still remain incompletely understood.

IL-33 is constitutively present in the nuclei of epithelial cells in barrier tissues and acts as a dual-function protein, with an intracellular role as a transcriptional regulator¹² distinct from its extracellular cytokine properties,^{13,14} the latter being mediated by interaction with the orphan IL-1 receptor family member ST2 (IL-33 receptor) on various target cells, including dendritic cells, mast cells, and a recently identified subtype of innate lymphoid cells.^{11,15} Being constitutively expressed in epithelia of barrier tissues, IL-33 can be released rapidly in response to diverse exogenous stimuli as an “alarmin,” which is commonly thought to involve passive release caused by cell necrosis.^{10,13} However, several recent lines of evidence indicate active mechanisms of IL-33 secretion, such as activation of

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Abbreviations used

BAL: Bronchoalveolar lavage
 BMDM: Bone marrow–derived macrophage
 DMSO: Dimethyl sulfoxide
 DUOX: Dual oxidase
 EC₅₀: Median effective concentration
 EGFR: Epidermal growth factor receptor
 ERK: Extracellular signal-regulated kinase
 HDM: House dust mite
 HNE: Human nasal epithelial
 MTE: Mouse tracheal epithelial
 NHBE: Normal human bronchial epithelial
 NS-siRNA: Nontarget siRNA
 poly(I:C): Polyinosinic:polycytidylic acid
 siRNA: Small interfering RNA
 TLR: Toll-like receptor
 TSLP: Thymic stromal lymphopoietin
 WT: Wild-type

purinoceptor-dependent signaling in response to the fungal allergen *Alternaria alternata*,^{16,17} although the mechanistic details of such regulated IL-33 secretion are still largely undefined.^{10,18}

We recently elucidated a critical role for ATP-mediated purinoceptor signaling and the NADPH oxidase dual oxidase 1 (DUOX1) in epithelial wound responses to injury,^{19,20} which were found to depend on H₂O₂-dependent activation of the nonreceptor tyrosine kinase Src and epidermal growth factor receptor (EGFR).^{21,22} In light of the proposed importance of type 2 immune responses in epithelial wound healing,³ we speculated that epithelial DUOX1 might also regulate epithelial IL-33 secretion in response to airborne allergens or other injurious stimuli. Indeed, the present studies demonstrate that DUOX1 critically contributes to airway epithelial secretion of IL-33 and initiation of subsequent type 2 immune responses in response to 2 common allergens that are strongly associated with asthma and identify the importance of redox-dependent activation Src/EGFR pathways and calpain activation in this process. Moreover, consistent with observations that epithelial DUOX1 is inducible by T_H2 cytokines,²³ we observed enhanced DUOX1 expression and allergen-induced IL-33 secretion in epithelial cells from patients with allergic asthma, indicating a potential role for DUOX1 in asthma pathology.

METHODS**Cell culture and treatments**

Normal human bronchial epithelial (NHBE) cells were obtained from Lonza (Allendale, NJ) and cultured according to established protocols. Immortalized bronchial epithelial HBE1 cells were cultured, as previously described.²⁴ MTE cells were isolated from either wild-type (WT) C57BL/6 mice or *Duox1*^{-/-} mice²⁵ and cultured, as described previously.^{26,27} Primary human nasal epithelial (HNE) cells were isolated from healthy volunteers and patients with allergic rhinitis and cultured, as recently described.²⁸ Atopy was confirmed based on positive skin test results and increased serum IgE levels (>100 IU/mL), asthma was confirmed by a positive response to bronchodilator (≥200 mL and 12% improvement in FEV₁, forced vital capacity, or both) or a positive methacholine challenge test result (PC₂₀ < 8 mg/mL), and all selected subjects had rhinitis with a sinonasal questionnaire (SNQ) score of greater than 1.²⁹ Human subject protocols were approved by the Institutional Review Board of the University of Vermont, and all subjects provided written informed consent. Cells were seeded at confluence in 24-well culture plates and treated with

extracts of *A alternata* (Greer Laboratories, Lenoir, NC) or *Dermatophagoides pteronyssinus* (house dust mite [HDM]; Greer Laboratories; 1.27 endotoxin units/mg) or with ATP (Sigma, St Louis, Mo), H₂O₂ (Sigma), LPS (from *Escherichia coli* 055:B5; Sigma; 1 μg), or polyinosinic:polycytidylic acid (poly[I:C]; Sigma), and conditioned media were collected at indicated times for cytokine analysis and other biochemical assays. Cell extracts were prepared for mRNA extraction and RT-PCR analysis, biochemical assays, or protein analysis by means of SDS-PAGE and Western blotting (see Table E1 in this article's Online Repository at www.jacionline.org). Pharmacologic inhibitors were administered 30 minutes before cell stimulation.

Mice

C57BL/6J mice (Jackson Laboratory, Bar Harbor, Me) or DUOX1-deficient mice (Lexicon Pharmaceuticals, The Woodlands, Tex)²⁵ at 8 to 12 weeks of age were subjected to brief isoflurane anesthesia for oropharyngeal administration of extracts of *A alternata* (50 μg), HDM (50 μg), LPS (1 μg), recombinant mouse IL-33 (Ser¹⁰⁹-Ile²⁶⁶, <0.1 ng/μg endotoxin; Invitrogen, Grand Island, NY; 1 μg), or ATP (250 μmol/L), all in 50 μL of PBS. Bronchoalveolar lavage (BAL) and lung tissues were collected at 1 or 6 hours after challenge for various analyses. In appropriate experiments mice were administered the EGFR tyrosine kinase inhibitor AG1478 (Sigma; 50 μL of a solution of 10 μmol/L in saline prepared from a 1 mmol/L stock solution in dimethyl sulfoxide [DMSO]) intranasally 1 hour before allergen challenge. All animal procedures were reviewed and approved by the Animal Care and Use Committee of the University of Vermont.

Small interfering RNA silencing

NHBE or HBE1 cells were transfected at 60% to 70% confluence with ON-TARGETplus SMARTpool small interfering RNAs (siRNAs) targeted against *P2RY2*, *CAPN1* (calpain 1), or *CAPN2* (calpain 2) or Non-Targeting siRNA Pool#1 as a control (Dharmacon, Lafayette, Colo) with DharmaFECT transfection reagent, according to the manufacturer's instructions, 72 hours before experimentation. Silencing of DUOX1 or DUOX2 was performed by means of transfection with the siRNA reagents DUOX1 (sense, GCUAUGCA-GAUGGCGUGUAtt; antisense, UACACGCCAUCUGCAUAGCtg) or DUOX2 (sense, CGCAGUCA AUGUCUACAUCtt; antisense, GAUGUAGA CAUUGACUGCGtg) or with Silencer Negative Control #1 siRNA (Invitrogen). MTE cells were transfected at 60% to 70% confluence with predesigned siRNA targets against DUOX1 (100 nmol/L each) or nontarget siRNA (NS-siRNA; Ambion, Austin, Tex; 200 nmol/L) by using DharmaFECT transfection reagent (Dharmacon, Lafayette, Colo) and used for experimentation after 72 hours.²² For *in vivo* siRNA silencing of DUOX1, 2 DUOX1 siRNA targets in sterile PBS (35 μg per target sequence per mouse) or NS-siRNA (70 μg per mouse) were instilled oropharyngeally in mice after achievement of brief isoflurane-induced anesthesia²² 48 hours before allergen challenge.

Biochemical assays

Cell-culture supernatants or BAL fluids were analyzed for IL-33, IL-1α, IL-17E (IL-25), IL-5, and IL-13 by using DuoSet ELISAs from R&D Systems (Minneapolis, Minn). Human thymic stromal lymphopoietin (TSLP) expression was determined by using an ELISA from eBioscience (San Diego, Calif), human IL-17E (IL-25) by using an Omnikine ELISA kit from Assay Biotechnology (Sunnyvale, Calif), and human IL-8/CXCL8 by using an ELISA from BD Biosciences (San Diego, Calif). ATP in BAL fluids was analyzed by using the luciferase-dependent bioluminescence assay (Life Technologies, Grand Island, NY). Extracellular H₂O₂ production was determined by using lactoperoxidase-dependent cross-linking tyrosine and measured by means of HPLC with fluorescence detection, as described previously.²¹ Calpain activity was determined with a Calpain Activity Assay Kit (Abcam, Cambridge, Mass). Calpain inhibitor I (Ac-LLnL-CHO, Invitrogen) or calpain inhibitor IV (Z-LLY-FMK, Abcam) were added 30 minutes before cell stimulation.

Analysis of protein cysteine oxidation

For analysis of protein reduced cysteine content, MTE cells were lysed in deoxygenated Western solubilization buffer containing 100 μmol/L

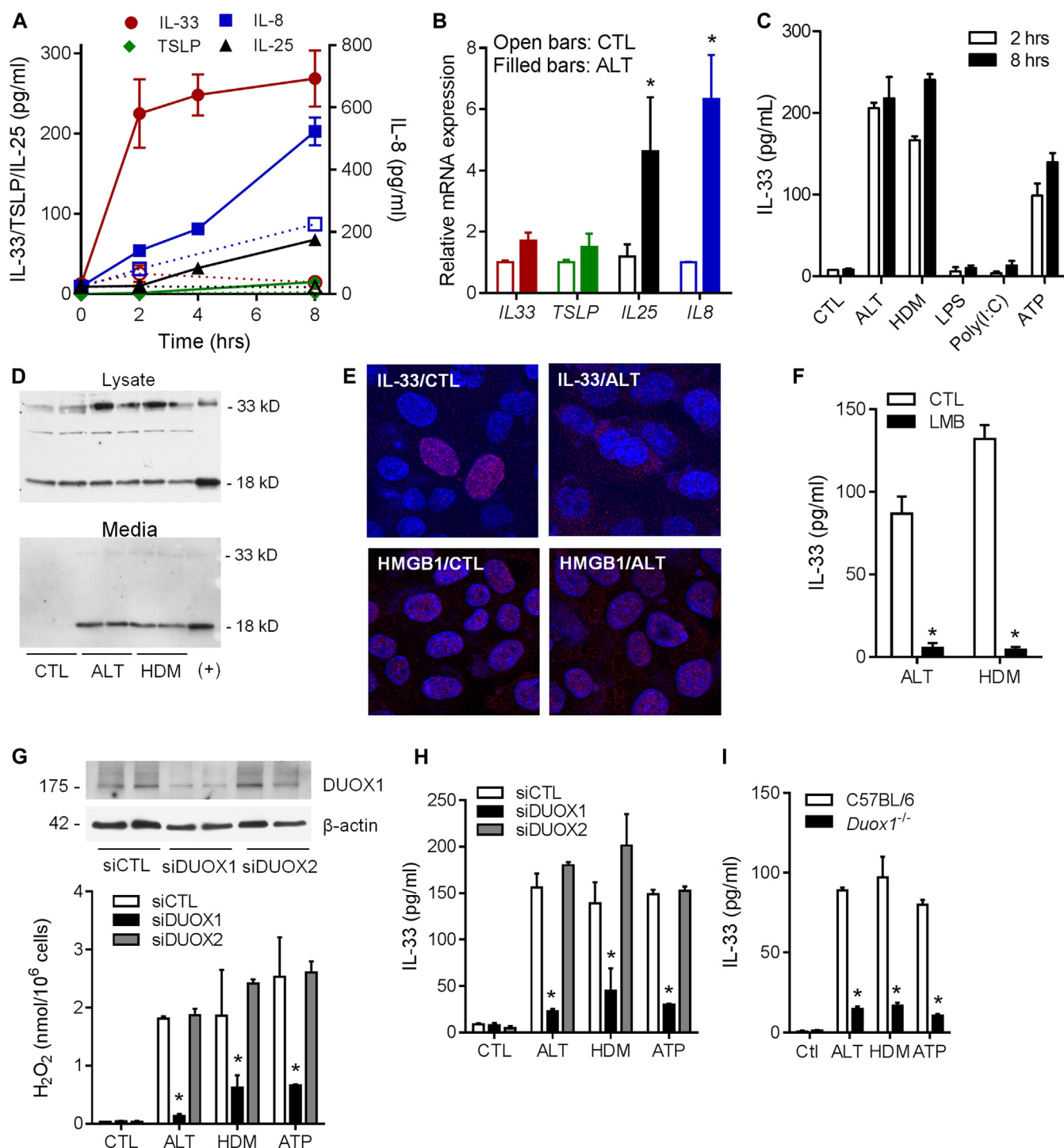


FIG 1. Allergen-induced airway epithelial secretion of IL-33 is mediated by DUOX1 activation. **A** and **B**, Cytokine/chemokine production and cytokine mRNA expression (8 hours) after NHBE cell stimulation with *A. alternata* (50 μ g/mL). Open symbols represent PBS controls. **C**, IL-33 release into NHBE cell supernatants after 2 or 8 hours of stimulation with *A. alternata* (50 μ g/mL), HDM (50 μ g/mL), LPS (1 μ g/mL), poly(I:C) (100 ng/mL), or ATP (100 μ mol/L). **D**, Western blot analysis of cell lysates and culture supernatants of stimulated NHBE cells (8 hours) for full-length (33 kDa) or mature (18 kDa) IL-33. +, Positive IL-33 (18 kDa) control. **E**, Immunofluorescence analysis of IL-33 or high-mobility group 1 (HMGB1) in HBE1 cells after 1 hour of stimulation with *A. alternata*. Nuclei are counterstained in blue. **F**, Effect of 15 minutes of preincubation with leptomycin B (100 nmol/L) on allergen-induced IL-33 secretion (2 hours) from HBE1 cells. **G** and **H**, Effects of siRNA-mediated silencing of DUOX1 or DUOX2 on NHBE cell DUOX protein and allergen-induced H₂O₂ production (15 minutes) and IL-33 secretion (2 hours). **I**, IL-33 secretion after allergen stimulation (2 hours) of MTE cells from WT C57BL/6 or *Duox1*^{-/-} mice. Means $\pm$ SEs from 4 to 6 replicates from 2 to 3 experiments are shown. **P* < .05. ALT, *A. alternata*; CTL, control; siCTL, non-targeting control siRNA; siDUOX, DUOX siRNA.

