HHS Public Access

Author manuscript

J Allergy Clin Immunol. Author manuscript; available in PMC 2017 May 01.

Published in final edited form as:

J Allergy Clin Immunol. 2016 May ; 137(5): 1545–1556.e11. doi:10.1016/j.jaci.2015.10.003.

Airway epithelial DUOX1 mediates allergen-induced IL-33 secretion and activation of type 2 immune responses

Milena Hristova, B.S.[1], **Aida Habibovic, B.S.**[1], **Carmen Veith, M.S.**[1], **Yvonne M.W. Janssen-Heininger, Ph.D.**[1], **Anne E. Dixon, M.D.**[2], **Miklos Geiszt, M.D., Ph.D.**[3], and **Albert van der Vliet, Ph.D.**[1,*]

[1]Department of Pathology, Vermont Lung Center, University of Vermont, Burlington, VT 05405, USA

[2]Department of Medicine, Vermont Lung Center, University of Vermont, Burlington, VT 05405, USA

[3]Department of Physiology and “Lendület” Peroxidase Enzyme Research Group, Semmelweis University, Budapest, Hungary

Abstract

Background—The interleukin (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 DUOX1 in epithelial wound responses, we explored the potential involvement of DUOX1 in allergen-induced IL-33 secretion and potential alterations in airways of subjects with asthma.

Methods—Cultured human or murine airway epithelial cells or mice were subjected to acute challenge with *Alternaria alternata* or house dust mite (HDM), and secretion of IL-33 and activation of subsequent type 2 responses were determined. The role of DUOX1 was explored using siRNA approaches and DUOX1-deficient mice. Cultured nasal epithelial cells from healthy or asthmatic subjects 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 critically depended on DUOX1-mediated activation of epithelial epidermal growth factor receptor (EGFR) and the protease calpain-2, via a redox-dependent mechanism involving cysteine oxidation within EGFR and the tyrosine kinase Src. Primary nasal epithelial cells from subjects

*To whom correspondence should be addressed: Phone: +1-802-656-8638; Fax: +1-802-656-8892; albert.van-der-vliet@uvm.edu.

Author contributions: M.H., A.H., and C.V. performed and analyzed the experiments; Y.M.W.J.-H., assisted with experimental design; A.E.D. was responsible for recruitment of human subjects and collection of nasal epithelial cells, and M.G. provided the DUOX1-deficient mice. A.v.d.V. was responsible for the conception and overall supervision of the project. All authors contributed to the planning, discussion and interpretation of experiments, and to the writing of the manuscript.

Conflicts of interest: None

with allergic asthma were found to express elevated DUOX1 and IL-33, and demonstrated enhanced IL-33 secretion in response to allergen challenge compared to nasal epithelial cells from non-asthmatic 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 may present important contributing features of allergic asthma.

Keywords

asthma; redox signaling; H_2O_2; Src; EGFR; IL-13

Introduction

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 non-microbial stimuli, allergens, and parasites (3, 4), and also as an important contributing mechanism to 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 interleukin (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 subjects (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 development of allergen-induced eosinophilic inflammation, mucus metaplasia, and airways hyperresponsiveness (10, 11). In spite of this, mechanisms mediating IL-33 expression and/or secretion 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 regulator (12) distinct from its extracellular cytokine properties (13, 14), the latter being mediated by interaction with the orphan IL-1 receptor family member ST2 (IL-33R) on various target cells, including dendritic cells, mast cells, and a recently identified subtype of innate lymphoid cells (ILC2) (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 due to cell necrosis (10, 13). However, several recent lines of evidence indicate active mechanisms of IL-33 secretion, such as activation of purinoceptor-dependent signaling in response 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 H_2O_2-dependent activation of the non-receptor 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. The present studies indeed demonstrate that DUOX1 critically contributes to airway epithelial secretion of IL-33 and initiation of subsequent type 2 immune responses in response to two 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 subjects 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). Murine tracheal epithelial (MTE) cells were isolated from either wild-type C57BL/6 mice or *Duox1*$^{-/-}$ mice (25), and cultured as described previously (26, 27). Primary human nasal epithelial (HNE) cells were isolated from healthy volunteers and subjects with allergic rhinitis and cultured as recently described (28). Atopy was confirmed by positive skin tests and elevated serum IgE (> 100 IU/ml), asthma was confirmed by positive response to bronchodilator (≥ 200 cc and 12 % improvement in FEV1 and/or FVC) or a positive methacholine challenge test (PC_{20}< 8mg/ml), and all selected subjects had rhinitis with an SNQ score >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 *Alternaria alternata* (ALT; Greer Laboratories, Lenoir, NC) or *D. pteronyssinus* (HDM; Greer Laboratories; 1.27 endotoxin units/mg), or with ATP (Sigma), H_2O_2 (Sigma), LPS (from *E. Coli* 055:B5; Sigma; 1 μg), or poly(I:C) (Sigma), and conditioned media was collected at indicated times for cytokine analysis and other biochemical assays. Cell extracts were prepared for mRNA extraction, biochemical assays or protein analysis by SDS-PAGE and Western blotting. Pharmacological inhibitors were administered 30 min prior to cell stimulation.