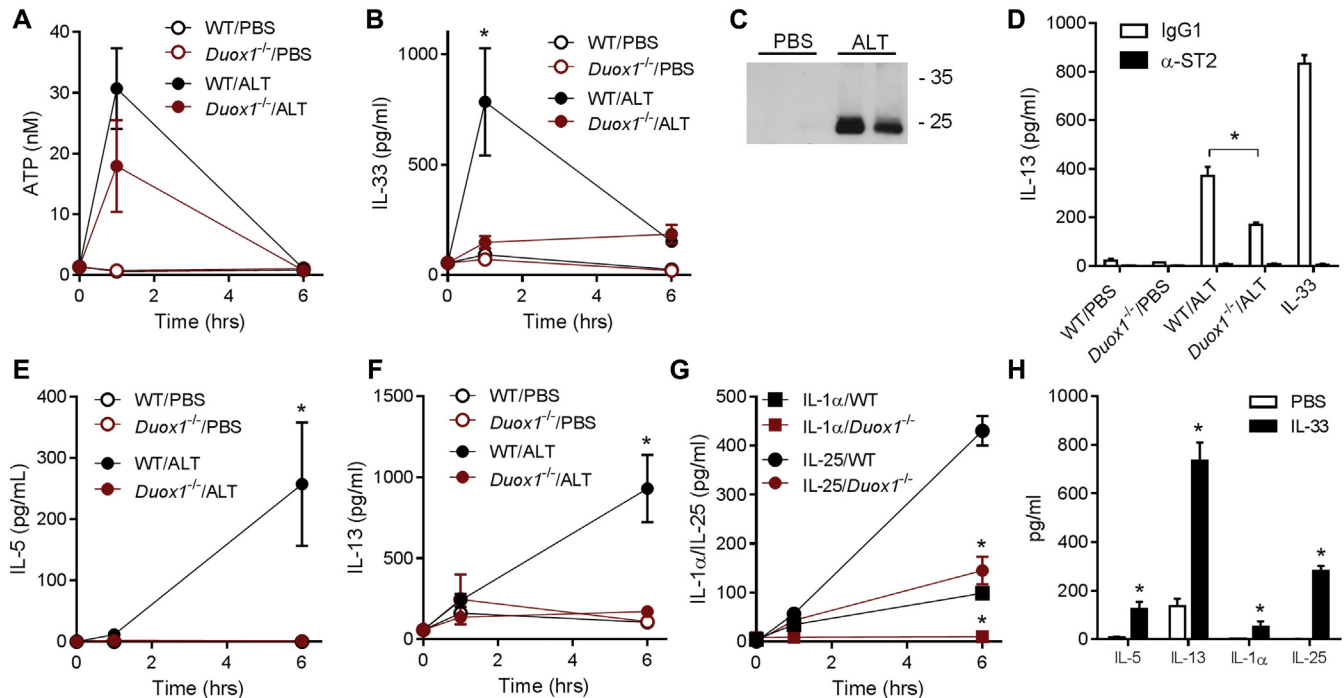


FIG 2. DUOX1 contributes to allergen-induced IL-33 secretion and type 2 responses *in vivo*. **A-C**, Analysis of ATP or IL-33 in BAL fluid from WT or *Duox1*^{-/-} mice after intratracheal instillation of *A alternata* (50 μ g). **D**, Analysis of IL-13 production from BMDM after 72 hours of exposure to BAL fluids from PBS- or *A alternata*-challenged mice (1 hour) or IL-33 (10 ng/mL) in the presence of α -ST2 blocking antibody or control IgG (1 μ g/mL). **E-G**, Analysis of BAL fluid IL-5, IL-13, IL-25, or IL-1 α levels after *A alternata* challenge of WT or *Duox1*^{-/-} mice. **H**, Analysis of BAL fluid cytokine levels after intratracheal instillation of recombinant IL-33 (1 μ g; 6 hours). Means $\pm$ SEs from 4 to 8 replicates from 2 to 3 experiments are shown. **P* < .05. ALT, *A alternata*.

EZ-link Iodoacetyl-LC-biotin (Pierce, Rockford, Ill) and 20 U/mL catalase in an N₂ atmosphere to avoid artificial cysteine oxidation during processing and incubated for 90 minutes at 37°C. For analysis of protein sulfenylation (-S-OH), MTE cells were lysed in deoxygenated Western solubilization buffer containing 1 mmol/L DCP-Bio1 (Kerafast, Boston, Mass), 200 U/mL catalase (Worthington, Lakewood, NJ), and 10 mmol/L *N*-ethylmaleimide (Sigma) and incubated for 1 hour at 4°C. After derivatization, excess biotinylating reagent was removed in both cases by 6 successive washes with 20 mmol/L Tris-HCl (pH 7.4) on Amicon Ultra-0.5 Centrifugal Filter Devices (Millipore, Temecula, Calif), and biotin-tagged proteins were subsequently collected with NeutrAvidin agarose beads (50 μ L of 50/50 slurry, Pierce) and washed successively with 1% SDS, 4 mol/L urea, 1 mol/L NaCl, and 100 mmol/L ammonium bicarbonate (with and without 10 mmol/L dithiothreitol) to remove nonspecifically bound proteins.³⁰ To elute biotinylated proteins, beads were suspended in 2 $\times$ reducing sample buffer containing β -mercaptoethanol and heated (for 10 minutes at 90°C), and eluted proteins were analyzed by means of SDS-PAGE and Western blotting for Src or EGFR. Whole-cell lysates were analyzed similarly as input controls. Alternatively, EGFR was immunoprecipitated from DCP-Bio1-derivatized cell lysates by means of overnight incubation with α -EGFR mAb (C74B9; Cell Signaling, Danvers, Mass) and analyzed by using SDS-PAGE and blotting with streptavidin-horseradish peroxidase (Sigma). To determine *in vivo* protein sulfenylation, mice were administered dimedone (5,5-dimethyl-1,3-cyclohexanedione) intranasally (50 μ L of 1 mmol/L solution in PBS with 1% DMSO) 1 hour before *A alternata* challenge, and lung tissues were collected 1 hour after allergen challenge for homogenization in Western solubilization buffer (containing 1 mmol/L DCP-Bio1, 200 U/mL catalase, and 10 mmol/L *N*-ethylmaleimide) and EGFR immunoprecipitation and streptavidin blotting, as described above.

Statistical analysis

All quantitative data are represented as means and SEs. Statistical differences between groups were evaluated by means of 2-way ANOVA with Bonferroni postanalysis in GraphPad Prism software (version 6.0; GraphPad Software, La Jolla, Calif) and were considered significant at a *P* value of less than .05.

Further details on the Methods used in this study are provided in this article's Online Repository at www.jacionline.org.

RESULTS

DUOX1 mediates airway epithelial secretion of IL-33 in response to allergen challenge

We evaluated epithelial cytokine production by NHBE cells in response to extracts of 2 important airborne allergens that have been associated with asthma: *A alternata* and *Dermatophagoides pteronyssinus* (HDM).^{31,32} As expected,¹⁶ exposure of NHBE cells to extracts of *A alternata* induced rapid release of IL-33, reaching maximal levels after 2 hours, whereas secretion of other T_H2-inducing cytokines (IL-25 and TSLP), as well as the neutrophil chemokine IL-8/CXCL8, was more modest or delayed (Fig 1, A). In contrast to IL-25 and IL-8, allergen-induced IL-33 release was not associated with significant induction of *IL33* mRNA (Fig 1, B) and likely reflected secretion of preformed IL-33 protein. IL-33 release was also evoked by HDM and exogenous ATP but not by the Toll-like receptor (TLR) agonists LPS or poly(I:C) (Fig 1, C), and secreted IL-33 in conditioned media was detected primarily in its mature (18 kDa) form