Mice

C57BL/6J mice (The Jackson Laboratory; Bar Harbor, ME) or DUOX1-deficient mice (Lexicon Pharmaceuticals, Inc., The Woodlands, TX, USA) (25), at 8–12 weeks were subjected to brief isofluorane anesthesia for oropharyngeal administration of extracts of ALT (50 μg), HDM (50 μg), LPS (1 μg), recombinant mouse IL-33 (Ser^{109}-Ile^{266}, <0.1 ng/μg endotoxin; Invitrogen; 1 μg), or ATP (250 μM), all in 50 μL PBS. BAL and lung tissues were collected at 1 or 6 hrs 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 μM in saline, prepared from a 1 mM stock solution in DMSO) intranasally, 1 hr prior to allergen challenge. All animal procedures were reviewed and approved by the Animal Care and Use Committee of the University of Vermont.

SiRNA silencing

NHBE or HBE1 cells were transfected at 60–70% confluence with ON-TARGET *plus* SMARTpool® siRNAs targeted against $P2Y_2R$, CAPN1 or CAPN2 or Non-Targeting siRNA Pool#1 as control (Dharmacon, Lafayette, CO) using DharmaFECT transfection reagent, according to the manufacturer's instructions, 72 hrs prior to expermentation. Silencing of DUOX1 or DUOX2 was performed by transfection with the following siRNA reagents: DUOX1: (sense) GCUAUGCAGAUGGCGUGUAtt, (antisense) UACACGCCAUCUGCAUAGCtg; DUOX2: (sense) CGCAGUCAAUGUCUACAUCtt, (antisense) GAUGUAGACAUUGACUGCGtg, or with Silencer Negative Control #1 siRNA (Invitrogen). MTE cells were transfected at 60–70% confluence with pre-designed siRNA targets against DUOX1 (100 nM each) or non-target (NS) siRNA (Ambion; 200 nM) using DharmaFECT® transfection reagent (Dharmacon) and used for experimentation after 72 hrs (22). For *in vivo* siRNA silencing of DUOX1, two DUOX1 siRNA targets in sterile PBS (35 μg/target sequence/mouse) or NS-siRNA (70 μg/mouse) were instilled oropharyngeally in mice under brief isofluorane anesthesia (22), 48 hrs prior to allergen challenge.

Biochemical assays

Cell culture supernatants or BAL fluids were analyzed for IL-33, IL-1α, IL-17E (IL-25), IL-5, IL-13, using DuoSet® ELISAs from R&D Systems (Mineapolis, MN). Human TSLP was determined by an ELISA from eBioscience (San Diego, CA); human IL-17E (IL-25) by an Omnikine™ ELISA kit from Assay Biotechology (Sunnyvale, CA); and human IL-8/CXCL8 by an ELISA from BD Biosciences (San Diego, CA). ATP in BAL fluids was analyzed by luciferase-dependent bioluminescence assay (Life Technologies, Grand Island, NY). Extracellular H_2O_2 production was determined by lactoperoxidase-dependent crosslinking tyrosine, measured by HPLC with fluorescence detection as described previously (21). Calpain activity was determined using a Calpain Activity Assay Kit (Abcam, Cambridge, MA). Calpain inhibitor I (Ac-LLnL-CHO; Invitrogen) or calpain inhibitor IV (Z-LLY-FMK; Abcam) were added 30 min prior to 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 μM EZ-link Iodoacetyl-LC-biotin (Pierce, Rockford, IL) and 20 U/ml catalase, under N_2 atmosphere to avoid artificial cysteine oxidation during processing, and incubated for 90 min at 37°C. For analysis of protein sulfenylation (-S-OH), MTE cells were lysed in deoxygenated Western solubilization buffer containing 1 mM DCP-Bio1 (Kerafast, Boston, MA), 200 U/ml catalase (Worthington, Lakewood, NJ), and 10 mM *N*-ethyl-maleimide (NEM; Sigma), and incubated for 1 hr t 4°C. Following derivatization, excess biotinylating reagent was in both cases removed by 6 successive washes with 20 mM Tris.HCl (pH 7.4) on Amicon Ultra-0.5 Centrifugal Filter Devices (Millipore), and biotin-tagged proteins were subsequently collected with

NeutrAvidin agarose beads (50 μl of 50/50 slurry; Pierce), washed successively with 1% SDS, 4 M urea, 1 M NaCl and 100 mM ammonium bicarbonate (with and without 10 mM DTT), to remove non-specifically bound proteins (30). To elute biotinylated proteins, beads were suspended in 2X reducing sample buffer containing β-mercaptoethanol and heated (10 min; 90°C), and eluted proteins were analyzed by 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 overnight incubation with α-EGFR mAb (C74B9; Cell Signaling) and analyzed by SDS-PAGE and blotting with streptavidin-HRP (Sigma). To determine *in vivo* protein sulfenylation, mice were administered dimedone (5,5-dimethyl-1.3-cyclohexanedione) intranasally (50 μl of 1 mM solution in PBS with 1% DMSO) 1 hr prior to ALT challenge, and lung tissues were collected 1 hr after allergen challenge for homogenization in Western solubilization buffer (containing 1 mM DCP-Bio1, 200 U/ml catalase, and 10 mM *N*-ethyl-maleimide) and EGFR immunoprecipation and streptavidin blotting as described above.

Statistical analysis

All quantitative data are represented as mean S.E. Statistical differences between groups were evaluated by 2-way ANOVA with Bonferroni post-analysis using GraphPad Prism version 6.0 (GraphPad, La Jolla, CA), and were considered significant when $P < 0.05$.

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 normal human bronchial epithelial (NHBE) cells in response to extracts of two important airborne allergens that have been associated with asthma, *Alternaria alternata* (ALT) or *Dermatophagoides pteronyssinus* (house dust mite; HDM) (31, 32). As expected (16), exposure of NHBE cells to extracts of ALT induced rapid release of IL-33, reaching maximal levels after 2 hrs, whereas secretion of other T_H2-inducing cytokines, IL-25 and thymic stromal lymphopoietin (TSLP), as well as 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 TLR agonists LPS or poly(I:C) (Fig. 1, *C*), and secreted IL-33 in conditioned media was detected primarily in its mature (18 kD) form (Fig. 1, *D*), indicating the involvement of proteolytic processing. Immunofluorescence analysis indicated export of nuclear IL-33 towards extranuclear regions in response to ALT, 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 pre-incubation 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 $P2Y_2R$ (see Fig. E1, *A* in this article's Online Repository at www.jacionline.org). Following previous findings that ATP-

mediated $P2Y_2R$ activation promotes epithelial production of H_2O_2 though activation of the NADPH oxidase DUOX1 (19), we detected rapid epithelial $P2Y_2R$-dependent H_2O_2 production in NHBE cells in response to either ALT or HDM (see Fig. E1, *A*). SiRNA-dependent silencing demonstrated that allergen- or ATP-induced H_2O_2 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 H_2O_2 in epithelial IL-33 secretion, comparable IL-33 secretion could be evoked by direct stimulation with H_2O_2 (see Fig. E1, *C*). Similar findings were obtained using primary mouse tracheal epithelial (MTE) cells, which similarly demonstrated transient IL-33 release in response to either ALT 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 $P2Y_2R$ activation and DUOX1-dependent production of H_2O_2.