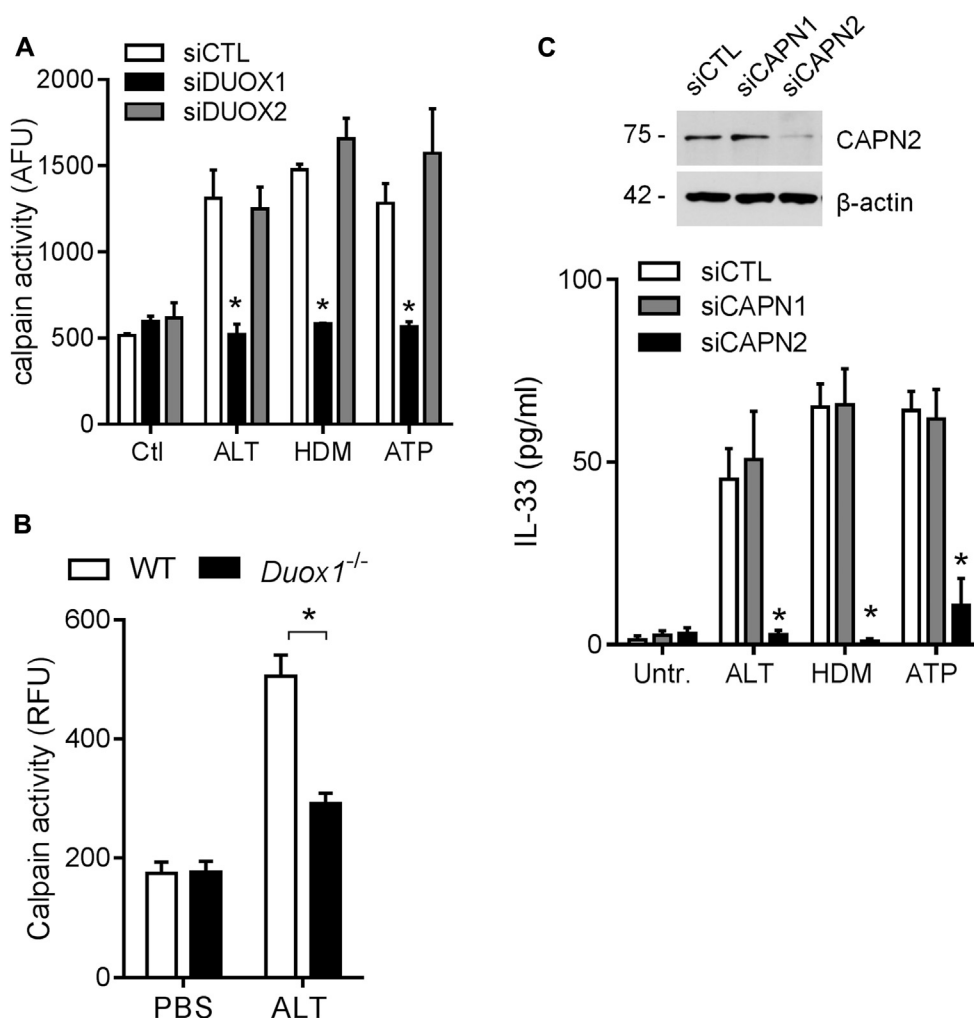


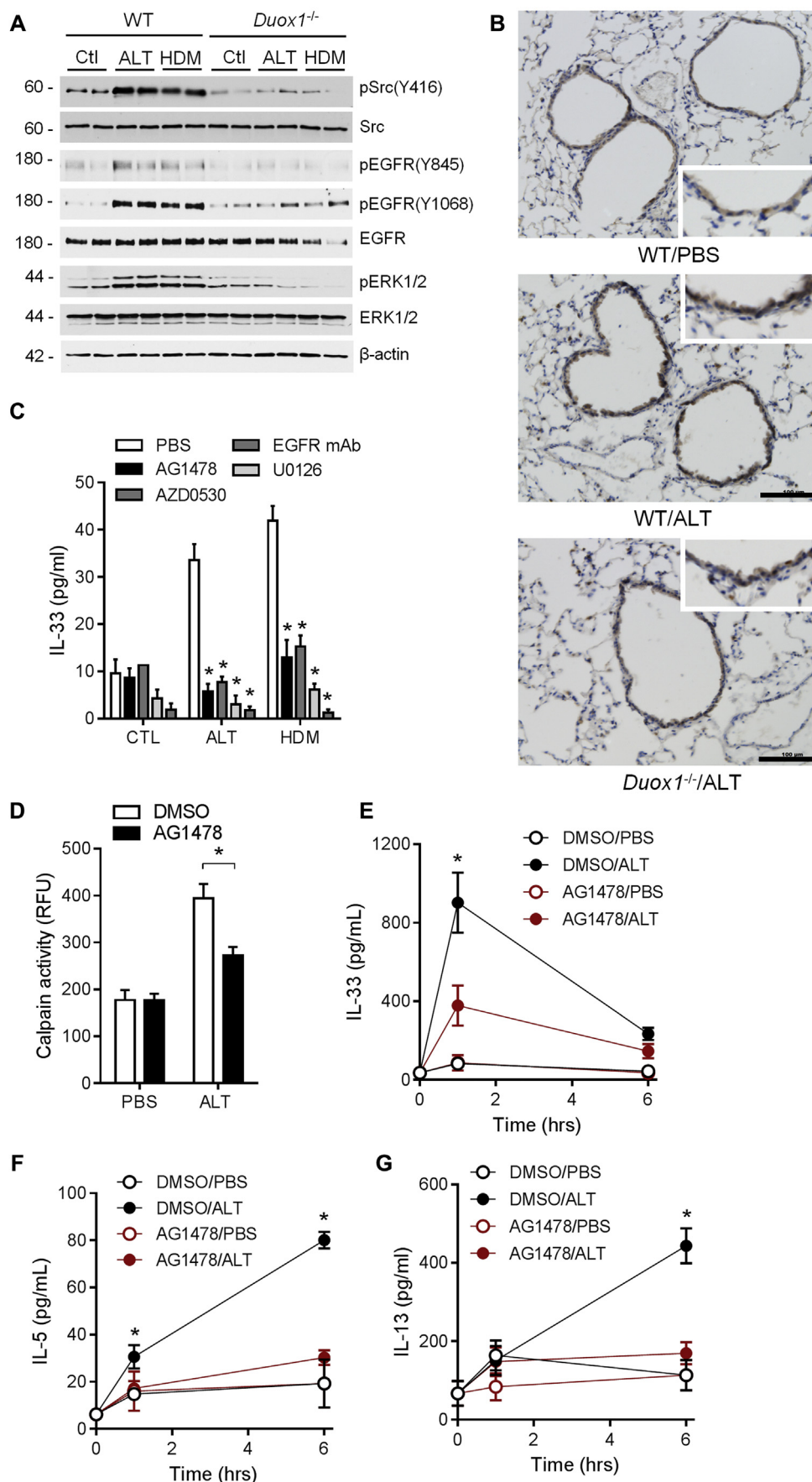
FIG 3. Allergen-mediated IL-33 secretion is mediated by activation of calpain. **A**, Effect of siRNA-mediated silencing of DUOX1 or DUOX2 on allergen-induced (15 minutes) activation of calpain in NHBE cells. **B**, PBS or *A alternata* were instilled intratracheally into mice (1 hour), and calpain activity was analyzed in lung homogenates. **C**, Effect of CAPN1- or CAPN2-targeted siRNA in HBE1 cells or allergen-induced IL-33 release (2 hours). Means $\pm$ SEs from 4 to 6 replicates from 2 to 3 experiments are shown. * $P < .05$. ALT, *A alternata*; CTL, control; siCAPN, CAPN-targeted siRNA; siCTL, non-targeting control siRNA.

(Fig 1, D), indicating the involvement of proteolytic processing. Immunofluorescence analysis indicated export of nuclear IL-33 toward extranuclear regions in response to *A alternata*, whereas high-mobility group 1 (HMGB1), an alternative nuclear protein, remained within the nucleus (Fig 1, E). Accordingly, allergen-induced IL-33 secretion was prevented after preincubation with the nuclear export inhibitor leptomycin B (Fig 1, F). Consistent with earlier findings,¹⁶ IL-33 secretion in response to either allergen was dependent on the epithelial P2Y receptor subtype P2Y₂ (see Fig E1, A, in this article's Online Repository at www.jacionline.org).

After previous findings that ATP-mediated P2Y₂ receptor activation promotes epithelial production of H₂O₂ through activation of the NADPH oxidase DUOX1,¹⁹ we detected rapid epithelial P2Y₂ receptor-dependent H₂O₂ production in NHBE cells in response to either *A alternata* or HDM (see Fig E1, A). siRNA-dependent silencing demonstrated that allergen- or ATP-induced H₂O₂ production was primarily mediated by

DUOX1 and not DUOX2 (Fig 1, G, and see Fig E1, B) and established its critical importance of DUOX1 in allergen-induced IL-33 secretion (Fig 1, H).

Supporting the importance of DUOX1-derived H₂O₂ in epithelial IL-33 secretion, comparable IL-33 secretion could be evoked by direct stimulation with H₂O₂ (see Fig E1, C). Similar findings were obtained by using primary mouse tracheal epithelial (MTE) cells, which similarly demonstrated transient IL-33 release in response to either *A alternata* or HDM (see Fig E1, D) that was mediated by Duox1 based on suppression of allergen-mediated IL-33 secretion after siRNA-mediated DUOX1 silencing (see Fig E1, E) or the absence of allergen-mediated IL-33 secretion in MTE cells from Duox1-deficient mice (Fig 1, G). Importantly, allergen-induced epithelial secretion of IL-33 was not associated with cell necrosis (results not shown), indicating that it involved a redox-dependent cell signaling mechanism initiated by ATP-mediated P2Y₂ receptor activation and DUOX1-dependent production of H₂O₂.



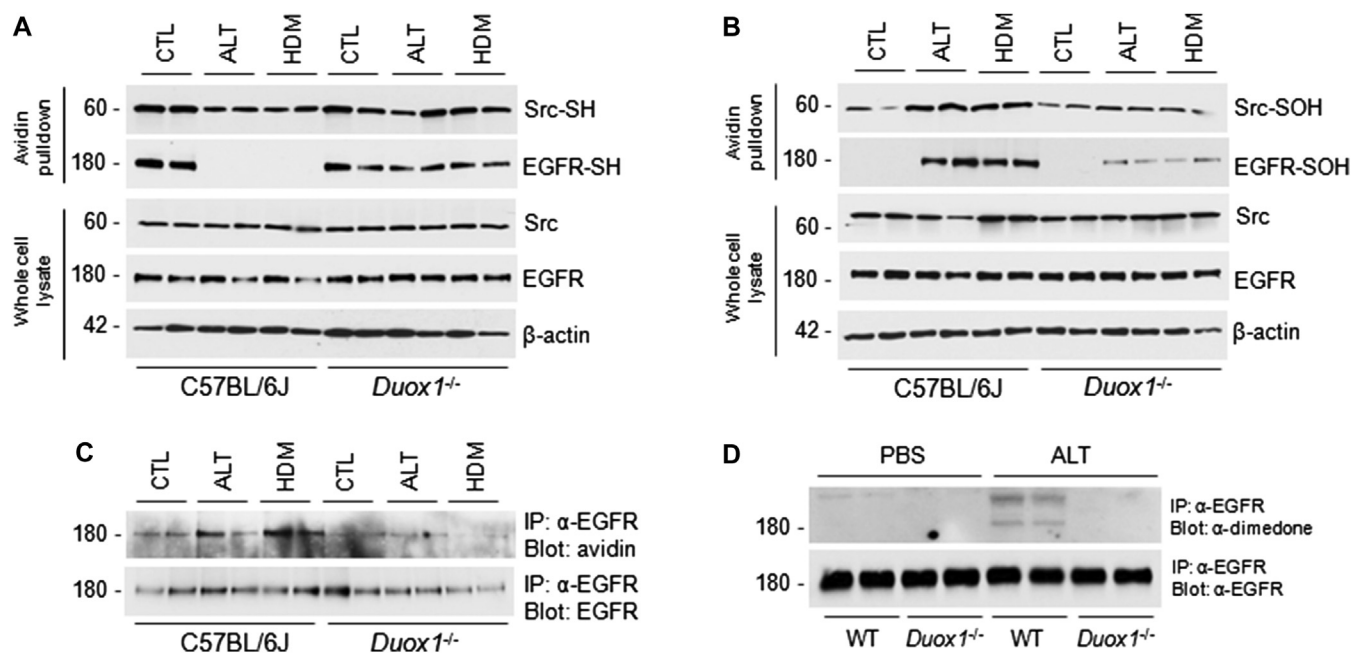


FIG 5. Allergen challenge induces DUOX1-dependent cysteine oxidation within Src and EGFR. **A**, Western blot analysis of Src and EGFR in avidin-purified proteins and corresponding whole-cell lysates from allergen-stimulated (10 minutes) MTE cells from WT or *Duox1*^{-/-} mice after cysteine-selective biotin labeling with Iodoacetyl-LC-Biotin. **B**, Western blot analysis of avidin-purified proteins and whole-cell lysates of allergen-stimulated MTE cells after lysis with DCP-Bio1 to detect protein sulfenylation (-S-OH). **C**, Analysis of immunoprecipitated EGFR from DCP-Bio1-derivatized MTE cell lysates treated as in Fig 5, B, for biotin incorporation by streptavidin blot. **D**, Identification of EGFR-sulfenic acid (SOH) in lung tissues from allergen-challenged WT mice or *Duox1*^{-/-} mice by trapping with dimedone, EGFR immunoprecipitation, and Western blotting with α-dimmedone or α-EGFR. Results are representative of 2 to 3 experiments. ALT, *A alternata*; CTL, control; IP, immunoprecipitation.

DUOX1 contributes to innate type 2 immune responses *in vivo*

C57BL/6 mice were subjected to intratracheal allergen challenge to evaluate IL-33 secretion and activation of subsequent type 2 immune responses within the airway to address the role of DUOX1 in innate allergen responses *in vivo*. As shown in Fig 2, instillation of *A alternata* resulted in rapid and transient increases in levels of BAL fluid ATP (Fig 2, A) and IL-33 (Fig 2, B), with the latter detected primarily as its mature 18-kDa form (Fig 2, C). Confirming the presence of active IL-33, addition of BAL fluids from *A alternata*-challenged mice to cultured bone marrow-derived macrophages (BMDMs) *in vitro* resulted in production of IL-13, which was almost completely dependent on ST2/IL-33 receptor (Fig 2, D). Whereas *A alternata*-induced ATP release was similar in *Duox1*^{-/-} mice compared with that in WT mice (Fig 2, A), allergen-induced IL-33 secretion was dramatically attenuated in *Duox1*^{-/-} mice (Fig 2, B and D).

Similarly, allergen-induced production of the type 2 cytokines IL-5 and IL-13 (Fig 2, E and F), as well as IL-25 (IL-17E) and IL-1α (Fig 2, G), additional epithelial mediators of allergen-induced airway inflammation,^{33,34} was also markedly suppressed in *Duox1*^{-/-} mice. Comparable levels of these latter cytokines were also generated after direct instillation of recombinant IL-33 (Fig 2, H), which is consistent with the suggested role of initial IL-33 secretion in allergen-induced production of these mediators. Highly similar findings were also observed on similar challenge with HDM instead of *A alternata* (see Fig E2, A-C, in this article's Online Repository at www.jacionline.org).

Consistent with a role for initial ATP release in these responses, similar DUOX1-dependent production of type 2 cytokines was observed after direct administration of ATP (see Fig E2, D). Allergen-induced IL-33 secretion and subsequent production of type 2 cytokines were also significantly attenuated after *in vivo* DUOX1 silencing by means of intratracheal administration of

FIG 4. DUOX1-dependent IL-33 secretion is mediated by EGFR signaling. **A**, Analysis for phosphorylation of Src, EGFR, and ERK1/2 after 15 minutes of stimulation of MTE cells from WT or *Duox1*^{-/-} mice with *A alternata* or HDM (representative experiment of 3). **B**, Immunohistochemical analysis of phosphorylated EGFR (Y1068) in lung tissues of C57BL/6J WT or *Duox1*^{-/-} mice after 1 hour of instillation of PBS or *A alternata* (representative of 4 animals). Bar = 100 μm. **C**, Analysis of allergen-induced IL-33 secretion (2 hours) from MTE cells after preincubation with AG1478 (1 μmol/L), α-EGFR mAb (4 μg/mL), U0126 (10 μmol/L), or ADZ0530 (1 μmol/L). **D-G**, C57BL/6J mice were administered AG1478 (intranasally) before *A alternata* challenge, calpain activity was analyzed in lung tissue homogenates (1 hour), and BAL fluid was analyzed for IL-33, IL-5, or IL-13. Means ± SEs of 4 to 6 replicates from 2 to 3 experiments are shown. **P* < .05. ALT, *A alternata*; CTL, control.

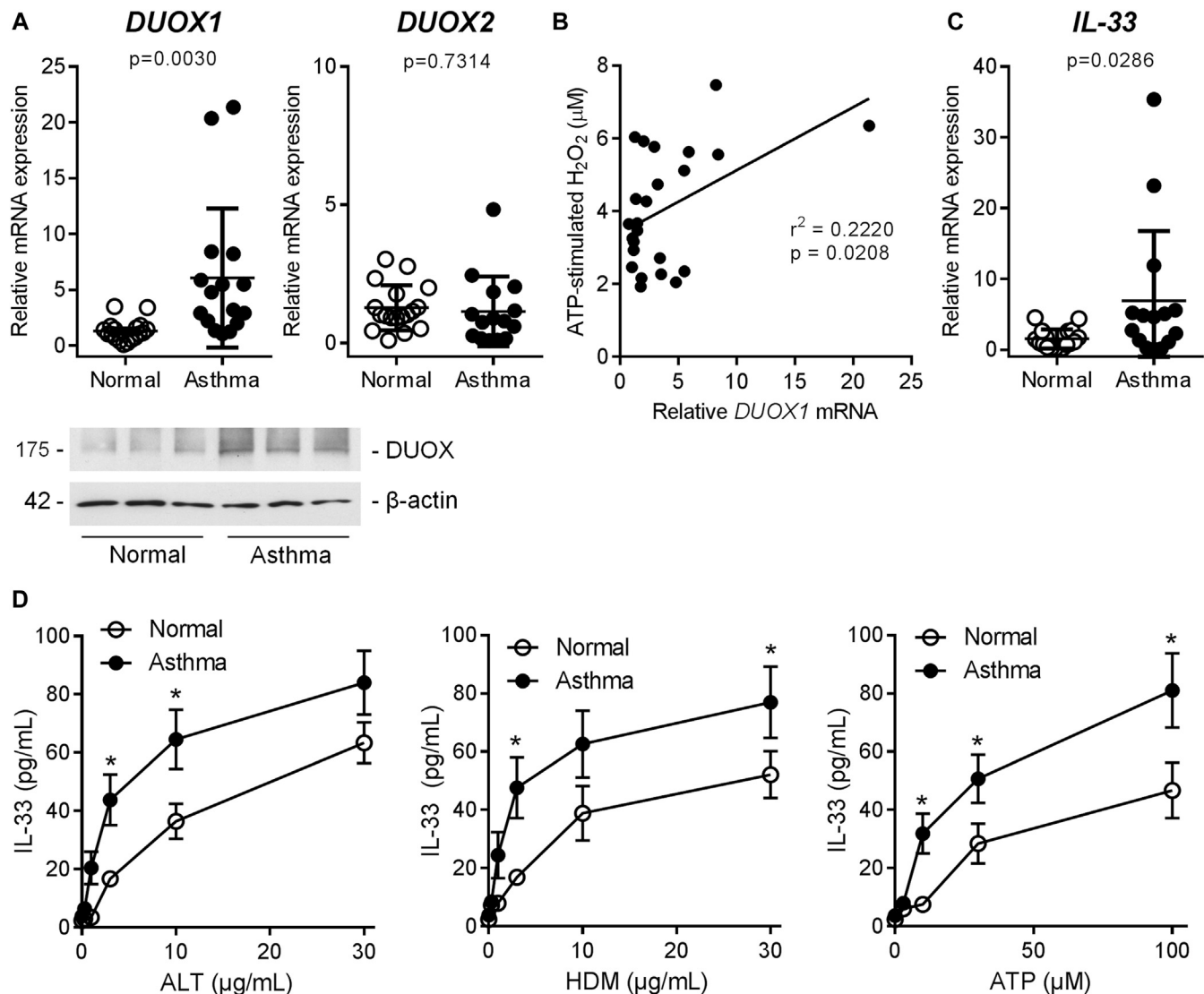


FIG 6. DUOX1 expression and allergen responses are enhanced in HNE cells from asthmatic patients. **A**, Analysis of *DUOX1* and *DUOX2* mRNA or DUOX protein in HNE cells from healthy subjects ($n = 18$) or asthmatic patients ($n = 17$). **B**, Correlation between *DUOX1* mRNA expression and ATP-stimulated ($100 \mu M$) production of H_2O_2 in HNE cells from healthy subjects and asthmatic patients. **C**, RT-PCR analysis of *IL33* mRNA expression in HNE cells from healthy subjects and asthmatic patients. **D**, Quantitation of allergen-induced IL-33 release (2 hours) by HNE cells from healthy subjects ($n = 10$) or asthmatic patients ($n = 11$). Means $\pm$ SEs are shown. * $P < .05$. ALT, *A. alternata*.

DUOX1-targeted siRNA (see Fig E2, E-H), further confirming the critical role of DUOX1 in these responses. Collectively, these findings extend our *in vitro* observations and demonstrate that allergen-induced IL-33 secretion and subsequent production of type 2 cytokines is mediated by initial ATP production and ATP-dependent activation of DUOX1. These allergen responses were likely independent of TLR4-dependent signaling because instillation of LPS ($1 \mu g$) did not induce significant increases in BAL fluid ATP, IL-33, or IL-5/IL-13 levels (results not shown). In contrast, LPS challenge promoted marked production into the BAL fluid of the T_H1 cytokines TNF- α , IL-1 β , and IL-12p40, but this response was identical in both WT and Duox1-deficient mice (see Fig E3, A, in this article's

Online Repository at www.jacionline.org). Conversely, although *A. alternata* induced significant production of IL-33 and type 2 cytokines (Fig 2), only modest and DUOX1-independent increases in IL-1 β (see Fig E3, B) or TNF- α (not shown) were observed. Finally, allergen challenge induced rapid and DUOX1-dependent secretion IL-33 (Fig 1, H) and IL-1 α (see Fig E3, C), whereas only minimal and DUOX1-independent production of IL-1 β was observed. These findings indicate that DUOX1 does not play a general role in innate T_H1 or T_H2 cytokine responses to diverse microbial or allergic triggers but rather plays a more specific role in activating selected IL-1 family members (IL-33 and IL-1 α) to promote type 2 immune responses to allergens or other injurious stimuli.

Allergen-induced IL-33 secretion involves Ca^{2+} signaling and DUOX1-dependent activation of calpain-2

Recent studies have implicated a role for Ca^{2+} signaling and activation of calpain in epithelial secretion of IL-1 α or IL-3.^{35,36} Accordingly, allergen-induced activation of DUOX1¹⁹ and secretion of IL-33¹⁶ were both dramatically suppressed by cell preloading with the Ca^{2+} chelator BAPTA-AM (see Fig E4, A and B, in this article's Online Repository at www.jacionline.org), indicating a critical role for Ca^{2+} -dependent signaling. Moreover, increased calpain activity was detectable in allergen-stimulated HBE1 cells (Fig 3, A) and in mouse lung tissues after *in vivo* allergen challenge (Fig 3, B), which was suppressed after siRNA-dependent DUOX1 silencing or in lungs of *Duox1*^{-/-} mice. To address the role of calpain in allergen-induced IL-33 secretion, we used the calpain inhibitors Ac-LLnL-CHO (ALLN, calpain inhibitor I) and Z-LLY-FMK (calpain inhibitor IV), which both were found to partially inhibit allergen-induced IL-33 secretion (see Fig E4, C). Furthermore, siRNA-mediated silencing experiments indicated that, among the 2 major constitutive calpain isoforms, allergen-induced calpain activation and IL-33 secretion were mediated primarily by calpain 2 (m-calpain; Fig 3, C, and see Fig E4, D and E).

Allergen-induced IL-33 secretion is mediated by DUOX1-dependent activation of EGFR signaling

We previously demonstrated that DUOX1 contributes to epithelial wound responses through redox-dependent activation of Src and EGFR-dependent signaling.^{21,22} Similarly, allergen stimulation of HBE1 cells (not shown) or MTE cells (Fig 4, A) induced rapid phosphorylation of Src, EGFR, and extracellular signal-regulated kinase (ERK) 1/2 and *in vivo* instillation of *A alternata*-enhanced EGFR phosphorylation within the airway epithelium (Fig 4, B), and these responses were largely absent in *Duox1*^{-/-} mice (Fig 4, A and B) or reduced after siRNA silencing of DUOX1 (see Fig E5, A, in this article's Online Repository at www.jacionline.org). Moreover, these kinases are critical in allergen-induced IL-33 secretion by HBE1 or MTE cells because they were dramatically suppressed by pharmacologic inhibition of Src, EGFR, or ERK (Fig 4, C, and see Fig E5, B). The specific role of EGFR signaling was also demonstrated by the inhibitory effects of an EGFR-blocking antibody (Fig 4, C, and see Fig E5, B). Neither AG1478 nor α -EGFR mAb affected ATP-dependent DUOX activation, as measured by H_2O_2 production (not shown). Consistent with recent studies indicating that activation of calpain-2 (m-calpain) requires calpain phosphorylation by EGFR/ERK,³⁷ inhibition of EGFR and/or ERK signaling also markedly prevented allergen-induced calpain activation in MTE cells (see Fig E5, C). Finally, administration of the EGFR tyrosine kinase inhibitor AG1478 before *in vivo* allergen (*A alternata*) challenge in mice prevented allergen-induced EGFR tyrosine phosphorylation (see Fig E5, D) and significantly suppressed allergen-induced increases in calpain activation (Fig 4, D), IL-33 secretion (Fig 4, E), and IL-5/IL-13 production (Fig 4, F and G). Collectively, these findings establish that allergen-induced IL-33 secretion from the airway epithelium is mediated by a concerted mechanism involving DUOX1-dependent EGFR/ERK signaling and calpain-2 activation.

DUOX1-dependent activation of Src/EGFR signaling involves protein cysteine oxidation

Recent studies have indicated that activation of Src family kinases, as well as EGFR, might involve specific oxidation of conserved cysteines within these proteins.^{38,39} To determine whether DUOX1-dependent activation of Src and EGFR was associated with cysteine oxidation, reduced protein cysteine residues (-SH) in cell lysates from allergen-exposed MTE cells were labeled by using thiol-specific biotinylation, after which biotin-tagged proteins were collected by means of avidin purification and probed by using Western blot analysis for the presence of -SH content within Src or EGFR. This revealed that allergen challenge of MTE cells resulted in partial loss of Src-SH content and dramatic loss of EGFR-SH, whereas no such changes were observed in MTE cells from *Duox1*^{-/-} mice (Fig 5, A), indicating the importance of DUOX1 in mediating cysteine oxidation within these kinases. Using a comparable approach to capture formation of sulfenic acids (-S-OH) as the initial product of H_2O_2 -mediated cysteine oxidation, by using chemical derivatization with biotin-tagged dimedone (DCP-Bio1), allergen stimulation of MTE cells was found to result in enhanced presence of both Src-sulfenic acid and EGFR-sulfenic acid (Fig 5, B). Again, allergen-dependent sulfenylation of these proteins was markedly attenuated in MTE cells from *Duox1*^{-/-} mice, illustrating the importance of DUOX1. To confirm specific sulfenylation within EGFR, this receptor was immunoprecipitated from DCP-Bio1-derivatized MTE cell lysates and probed by using streptavidin blotting, which demonstrated increased biotin labeling of EGFR in allergen-stimulated MTE cells from WT mice but not from *Duox1*^{-/-} mice (Fig 5, C). Finally, to demonstrate whether DUOX1 also mediates allergen-induced EGFR sulfenylation *in vivo*, WT or *Duox1*^{-/-} mice were instilled intranasally with dimedone to trap intermediate -S-OH formation in response to subsequent allergen challenge. Analysis of immunoprecipitated EGFR from lung homogenates for incorporation of dimedone indicated increased dimedone labeling in response to allergen challenge in WT but not *Duox1*^{-/-} mice (Fig 5, D). Collectively, these findings demonstrate that DUOX1-dependent secretion of epithelial IL-33 is associated with redox-dependent activation of Src and EGFR signaling through cysteine oxidation.

DUOX1 expression and allergen responses are increased in nasal epithelial cells from asthmatic patients

A growing number of recent studies indicate increased expression and secretion of IL-33 in the airways of asthmatic patients.^{8,40,41} Moreover, because $\text{T}_\text{H}2$ -associated cytokines, such as IL-13, are capable of inducing epithelial DUOX1 expression,²³ we hypothesized that airway DUOX1 expression is enhanced in patients with allergic asthma and potentially contributes to amplified IL-33 secretion and subsequent type 2 responses to allergen challenge. Consistent with this notion, allergen-induced IL-33 secretion by MTE cells was enhanced after prior induction of DUOX1 by means of prestimulation with IL-13 (see Fig E6, A, in this article's Online Repository at www.jacionline.org). We collected HNE cells from 18 healthy human subjects and 17 patients with allergic asthma and rhinitis and evaluated mRNA expression of DUOX1/2, IL-33, and P2YR

subtypes, as well as H₂O₂ production and IL-33 secretion, in response to *in vitro* allergen challenge. Subjects' characteristics are summarized in Table E2 in this article's Online Repository at www.jacionline.org. *DUOX1* mRNA expression was found to be significantly higher in HNE cells from asthmatic patients compared with nonasthmatic control subjects, whereas *DUOX2* mRNA expression was relatively similar (Fig 6, A). Increased *DUOX1* mRNA expression was associated with increased DUOX protein levels, and *DUOX1* mRNA expression correlated positively with ATP-induced H₂O₂ production, a measure of DUOX1 activation (Fig 6, B). In agreement with earlier reports,^{8,40} HNE cells from asthmatic patients also expressed increased *IL33* mRNA expression compared with HNE cells from nonasthmatic control subjects (Fig 6, C). HNE cells from asthmatic patients also expressed increased P2YR2 mRNA expression, although mRNA expression of P2YR6 was comparable between normal and asthmatic HNE cells (see Fig E6, B). Analysis of dose-dependent production of H₂O₂ or IL-33 secretion in response to *A alternata*, HDM, or ATP revealed that allergen-induced H₂O₂ production was consistently higher in HNE cells from asthmatic patients compared with that seen in nonasthmatic control subjects (see Fig E6, C). Moreover, significant increases in allergen-induced IL-33 secretion were observed in HNE cells from asthmatic patients compared with levels in nonasthmatic control subjects at most doses studied (Fig 6, D). Calculation of median effective concentration (EC₅₀) values indicated enhanced sensitivity of HNE cells from asthmatic patients to allergen challenge, as demonstrated by significantly lower EC₅₀ values for HDM and ATP and near-significant lower EC₅₀ values for *A alternata* in HNE cells from asthmatic patients compared with healthy control subjects (see Fig E6, D). Interestingly, although we observed enhanced *IL33* mRNA expression in HNE cells from asthmatic patients, no significant correlation was observed between *IL33* mRNA expression and allergen-induced IL-33 secretion (see Fig E6, E), which would suggest that the increased allergen-dependent IL-33 secretion in epithelia of asthmatic patients is primarily driven by enhanced DUOX1 expression and activation.