DUOX1 contributes to innate type 2 immune responses in vivo

To address the role of DUOX1 in innate allergen 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. As shown in Fig. 2, instillation of ALT resulted in rapid and transient increases in BAL ATP (Fig. 2, *A*) as well as IL-33 (Fig. 2, *B*), the latter detected primarily as its mature 18-kD form (Fig. 2, *C*). Confirming the presence of active IL-33, addition of BAL fluids from ALT-challenged mice to cultured bone marrow-derived macrophages (BMDM) *in vitro* resulted in production of IL-13 which was almost completely dependent on ST2/IL-33R (Fig. 2, *D*). Whereas ALT-induced ATP release was similar in *Duox1*$^{-/-}$ mice compared to wild-type mice (Fig. 2, *A*), allergen-induced IL-33 secretion was dramatically attenuated in *Duox1*$^{-/-}$ mice (Fig. 2, *B,D*). Similarly, allergen-induced production of the type 2 cytokines IL-5 and IL-13 (Fig. 2, *E,F*) as well as IL-25 (IL-17E) and IL-1α (Fig. 2, *G*), additional epithelial mediators of allergen-induced airway inflammation (33, 34), were 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*), consistent with the suggested role of initial IL-33 secretion in allergen-induced production of these mediators. Highly similar findings were also observed upon similar challenge with HDM instead of ALT (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 intratracheal administration of 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, since instillation of LPS (1 μg) failed to induce significant increases in BAL ATP, IL-33 or IL-5/IL-13 (results not shown). In contrast, LPS challenge promoted marked production into the BAL of the T_H1 cytokines TNF-α, IL-1β, and IL-12p40), but this response was identical in both WT and Duox1-deficient mice (see Fig. E3, *A*). Conversely, while ALT induced significant production of IL-33 and type 2 cytokines (Fig. 2), only modest and DUOX1-independent increases in IL-1β (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-1α (see Fig. E3, *C*), whereas only minimal and DUOX1-independent production of IL-1β 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, IL-1α) 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-1α or IL-3 (35, 36). Accordingly, allergen-induced activation of DUOX1 (19) and secretion of IL-33 (16) were both dramatically suppressed by cell preloading with the Ca^{2+} chelator BAPTA-AM (see Fig. E4, *A,B*), 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 from 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*). SiRNA-mediated silencing experiments furthermore indicated that, among the two 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,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 by 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, as well as ERK1/2, and *in vivo* instillation of ALT enhanced EGFR phosphorylation within the airway epithelium (Fig. 4, *B*), and these responses were largely absent in *Duox1*$^{-/-}$mice (Fig. 4, *A,B*) or reduced after siRNA silencing of DUOX1 (see Fig. E5, *A*). Moreover, these kinases are critical in allergen-induced IL-33 secretion by HBE1 cells or MTE cells, as they were dramatically suppressed by pharmacological inhibition of either Src, EGFR, or ERK (Fig. 4C and see Fig. E5, *B*). The specific role of EGFR signaling was also demonstrated by 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. S5, *C*). Finally, administration of the EGFR tyrosine kinase inhibitor AG1478 prior to *in vivo* allergen (ALT) 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 production of IL-5/IL13 (Fig. 4, *F,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 may 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 thiol-specific biotinylation, after which biotin-tagged proteins were collected by avidin purification and probed by Western blot analysis for the presence of -SH content within Src or EGFR. This revealed that allergen challenge of MTE cells indeed resulted in partial loss of Src-SH content and dramatic loss of EGFR-SH, while 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 chemical derivatization with biotin-tagged dimedone (DCP-Bio1), allergen stimulation of MTE cells was found to result in enhanced presence of both Src-SOH and EGFR-SOH (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 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 indeed indicated increased dimedone labeling in response to allergen challenge, only in WT but not in *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 subjects

A growing number of recent studies indicate elevated expression and secretion of IL-33 in the airways of asthmatic subjects (8, 40, 41). Moreover, since T_H2-associated cytokines such as IL-13 are capable of inducing epithelial DUOX1 expression (23), we hypothesized that