DISCUSSION

The IL-1 family member IL-33 has emerged as an important cytokine in innate type 2 immune responses to various allergens and helminths and is thought to play a major role in the pathophysiology of allergic diseases, such as asthma and rhinosinusitis.^{11,42} However, the molecular mechanisms involved in cellular release of IL-33 have remained largely elusive and might involve regulated mechanisms rather than passive release caused by cell necrosis.^{16,17} The present findings are highly significant because they define a previously unrecognized signaling pathway as a common mechanism of epithelial IL-33 secretion in response to diverse allergens, which depends on activation of the NADPH oxidase DUOX1 and redox-dependent activation of Src/EGFR signaling. Although airway responses to diverse airborne allergens likely involve activation of a range of pattern recognition receptors, TLRs, and other receptor types,^{1,32} our findings indicate a common concerted mechanism by which these diverse stimuli evoke epithelial IL-33 secretion and subsequently activate type 2 responses, involving initial ATP release as a cellular damage signal and

purinoceptor-dependent activation of epithelial NADPH oxidase DUOX1. In agreement with studies by Snelgrove et al,⁴³ allergen-induced IL-33 secretion was found to be related to intrinsic protease activity, and effects of *A alternata* and HDM were in both cases suppressed by common serine protease inhibitors, such as phenylmethylsulfonyl fluoride and antipain (results not shown), suggesting that protease-dependent mechanisms are responsible for initial ATP release and activation of DUOX1-dependent signaling.

Our studies also provide novel insight into the as yet incompletely understood biological function of airway DUOX1^{19,20} and highlight its specific role in activation of type 2 immune responses as a key mechanism to enhance host tolerance or resistance against diverse parasites, toxins, or irritants.⁴⁴ Previous studies indicating a role for DUOX1 in epithelial wound responses and repair pathways^{21,22,45} fit this general paradigm, and our present findings indicate that DUOX1-dependent IL-33 secretion and activation of type 2 immune responses form an important component of such host protective responses, serving to minimize tissue injury and enhance tolerance to parasitic infections.^{3,4} Intriguingly, helminth parasites were recently demonstrated to suppress allergen-induced epithelial IL-33 secretion and type 2 host responses; these were attributed to secretion of helminth-derived apyrases,⁴⁶ which would minimize ATP- and DUOX1-mediated host responses. Our findings also indicate that DUOX1 is uniquely involved in activating release of IL-33 or IL-1 α as mediators of injury or sterile inflammation and does not mediate activation of IL-1 β or type 1 immune responses to infectious stimuli, such as LPS. In this regard the host defense function of DUOX1 appears to be distinct from that of its closely related homolog, DUOX2, which has been linked primarily to antimicrobial and antiviral responses mediated by type 1 immune responses^{23,47} and would suggest that the 2 separate DUOX isoforms might have evolved to play unique specific roles in these distinct immune responses.

A final major finding of the present studies is that DUOX1 and allergen-induced IL-33 secretion are markedly increased within the epithelia of asthmatic patients. Given the increasingly recognized role for IL-33 in persistent or steroid-resistant asthma^{40,48} and allergen-induced bronchoconstriction,³⁴ enhanced DUOX1 expression might contribute to exaggerated airway responses to commonly inhaled allergens and mediate exacerbations in certain asthma phenotypes. Thus although DUOX1-dependent activation of IL-33 and type 2 immune responses might be important in host defense by promoting tissue restoration and homeostasis, exaggerated or chronic DUOX1 activation and IL-33 secretion in the context of allergic airway disease might contribute to disease pathology by promoting production of type 2 cytokines (IL-5 and IL-13) known to mediate eosinophilic inflammation and mucus metaplasia and related to presence of increased numbers of IL-33 target cells in the asthmatic airway, such as type 2 innate lymphoid cells⁴⁹ or mast cells,⁵⁰ which mediate type 2 cytokine production and bronchoconstriction. Although the present studies are the first to demonstrate increased DUOX1 expression in human asthma, a recent report highlighted enhanced expression of DUOX2 and its potential involvement in oxidative mechanisms in the airways of patients with severe asthma.⁵¹ Because asthma is a highly heterogeneous disease, it is plausible that both DUOX1 and DUOX2 might be of variable importance in different asthma

subgroups.⁵² Our studies would argue that DUOX1 might be particularly important in asthma subgroups with a high T_H2 signature in which IL-33 could contribute importantly to persistent steroid-resistant asthma or asthma exacerbations.^{40,41,43} Recent findings of increased DUOX1-and/or IL-33-mediated type 2 responses in patients with nasal chronic rhinosinusitis^{42,53} or cutaneous atopic disease^{54,55} would also suggest a more common role for DUOX1 in several allergen-related pathologies.

Contrasting claims that epithelial secretion of IL-33 results primarily from passive release during necrosis, our findings reveal the involvement of DUOX1-mediated and redox-dependent activation of Src- and EGFR-dependent signaling and activation of calpain-2 as critical steps in allergen-induced secretion of IL-33 (see Fig E7 in this article's Online Repository at www.jacionline.org). Although we determined that allergen-induced calpain activation was dependent on Ca²⁺ signaling, calpain-2 (also known as m-calpain) requires millimolar concentrations of Ca²⁺ for activation *in vitro*, and *in vivo* activation of calpain-2 was found to also depend on serine phosphorylation by EGFR/ERK pathways.³⁷ Hence the observed requirement of Ca²⁺ for calpain activation in our studies likely points to its importance in DUOX1 activation and subsequent EGFR/ERK activation rather than direct regulation of calpain-2 activity. However, the mechanism or mechanisms by which calpain-2 promotes allergen-induced IL-33 secretion remains unclear and might include a direct role in proteolytic IL-33 processing^{10,36} but could also include less defined roles in cytoskeletal organization and secretory pathways.^{56,57} Furthermore, DUOX1-dependent activation of Src or EGFR might also promote IL-33 secretion through actions independent of calpain, such as by promoting nuclear export or through other cytoskeletal mechanisms involved in nonclassical secretion pathways.

Our studies indicate a direct role for DUOX1 in the activation of Src and EGFR caused by DUOX1-dependent reversible cysteine oxidation within these kinases, which is consistent with previous studies demonstrating a critical role for oxidation of critical conserved cysteines in regulating activation of these tyrosine kinases.^{38,39} However, the precise relationship or relationships between cysteine oxidation and tyrosine phosphorylation and kinase activation still remain to be fully elucidated and would require more detailed structure-function analysis. Sulfenylation of C797 in the tyrosine kinase domain of EGFR has been suggested to enhance intrinsic kinase activity,³⁹ and several conserved cysteines have been implicated in oxidant-dependent activation of Src,³⁸ but the precise structural and functional consequences of cysteine oxidation for kinase activation and (auto)phosphorylation are still unclear. Our recent studies indicating a direct association between DUOX1 and Src in response to ATP stimulation suggest a direct role for DUOX1 in oxidative Src activation in EGFR transactivation,²¹ and our observation of DUOX1-dependent cysteine oxidation of EGFR might imply a similar direct role for DUOX1 in regulating EGFR activation. However, DUOX1-mediated EGFR transactivation also involves intermediate production of EGFR ligands,²¹ which might promote EGFR activation and cysteine oxidation through the alternative NADPH oxidase NOX2.³⁹ Therefore the precise oxidant-dependent mechanisms involved in allergen-induced EGFR activation are likely to be more complex and might involve the contribution of indirect oxidative mechanisms, possibly triggered by initial DUOX1 activation. Finally, DUOX1

activation might also affect additional redox-sensitive pathways independent of EGFR, as indicated by our recent proteomic studies.⁵⁸

In summary, our findings highlight a critical and common role for airway epithelial DUOX1 in initiation of innate airway type 2 inflammatory responses to diverse airborne protease allergens through activation of redox-dependent signaling pathways that mediate epithelial secretion of IL-33. Although DUOX1-dependent activation of IL-33 likely represents an important component of the host protective properties associated with type 2 immunity, excessive or inappropriate DUOX1 expression or activation might also contribute to allergic disease caused by sensitized or augmented IL-33 production, which might be especially relevant in specific subgroups with persistent or steroid-resistant asthma.^{40,48} In these cases DUOX1 might present a novel and highly attractive therapeutic target to increase asthma control or minimize risk of exacerbations.

Key messages

- Allergen-induced epithelial secretion of IL-33 is mediated by redox-dependent activation of EGFR-dependent signaling caused by initial activation of the epithelial NADPH oxidase isoform DUOX1.
- Nasal epithelial cells from human subjects with allergic asthma display enhanced DUOX1 expression, as well as allergen-induced IL-33 secretion.
- Increased expression and activation of DUOX1 might be an important feature of enhanced IL-33 secretion in patients with severe and persistent asthma.

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METHODS

Isolation and culture of MTE cells

MTE cells were isolated from tracheas by using overnight incubation with 0.1% protease 14 (Sigma-Aldrich, St Louis, Mo) and cultured on rat tail collagen I gel (BD Biosciences) in DMEM/F12 media (Invitrogen) supplemented with 20 ng/mL cholera toxin (List Biological Laboratories, Campbell, Calif), 10 ng/mL EGF (Calbiochem, San Diego, Calif), 5 µg/mL insulin (Sigma), 5 µg/mL transferrin (Sigma), 100 nmol/L dexamethasone (Sigma), 15 µg/mL bovine pituitary extract (Invitrogen), 2 mmol/L L-glutamine, and 50 U/50 µg/mL penicillin/streptomycin (Invitrogen).^{E1,E2} MTE cells were used at passages 2 to 3 for experiments.

Cell culture and treatments

Experiments were performed with NHBE cells obtained from Lonza (Allendale, NJ) and cultured according to established protocols, as well as immortalized bronchial epithelial HBE1 cells^{E3} and MTE cells obtained from either WT C57BL/6 mice or *Duox1*^{-/-} mice.^{E4} Cells were seeded at confluence in 24-well culture plates and treated with extracts of *A alternata* (Greer Laboratories, Lenoir, NC) or *Dermatophagoides pteronyssinus* (HDM; Greer Laboratories; 1.27 endotoxin units/mg) or with ATP (Sigma), H₂O₂ (Sigma), LPS (from *Escherichia coli* 055:B5; Sigma; 1 µg), or poly(I:C) (Sigma), and conditioned media were collected at indicated times for cytokine analysis and other biochemical assays. Cell extracts were prepared for mRNA extraction, biochemical assays, or protein analysis by using SDS-PAGE and Western blotting. Pharmacologic inhibitors were administered 30 minutes before cell stimulation.

Mice

C57BL/6J mice were purchased from the Jackson Laboratory (Bar Harbor, Me). DUOX1-deficient mice were generated by using a retroviral-based gene-trapping method (Lexicon Pharmaceuticals, The Woodlands, Tex) and backcrossed onto a C57BL/6J background.^{E4} Both male and female mice 8 to 12 weeks of age were used for experimentation. Mice were subjected to brief isoflurane-induced anesthesia for oropharyngeal administration of extracts of *A alternata* (50 µg), HDM (50 µg), LPS (1 µg), recombinant mouse IL-33 (Ser¹⁰⁹-Ile²⁶⁶, <0.1 ng/µg endotoxin; Invitrogen; 1 µg), or ATP (250 µmol/L), all in 50 µL of PBS, and BAL fluid and lung tissues were collected at 1 or 6 hours after challenge for various analyses. In appropriate experiments mice were administered the EGFR tyrosine kinase inhibitor AG1478 (Sigma; 50 µL of a solution of 10 µmol/L in saline prepared from a 1 mmol/L stock solution in DMSO) intranasally 1 hour before allergen challenge. All animal procedures were reviewed and approved by the Animal Care and Use Committee of the University of Vermont.

Recruitment of human subjects and isolation of HNE cells

Healthy volunteers and patients with allergic rhinitis and asthma were recruited to the study. Atopy was confirmed based on positive skin test results and increased serum IgE levels (>100 IU/mL), asthma was confirmed by a positive response to bronchodilator (≥200 mL and 12% improvement in FEV₁, forced vital capacity, or both) or a positive methacholine challenge test result (PC₂₀ < 8 mg/mL), and all selected subjects had rhinitis with an SNQ score of greater than 1.^{E5} Healthy volunteers had no history of rhinitis (SNQ <1) or asthma, negative skin test results, and negative methacholine challenge test results. Human subject protocols were approved by the Institutional Review Board of the University of Vermont, and all subjects provided written informed consent. Primary HNE cells were collected by means of gently stroking the inferior turbinate surface with a Rhino-Probe curette (Arlington Scientific, Arlington, Tex). Primary HNE cells were cultured, as recently described,^{E6} and expanded to passage 2 in bronchial epithelial growth medium (BEGM; Lonza, Walkersville, Md). For experiments, HNE cells were plated on collagen-coated 24-well culture plates (Corning Laboratories, Corning, NY) and cultured in a 1:1 mixture of bronchial epithelial cell basic medium

(BEBM, Lonza) and DMEM-H (Gibco) with SingleQuot supplements (Lonza), bovine pituitary extracts (12.5 µg/mL), and BSA (1.5 µg/mL, Invitrogen) and grown to confluence.