airway DUOX1 expression is enhanced subjects 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 pre-stimulation with IL-13 (see Fig. E6, *A*). We collected nasal epithelial (HNE) cells from 18 healthy human subjects and 17 subjects with allergic asthma and rhinitis and evaluated mRNA levels of DUOX1/2, IL-33 and P2YR subtypes, as well as H_2O_2 production and IL-33 secretion in response to *in vitro* allergen challenge. Subject characteristics are summarized in Table EI in this article's Online Repository at www.jacionline.org. DUOX1 mRNA levels were found to be significantly higher in HNE cells from asthmatic subjects compared to non-asthmatic controls, whereas DUOX2 mRNA levels were 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 H_2O_2 production, a measure of DUOX1 activation (Fig. 6, *B*). In agreement with earlier reports (8, 40), HNE cells from asthmatic subjects also expressed increased IL-33 mRNA compared to HNE cells from non-asthmatic controls (Fig. 6, *C*). NHE cells from asthmatic subjects also expressed elevated P2YR2 mRNA levels, although mRNA levels of P2YR6 were comparable between normal and asthmatic HNE cells (see Fig. E6, *B*). Analysis of dose-dependent production of H_2O_2 or IL-33 secretion in response to ALT, HDM, or ATP, revealed that allergen-induced H_2O_2 production was consistently higher in HNE cells from asthmatics compared to those of non-asthmatic controls (see Fig. E6, *C*). Moreover, significant increases in allergen-induced IL-33 secretion were observed in HNE cells from asthmatic subjects compared to non-asthmatic controls, at most doses studied (Fig. 6, *D*). Calculation of EC_{50} values indicated enhanced sensitivity of asthmatic HNE cells to allergen challenge, demonstrated by significantly lower EC_{50} values for HDM and ATP and near-significant lower EC_{50} for ALT in HNE cells from asthmatic subjects compared to healthy controls (see Fig. E6, *D*). Interestingly, although we observed enhanced IL-33 mRNA expression in HNE from asthmatic subjects, no significant correlation was observed between IL-33 mRNA and allergen-induced IL-33 secretion (see Fig. E6, *E*), which would suggest that the increased allergen-dependent IL-33 secretion in asthmatic epithelia 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 helminthes, 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 may involve regulated mechanisms rather than passive release due to cell necrosis (16, 17). The present findings are highly significant as 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 (PPRs), toll-like 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 ALT and HDM were in both cases suppressed by common serine protease inhibitors, such as PMSF 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, which was 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-1α, as mediators of injury or sterile inflammation, and does not mediate activation of IL-1β 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 two separate DUOX isoforms may have evolved to play unique specific roles in these distinct immune responses.

A final major finding of the present studies is that DUOX1 as well as allergen-induced IL-33 secretion are markedly elevated within the epithelium from asthmatic subjects. Given the increasingly recognized role for IL-33 in persistent or steroid-resistant asthma (40, 48) and allergen-induced bronchoconstriction (34), enhanced DUOX1 expression may contribute to exaggerated airway responses to commonly inhaled allergens and mediate exacerbations in certain asthma phenotypes. Thus, while DUOX1-dependent activation of IL-33 and type 2 immune responses may 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 may likely contribute to disease pathology, by promoting production of type 2 cytokines (IL-5, IL-13) that are 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 ILC2 (49) or mast cells (50), which mediate type 2 cytokine production and bronchoconstriction. Although the present studies are the first to demonstrate elevated DUOX1 in human asthma, a recent report highlighted enhanced expression of DUOX2 and its potential involvement in oxidative mechanisms the airway of severe asthmatics (51). Since asthma is a highly heterogeneous disease, it is plausible that both DUOX1 and DUOX2 may 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 may contribute importantly to persistent steroid-resistant asthma or asthma exacerbations (40, 41, 43). Recent findings of elevated DUOX1 and/or IL-33-mediated type 2 responses in 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. Although we determined that allergen-induced calpain activation was dependent on Ca^{2+} signaling, calpain-2 (also known as m-calpain) requires mM Ca^{2+} 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 Ca^{2+} 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. The mechanism(s) by which calpain-2 promotes allergen-induced IL-33 secretion remains unclear, however, and may include a direct role in proteolytic IL-33 processing (10, 36), but might also include less defined roles in cytoskeletal organization and in secretory pathways (56, 57). Furthermore, DUOX1-dependent activation of Src or EGFR may also promote IL-33 secretion by actions independent of calpain, e.g. by promoting nuclear export or through other cytoskeletal mechanisms involved in non-classical secretion pathways.