Isolation and culture of BMDMs

Isolation of BMDMs was performed, as described previously.^{E7} Briefly, femurs of C57BL/6 mice were excised, placed in a dish containing sterile PBS, and flushed with 1 mL of HyClone RPMI 1640 culture medium (Thermo Scientific, Waltham, Mass) containing 20% FBS (Invitrogen) and 1% penicillin/streptomycin (Invitrogen). Flushed material was centrifuged and resuspended in fresh medium and seeded on a culture Petri dish. After overnight culture, nonadherent cells were collected and seeded in 24-well plates at 10⁶ cells/well and cultured for 7 to 10 days in the presence of 20 ng/mL macrophage colony-stimulating factor (R&D Systems, Minneapolis, Minn). Thus obtained BMDMs (in 500 µL of RPMI media without macrophage colony-stimulating factor) were stimulated with BAL fluid from PBS- or *A alternata*-stimulated mice (100 µL) or with recombinant IL-33 (10 ng/mL) for 72 hours in the presence of anti-mST-2/IL-1 R4 or IgG_{2B} isotype control (R&D Systems, Minneapolis, Minn) for analysis of IL-33-dependent production of IL-13.

siRNA silencing

HBE cells were transfected at 60% to 70% confluence with ON-TARGETplus SMARTpool siRNAs targeted against *P2RY2*, *CAPN1*, or *CAPN2* or Non-Targeting siRNA Pool#1 as a control (Dharmacon) by using DharmaFECT transfection reagent, according to the manufacturer's instructions, 72 hours before experimentation. Silencing of DUOX1 or DUOX2 was performed by means of transfection with the following siRNA reagents (DUOX1, [sense] GCUAUGCAGAUGGCGUGUAtt and [antisense] UACACGCCAUCUGCAUAGCtg; DUOX2, [sense] CGCAGUCAUGU CUACAUCtt and [antisense] GAUGUAGACAUUGACUGCGtg) or with Silencer Negative Control #1 siRNA (Invitrogen). MTE cells were transfected at 60% to 70% confluence with predesigned siRNA targets against DUOX1 (100 nmol/L each) or NS-siRNA (200 nmol/L; Ambion, Austin, Tex) by using DharmaFECT transfection reagent (Dharmacon) and used for experimentation after 72 hours.^{E8} For *in vivo* siRNA silencing of DUOX1, 2 DUOX1 siRNA targets in sterile PBS (35 µg/target sequence/mouse) or NS-siRNA (70 µg/mouse) were instilled oropharyngeally in mice after achievement of brief isoflurane anesthesia^{E8} 48 hours before allergen challenge.

ELISAs

Cell-culture supernatants or BAL fluid were analyzed for murine or human IL-33, IL-1α, IL-17E (IL-25), IL-5, or IL-13 by using DuoSet ELISAs from R&D Systems. Human TSLP expression was determined by using an ELISA from eBioscience (San Diego, Calif), human IL-17E (IL-25) by using an Omnikine ELISA kit from Assay Biotechnology (Sunnyvale, Calif), and human IL-8/CXCL8 by using an ELISA from BD Biosciences (San Diego, Calif).

Western blotting

Cell lysates or lung homogenates were prepared by using Western solubilization buffer (50 mmol/L HEPES, 250 mmol/L NaCl, 1.5 mmol/L MgCl₂, 1% Triton-X100, 10% glycerol, 1 mmol/L ethyleneglycol-bis-(β-aminoethylether)-N,N,N',N'-tetraacetic acid, 1 mmol/L phenylmethylsulfonyl fluoride, 2 mmol/L Na₃VO₄, 10 µg/mL aprotinin, and 10 µg/mL leupeptin; pH 7.4), and samples containing equal amounts of protein (measured by using BCA protein assay kit; Pierce) were separated on 10% SDS-PAGE gels, transferred to nitrocellulose membranes, and probed with antibodies against P2Y2R (Invitrogen; 1:250), EGFR (C74B9; 1:1000), pEGFR Tyr845 (D63B4; 1:500), pEGFR Tyr1068 (D7A5; 1:1000), p-Src Tyr416 (1:1000), Src (L4A1; 1:1000; all from Cell Signaling, Danvers, Mass), DUOX1 (1:1000; kindly provided by F. Miot^{E9}), IL-33 (Nessy-1; Enzo Life Sciences, Farmingdale, NY; 1:1000), or Calpain-2 (Abcam,

Cambridge, United Kingdom; 1:1000). Primary antibodies were probed with rabbit or mouse-specific secondary antibodies conjugated with horseradish peroxidase (Cell Signaling) and detected by means of chemiluminescence with SuperSignal West Pico Chemiluminescent Substrate (Pierce).

Immunohistochemistry/immunofluorescence

Mouse left lung lobes were fixed in paraformaldehyde and paraffin embedded, and 5- μ m sections were deparaffinized and subjected to antigen retrieval by 15 minutes of boiling in 1 mmol/L EDTA (pH 8.0). After quenching of endogenous peroxidase activity with 3% H₂O₂, sections were incubated overnight with Phospho-EGF Receptor (Tyr1068) Rabbit mAb (D7A5; Cell Signaling; 1:125), which was visualized by means of horseradish peroxidase-conjugated secondary antibody (Vectastain ABC kit PK-4001; Vector Laboratories, Burlingame, Calif) and counterstaining with hematoxylin. HBE1 cells on chamber slides were fixed in Z-Fix (Anatech, Battle Creek, Mich), permeabilized in 0.2% Triton/1% BSA, blocked with 10% donkey serum, and probed with either goat anti-human IL-33 (R&D Systems; 5 μ g/mL) or high-mobility group 1 (HMGB1; Abcam; 1:200), followed by Alexa Fluor 555 anti-rabbit or anti-goat IgG (Invitrogen, 1:500), and analyzed by using confocal microscopy with a Zeiss LSM 510 META confocal scanning laser microscope (Carl Zeiss Microimaging, Thornwood, NY).

Biochemical assays

ATP in BAL fluid was analyzed by using a luciferase-dependent bioluminescence assay (Life Technologies, Grand Island, NY). For analysis of H₂O₂ production, culture media were replaced with HBSS before cell stimulation, and aliquots of culture supernatants were mixed with tyrosine and lactoperoxidase for analysis of H₂O₂-dependent tyrosine cross-linking, as measured by using HPLC with fluorescence detection, as described previously.^{E10} Calpain activity in cell lysates was determined with a Calpain Activity Assay Kit (Abcam). Calpain inhibitor I (Ac-LLN-CHO; Invitrogen) or calpain inhibitor IV (Z-LLY-FMK; Abcam) were added 30 minutes before cell stimulation.

RT-PCR

Total cell or tissue RNA was extracted with TRIzol (Invitrogen) and the RNeasy Mini Kit (Qiagen, Germantown, Md) and reverse transcribed with M-MLV reverse transcriptase and Oligo(dT)12-16 primer. PCR amplification was performed with SYBR Green PCR Supermix (Bio-Rad Laboratories, Hercules, Calif) with 1 μ L of cDNA and 0.5 μ mol/L predesigned primers (see Table E1), and quantification of relative gene expression was performed with the $\Delta\Delta$ cycle threshold method, normalizing the cycle threshold value to the housekeeping gene glyceraldehyde-3-phosphate dehydrogenase (*GAPDH*).

Analysis of protein cysteine oxidation

For analysis of protein-reduced cysteine content, MTE cells were lysed in deoxygenated Western solubilization buffer containing 100 μ mol/L EZ-link Iodoacetyl-LC-biotin (Pierce) and 20 U/mL catalase in an N₂ atmosphere to avoid artificial cysteine oxidation during processing and incubated for 90 minutes at 37°C. For analysis of protein sulfenylation (-S-OH), MTE cells were lysed in deoxygenated Western solubilization buffer containing 1 mmol/L DCP-Bio1 (Kerafast, Boston, Mass), 200 U/mL catalase (Worthington, Lakewood, NJ), and 10 mmol/L *N*-ethylmaleimide (Sigma) and incubated for 1 hour at 4°C. After derivatization, excess biotinylating reagent was removed in both cases by means of 6 successive washes with 20 mmol/L Tris-HCl (pH 7.4) on Amicon Ultra-0.5 Centrifugal Filter Devices

(Millipore, Temecula, Calif), and biotin-tagged proteins were subsequently collected with NeutrAvidin agarose beads (50 μ L of a 50/50 slurry; Pierce) and washed successively with 1% SDS, 4 mol/L urea, 1 mol/L NaCl, and 100 mmol/L ammonium bicarbonate (with and without 10 mmol/L dithiothreitol) to remove nonspecifically bound proteins.^{E11} To elute biotinylated proteins, beads were suspended in 2 $\times$ reducing sample buffer containing β -mercaptoethanol and heated (10 minutes at 90°C), and eluted proteins were analyzed by using SDS-PAGE and Western blotting for Src or EGFR. Whole-cell lysates were analyzed similarly as input controls. Alternatively, EGFR was immunoprecipitated from DCP-Bio1-derivatized cell lysates by means of overnight incubation with α -EGFR mAb (C74B9; Cell Signaling) and analyzed by using SDS-PAGE and blotting with streptavidin-horseradish peroxidase (Sigma). To determine *in vivo* protein sulfenylation, mice were administered dimedone (5,5-dimethyl-1,3-cyclohexanedione) intranasally (50 μ L of 1 mmol/L solution in PBS with 1% DMSO) 1 hour before *A. alternata* challenge, and lung tissues were collected 1 hour after allergen challenge for homogenization in Western solubilization buffer (containing 1 mmol/L DCP-Bio1, 200 U/mL catalase, and 10 mmol/L *N*-ethylmaleimide) and EGFR immunoprecipitation and streptavidin blotting, as described above.

Statistical analysis

All quantitative data are represented as means and SEs. Statistical differences between groups were evaluated by using 2-way ANOVA with Bonferroni postanalysis in GraphPad Prism software (version 6.0; GraphPad Software, La Jolla, Calif) and were considered significant at a *P* value of less than .05.

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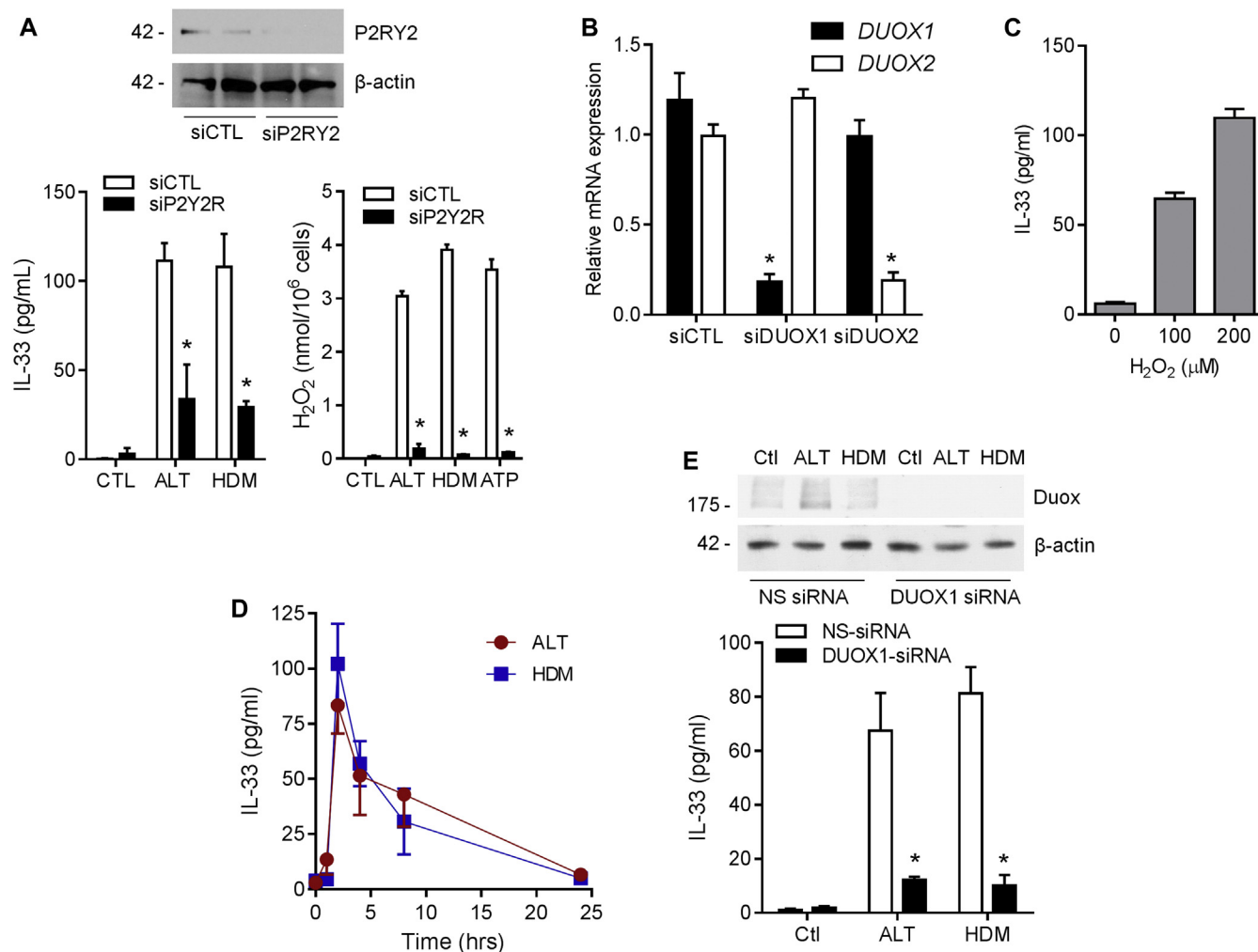


FIG E1. DUOX1- and P2Y2R-dependent responses to allergen challenge in human bronchial epithelial cells or MTE cells. **A**, Effect of siRNA-mediated targeting of P2Y2R in NHBE cells on P2Y2R protein expression (top) and allergen-induced extracellular H_2O_2 production (15 minutes, bottom left) or IL-33 secretion (2 hours, bottom right). **B**, Evaluation of efficacy of siRNA-dependent silencing of *DUOX1* or *DUOX2* in NHBE cells by using quantitative PCR. **C**, Effect of NHBE cell stimulation with H_2O_2 (2 hours) on IL-33 secretion, as measured by using ELISA. **D**, Time-dependent release of IL-33 in culture supernatants after stimulation of MTE cells with *A. alternata* or HDM. **E**, Effect of DUOX1 siRNA targeting of MTE cells on DUOX protein levels (top) and allergen-induced IL-33 secretion measured after 2 hours of stimulation (bottom). Data represent means $\pm$ SEs of 3 to 6 replicates from 2 to 3 independent experiments. * $P < .05$ compared with siCTL. ALT, *A. alternata*; CTL, control. siCTL, Non-targeting control siRNA; siP2Y2R, P2Y2R-targeted siRNA.