Our studies indicate a direct role for DUOX1 in activation of Src and EGFR, due to DUOX1-dependent reversible cysteine oxidation within these kinases, consistent with previous studies demonstrating a critical role for oxidation of critical conserved cysteines in regulating activation of these tyrosine kinases (38, 39). The precise relationship(s) between between cysteine oxidation and tyrosine phosphorylation and kinase activation, however, 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 may 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 may promote EGFR activation and cysteine oxidation via 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 may involve the contribution of indirect oxidative mechanisms, possibly triggered by initial DUOX1 activation. Finally, DUOX1 activation may 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, by activation 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 may also contribute to allergic disease due to sensitized or augmented IL-33 production, which may be especially relevant in specific subgroups with persistent or steroid-resistant asthma (40, 48). In these cases, DUOX1 may present a novel and highly attractive therapeutic target to increase asthma control or minimize risk of exacerbations.

Methods

The materials and methods used in this study are described in the Methods section in this article's Online Repository at www.jacionline.org.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

This work was supported by NIH grants R01 HL085646 and ES021476 (AvdV), R01 HL060014 (YJ-H), and IDeA-COBRE grant P30 GM103532.

List of abbreviations

ALT	Alternaria alternata
BAL	Bronchoalveolar lavage
BMDM	Bone marrow-derived macrophages
DUOX	Dual oxidase
EGFR	Epidermal growth factor receptor
ERK	Extracellular signal-regulated kinase
ILC2	Type 2 innate lymphoid cells
NHE	Human nasal epithelial
NHBE	Normal human bronchial epithelial
MTE	Mouse tracheal epithelial
SH	Thiol
SOH	Sulfenic acid

References

1. Lambrecht BN, Hammad H. Allergens and the airway epithelium response: gateway to allergic sensitization. J Allergy Clin Immunol. 2014; 134(3):499–507. [PubMed: 25171864]

2. Holtzman MJ, Byers DE, Alexander-Brett J, Wang X. The role of airway epithelial cells and innate immune cells in chronic respiratory disease. Nat Rev Immunol. 2014; 14(10):686–698. [PubMed: 25234144]

3. Gause WC, Wynn TA, Allen JE. Type 2 immunity and wound healing: evolutionary refinement of adaptive immunity by helminths. Nat Rev Immunol. 2013; 13(8):607–614. [PubMed: 23827958]

4. Pulendran B, Artis D. New paradigms in type 2 immunity. Science. 2012; 337(6093):431–435. [PubMed: 22837519]

5. Holgate ST. The sentinel role of the airway epithelium in asthma pathogenesis. Immunol Rev. 2011; 242(1):205–219. [PubMed: 21682747]

6. Hung LY, et al. IL-33 drives biphasic IL-13 production for noncanonical Type 2 immunity against hookworms. Proc Natl Acad Sci U S A. 2013; 110(1):282–287. [PubMed: 23248269]

7. Neill DR, et al. Nuocytes represent a new innate effector leukocyte that mediates type-2 immunity. Nature. 2010; 464(7293):1367–1370. [PubMed: 20200518]

8. Prefontaine D, et al. Increased IL-33 expression by epithelial cells in bronchial asthma. J Allergy Clin Immunol. 2010; 125(3):752–754. [PubMed: 20153038]

9. Moffatt MF, et al. A large-scale, consortium-based genomewide association study of asthma. N Engl J Med. 2010; 363(13):1211–1221. [PubMed: 20860503]

10. Cayrol C, Girard JP. IL-33: an alarmin cytokine with crucial roles in innate immunity, inflammation and allergy. Curr Opin Immunol. 2014; 31:31–37. [PubMed: 25278425]

11. Lloyd CM, Saglani S. Epithelial cytokines and pulmonary allergic inflammation. Curr Opin Immunol. 2015; 34C:52–58. [PubMed: 25705788]

12. Carriere V, et al. IL-33, the IL-1-like cytokine ligand for ST2 receptor, is a chromatin-associated nuclear factor in vivo. Proc Natl Acad Sci U S A. 2007; 104(1):282–287. [PubMed: 17185418]

13. Smith DE. The biological paths of IL-1 family members IL-18 and IL-33. J Leukoc Biol. 2011; 89(3):383–392. [PubMed: 20952658]

14. Dinarello CA. Interleukin-1 in the pathogenesis and treatment of inflammatory diseases. Blood. 2011; 117(14):3720–3732. [PubMed: 21304099]

15. Sanos SL, Diefenbach A. Innate lymphoid cells: from border protection to the initiation of inflammatory diseases. Immunol Cell Biol. 2013; 91(3):215–224. [PubMed: 23357882]