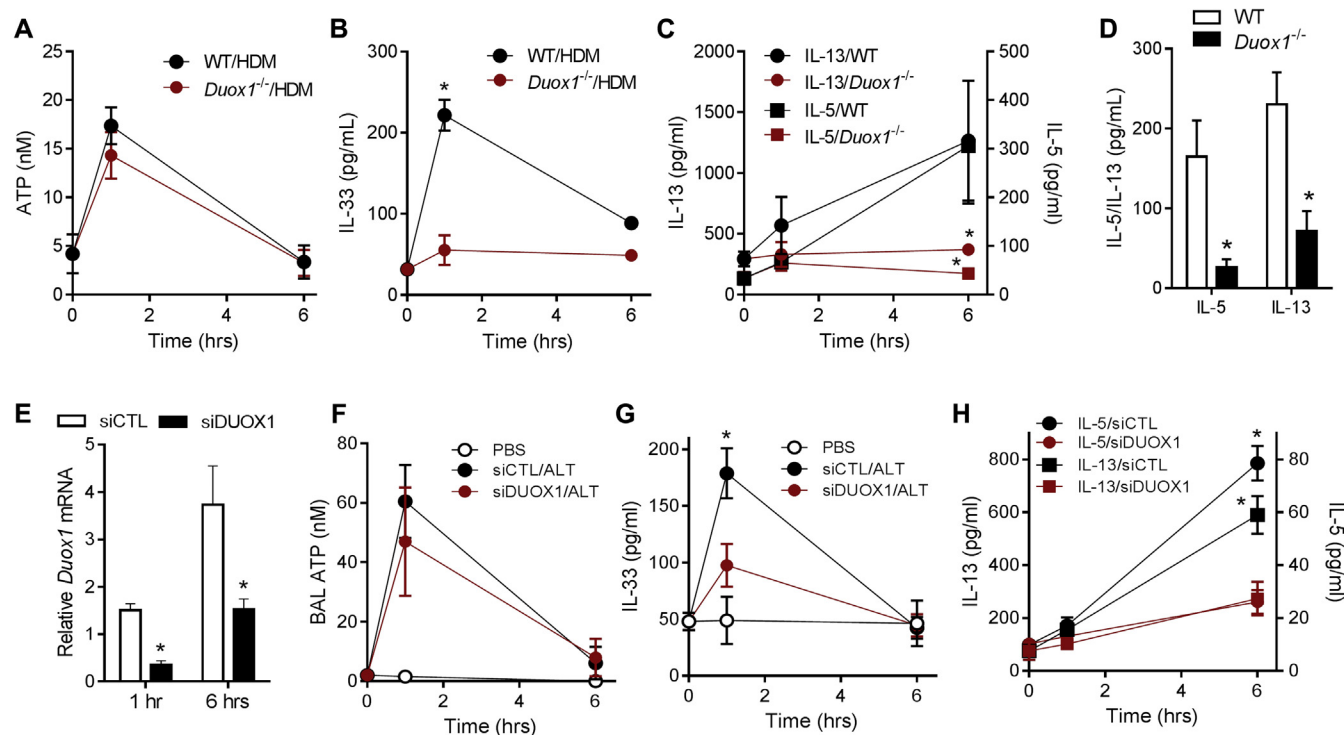


FIG E2. DUOX1 mediates allergen-induced IL-33 secretion and type 2 immune responses *in vivo*. **A-C**, C57BL/6 WT or *Duox1*^{-/-} mice were challenged with HDM extract (intratracheal; 50 μ g in 50 μ L of PBS), and BAL fluid was collected after 1 or 6 hours for analysis of ATP (Fig E2, A), IL-33 (Fig E2, B) and IL-5 and IL-13 (Fig E2, C) levels. **D**, Analysis of BAL fluid IL-5 and IL-13 levels 6 hours after intratracheal instillation of ATP (50 μ L; 250 μ mol/L) into C57BL/6 WT or *Duox1*^{-/-} mice. **E-H**, C57BL/6 mice were instilled with DUOX1-targeted siRNA (*siDUOX1*) or nontargeting siRNA (*siCTL*) 3 days before intratracheal challenge with *A. alternata* (50 μ g in 50 μ L of PBS), and lung tissues or BAL fluids were collected 1 or 6 hours after challenge for analysis of lung tissue *DUOX1* mRNA expression (Fig E2, E) or BAL fluid levels of ATP (Fig E2, F), IL-33 (Fig E2, G), and IL-5 and IL-13 (Fig E2, H). Data represent means $\pm$ SEs of 4 to 8 replicates from 2 to 3 independent experiments. * P < .05 compared with corresponding controls. ALT, *A. alternata*; CTL, control.

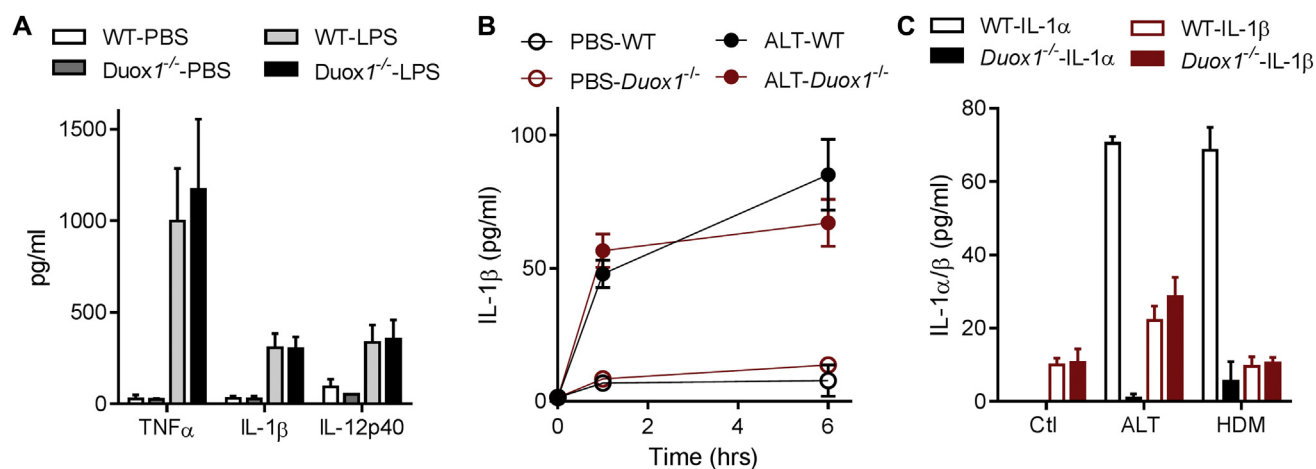


FIG E3. DUOX1 does not mediate innate T_H1 responses to LPS- or allergen-induced production of IL-1 β . **A**, C57BL/6 WT or Duox1^{-/-} mice were challenged with intratracheal LPS (1 μ g) or PBS for 6 hours, and BAL fluid was analyzed for TNF- α , IL-1 β , or IL-12p40 by using ELISA. **B**, C57BL/6 WT (black symbols) or Duox1^{-/-} mice (red symbols) were challenged with intratracheal *A alternata* (50 μ g) or PBS, and BAL fluid collected at indicated times was analyzed for IL-1 β . **C**, MTE cells from C57BL/6 WT or Duox1^{-/-} mice were stimulated with *A alternata* or HDM (50 μ g) for 2 hours, and production of IL-1 α (black bars) or IL-1 β (red bars) was measured in culture supernatants. Data represent means $\pm$ SEs of 4 to 8 replicates from 2 to 3 independent experiments. ALT, *A alternata*; CTL, control.

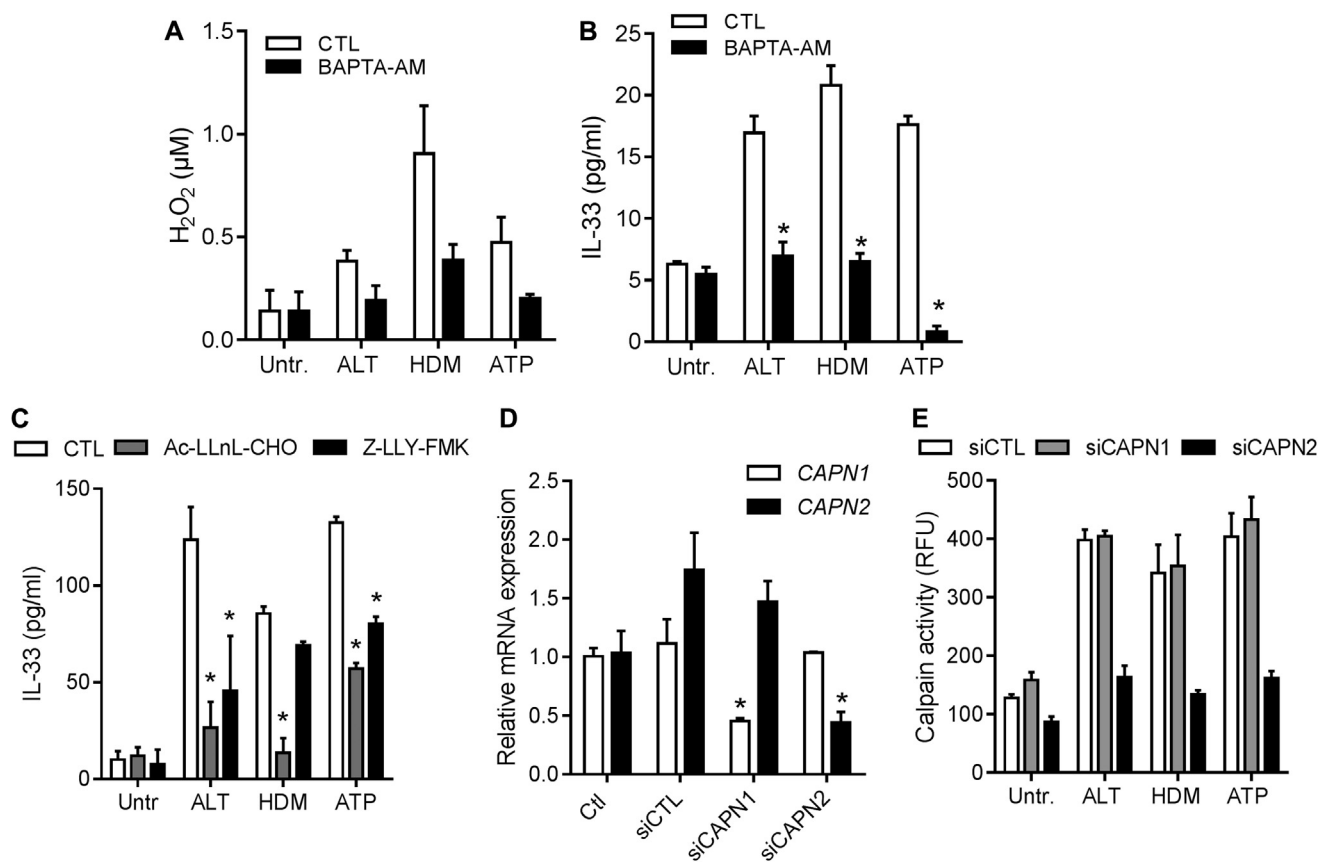


FIG E4. Involvement of Ca^{2+} and calpain in epithelial responses to allergen. **A** and **B**, HBE1 cells were preloaded with the Ca^{2+} chelator BAPTA-AM (100 $\mu\text{mol/L}$) for 30 minutes before challenge with *A. alternata* or HDM (50 μg) or ATP (100 $\mu\text{mol/L}$) for 15 minutes or 2 hours for analysis of extracellular H_2O_2 production (Fig E4, A) and IL-33 secretion (Fig E4, B), respectively. **C**, HBE1 cells were pretreated with the calpain inhibitors Ac-LLnL-CHO (10 $\mu\text{mol/L}$) or Z-LLY-FMK (4 $\mu\text{mol/L}$) before stimulation with allergens or ATP (2 hours) for analysis of IL-33 secretion. **D** and **E**, *CAPN1* or *CAPN2* were silenced in HBE1 cells by using siRNA and analyzed for *CAPN1* or *CAPN2* mRNA expression (Fig E4, D) or calpain activation after 15 minutes of stimulation with allergens or ATP (Fig E4, E). Data represent means $\pm$ SEs of 4 to 6 replicates from 2 independent experiments. * $P < .05$ compared with corresponding controls. ALT, *A. alternata*; CTL, control; siCAPN, calpain-targeted siRNA; siCTL, non-targeting control siRNA.

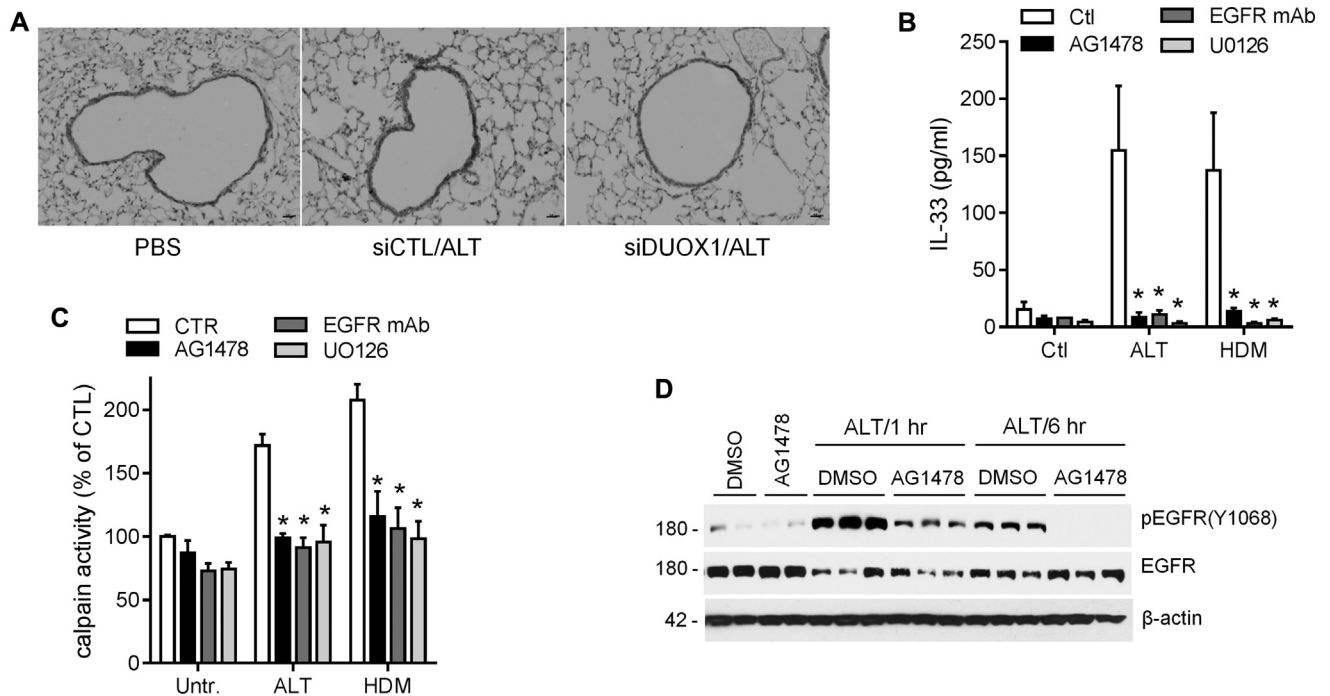


FIG E5. Role of EGFR in allergen-induced activation of calpain and IL-33 secretion. **A**, Immunohistochemical analysis of lung tissues from C57BL/6 mice for phosphorylated EGFR (pY1068) after 1 hour of challenge with *A. alternata* and after previous instillation of DUOX1-targeted siRNA on nontargeting siRNA controls. Representative images of 3 to 4 replicates are shown. **B** and **C**, Effect of 30 minutes of preincubation of HBE1 cells with inhibitors of EGFR (1 μ mol/L AG1478 or 2 μ g/mL α -EGFR mAb) or ERK (10 μ mol/L U0126) on allergen-induced IL-33 secretion (2 hours; Fig E5, **B**) or activation of calpain (15 minutes; Fig E5, **C**). Data represent means $\pm$ SEs of 4 to 8 replicates from 2 to 3 independent experiments. * P < .05 compared with corresponding controls. **D**, Effect of preinstillation of AG1478 (1 hour; 3 mg/kg in 1% DMSO; intratracheal) on *A. alternata*-induced EGFR phosphorylation in lung tissues from C57BL/6 mice collected 1 hour after challenge and analyzed by using Western blotting. A representative experiment of 2 is shown. ALT, *A. alternata*; CTL, control; siCTL, non-targeting control siRNA; siDUOX, DUOX-targeted siRNA.

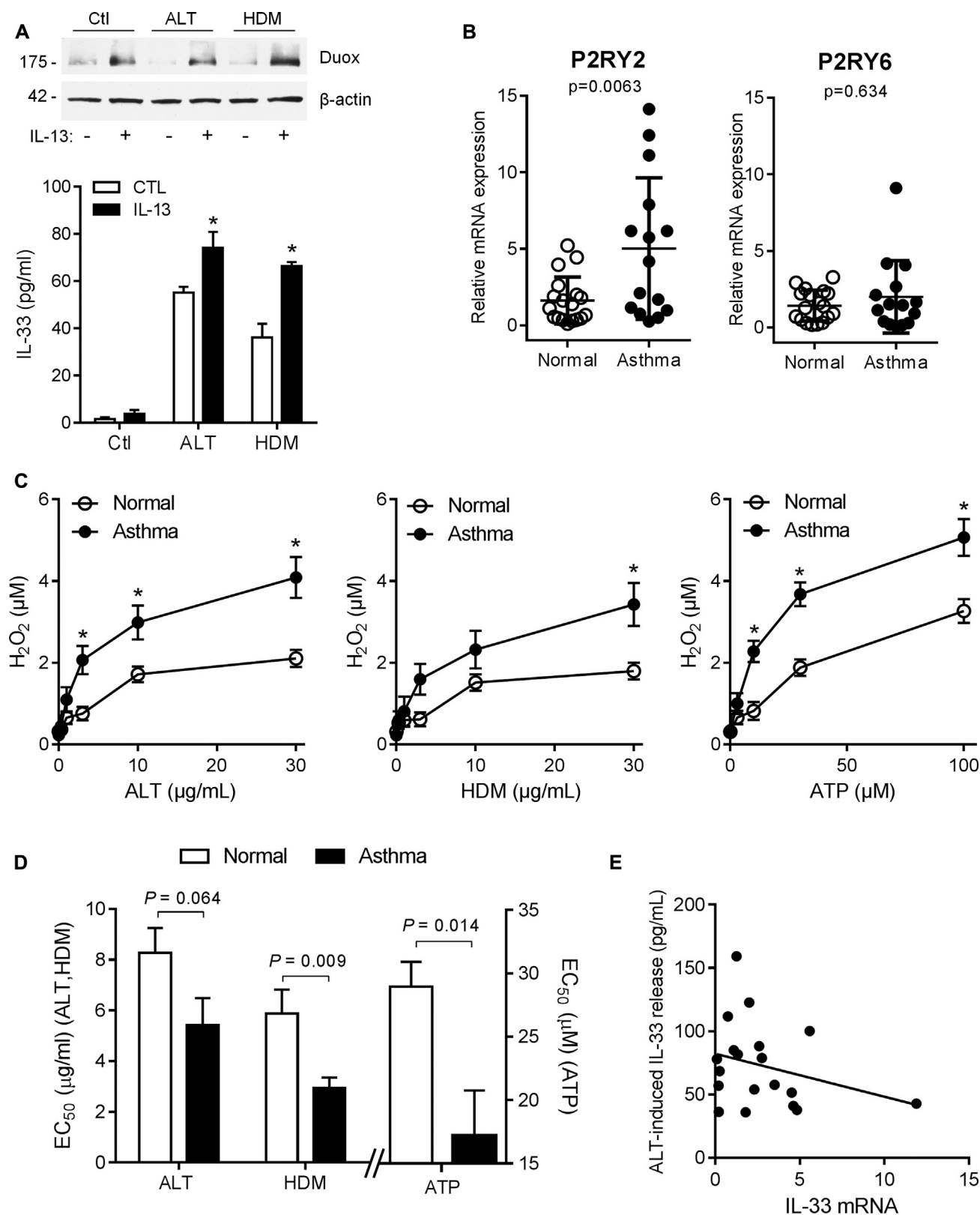


FIG E6. Allergen responses are enhanced in nasal epithelial cells from asthmatic patients. **A**, MTE cells were preincubated with human IL-13 (Calbiochem; 100 ng/mL) for 96 hours for analysis of DUOX protein expression (top) and allergen-induced IL-33 secretion (2 hours; bottom). Data represent means $\pm$ SEs of 6 replicates from 3 independent experiments. * $P < .05$ compared with corresponding controls. **B**, HNE cells from healthy subjects or asthmatic patients were analyzed for mRNA expression of purinergic receptor subtypes P2RY2 and P2RY6 by using quantitative PCR. **C**, HNE cells (p.2) from healthy subjects ($n = 10$)

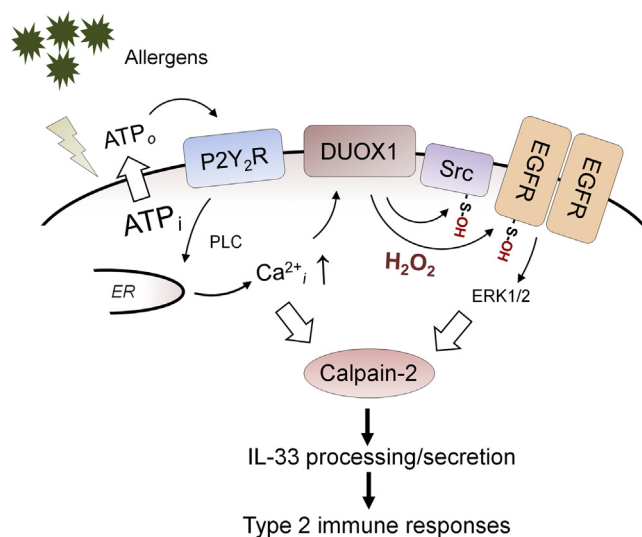


FIG E7. Schematic representation of proposed DUOX1-dependent signaling cascade in allergen-induced epithelial IL-33 secretion and activation of T_H2 type responses. ER, Endoplasmic reticulum; PLC, phospholipase C.

or asthmatic patients (n = 11) were stimulated for 15 minutes with increasing doses of *A alternata*, HDM, or ATP, for analysis of extracellular H₂O₂ production by using HPLC. **D**, Dose-dependent IL-33 production in response to *A alternata*, HDM, or ATP challenge (2 hours) of HNE cells from healthy subjects or asthmatic patients was expressed as EC₅₀ values calculated based on maximal responses induced by either 30 µg/mL *A alternata* or HDM or 100 µmol/L ATP. Data represent means ± SEs from 10 healthy subjects and 11 asthmatic patients. *P < .05 compared with nonasthmatic control subjects. **E**, Correlation of *IL33* mRNA expression in HNE cells from control subjects and asthmatic patients with IL-33 secretion induced by stimulation with *A alternata* (30 µg/mL). ALT, *A alternata*; CTL, control.

TABLE E1. Quantitative PCR primer sequences used in this study

Human	
<i>DUOX1</i>	
Forward:	ttcacgcagctctgtgtcaa
Reverse:	agggacagatcatatcctggct
<i>DUOX2</i>	
Forward:	acgcagctctgtgtcaaaggt
Reverse:	tgatgaacgagactcgacagc
<i>P2RY2</i>	
Forward:	gccagtgtgaggctgtaact
Reverse:	agccaactggctttacagt
<i>P2RY6</i>	
Forward:	gtctaccgcgagaactcaa
Reverse:	tgtcaccttgggcatagtt
<i>IL33</i>	
Forward:	caaagaagttgccccatgt
Reverse:	aaggcaaagcactccacagt
<i>TSLP</i>	
Forward:	cccaggctattcggaaactca
Reverse:	acgccacaatcctgttaattgtg
<i>IL25</i>	
Forward:	tggctgtaggtgtgggtcc
Reverse:	tggctgtaggtgtgggtcc
<i>IL8</i>	
Forward:	tagcaaaattgagccaagg
Reverse:	agcagactagggttgccaga
<i>GAPDH</i>	
Forward:	gaaggctggggctcatttg
Reverse:	aggctgtgtcatacttctcatgg
Mouse	
<i>Duox1</i>	
Forward:	atctgggtgacaaggactca
Reverse:	cagactcctgttcagcacct
<i>Duox2</i>	
Forward:	ggcctaagaagaggttgga
Reverse:	gcctccgtgtacagccggg
<i>Il33</i>	
Forward:	gatgggaagaaggtgatgggtg
Reverse:	ttgtgaaggacgaagaagc
<i>Gapdh</i>	
Forward:	ctggagaaacctgccaagta
Reverse:	tgtgtctgtaccgtattca

GAPDH, Glyceraldehyde-3-phosphate dehydrogenase.

TABLE E2. Baseline characteristics of study subjects

Characteristic	Healthy subjects (n = 18)	Asthmatic (n = 17)	<i>P</i> value
Age (y)	33.4 ± 14.6	35.0 ± 16.1	NS
Sex (%)			
Male	9 (50)	7 (41)	NS
Female	9 (50)	10 (59)	
FEV ₁ (% predicted)	ND	77.5 ± 20.5	
FVC (%)	ND	92.5 ± 17.6	
IgE (IU/mL), median (IQR)	8 (4-40)	131 (49-277)	<.001
SNQ score	0.42 ± 0.44	1.83 ± 0.75	<.001
ACT score	—	17.5 ± 6.8	
BMI (kg/m ²), mean (IQR)	24.3 (22.6-27.2)	25.3 (22.0-33.0)	NS

BMI, Body mass index; *FVC*, forced vital capacity; *IQR*, interquartile range; *ND*, not determined; *NS*, not significant; *SNQ*, sinonasal questionnaire.