16. Kouzaki H, Iijima K, Kobayashi T, O'Grady SM, Kita H. The danger signal, extracellular ATP, is a sensor for an airborne allergen and triggers IL-33 release and innate Th2-type responses. J Immunol. 2011; 186(7):4375–4387. [PubMed: 21357533]

17. Kakkar R, Hei H, Dobner S, Lee RT. Interleukin 33 as a mechanically responsive cytokine secreted by living cells. J Biol Chem. 2012

18. Carta S, Lavieri R, Rubartelli A. Different Members of the IL-1 Family Come Out in Different Ways: DAMPs vs. Cytokines? Front Immunol. 2013; 4:123. [PubMed: 23745123]

19. van der Vliet A. NADPH oxidases in lung biology and pathology: host defense enzymes, and more. Free Radic Biol Med. 2008; 44(6):938–955. [PubMed: 18164271]

20. De Deken X, Corvilain B, Dumont JE, Miot F. Roles of DUOX-mediated hydrogen peroxide in metabolism, host defense, and signaling. Antioxid Redox Signal. 2014; 20(17):2776–2793. [PubMed: 24161126]

21. Sham D, Wesley UV, Hristova M, van der Vliet A. ATP-mediated transactivation of the epidermal growth factor receptor in airway epithelial cells involves DUOX1-dependent oxidation of Src and ADAM17. PLoS One. 2013; 8(1):e54391. [PubMed: 23349873]

22. Gorissen SH, et al. Dual oxidase-1 is required for airway epithelial cell migration and bronchiolar reepithelialization after injury. Am J Respir Cell Mol Biol. 2013; 48(3):337–345. [PubMed: 23239498]

23. Harper RW, et al. Differential regulation of dual NADPH oxidases/peroxidases, Duox1 and Duox2, by Th1 and Th2 cytokines in respiratory tract epithelium. FEBS Lett. 2005; 579(21):4911–4917. [PubMed: 16111680]

24. Wesley UV, Bove PF, Hristova M, McCarthy S, van der Vliet A. Airway epithelial cell migration and wound repair by ATP-mediated activation of dual oxidase 1. J Biol Chem. 2007; 282(5):3213–3220. [PubMed: 17135261]

25. Donko A, et al. Urothelial cells produce hydrogen peroxide through the activation of Duox1. Free Radic Biol Med. 2010; 49(12):2040–2048. [PubMed: 21146788]
26. Wu R, Smith D. Continuous multiplication of rabbit tracheal epithelial cells in a defined, hormone-supplemented medium. In Vitro. 1982; 18(9):800–812. [PubMed: 6757109]
27. Alcorn JF, et al. Jun N-terminal kinase 1 regulates epithelial-to-mesenchymal transition induced by TGF-beta1. J Cell Sci. 2008; 121(Pt 7):1036–1045. [PubMed: 18334556]
28. Muller L, Brighton LE, Carson JL, Fischer WA 2nd, Jaspers I. Culturing of human nasal epithelial cells at the air liquid interface. J Vis Exp. 2013; (80)
29. Dixon AE, et al. Criteria to screen for chronic sinonasal disease. Chest. 2009; 136(5):1324–1332. [PubMed: 19581356]
30. Klomsiri C, et al. Use of dimedone-based chemical probes for sulfenic acid detection evaluation of conditions affecting probe incorporation into redox-sensitive proteins. Methods Enzymol. 2010; 473:77–94. [PubMed: 20513472]
31. Denning DW, O'Driscoll BR, Hogaboam CM, Bowyer P, Niven RM. The link between fungi and severe asthma: a summary of the evidence. Eur Respir J. 2006; 27(3):615–626. [PubMed: 16507864]
32. Gregory LG, Lloyd CM. Orchestrating house dust mite-associated allergy in the lung. Trends Immunol. 2011; 32(9):402–411. [PubMed: 21783420]
33. Willart MA, et al. Interleukin-1alpha controls allergic sensitization to inhaled house dust mite via the epithelial release of GM-CSF and IL-33. J Exp Med. 2012; 209(8):1505–1517. [PubMed: 22802353]
34. Barlow JL, et al. IL-33 is more potent than IL-25 in provoking IL-13-producing nuocytes (type 2 innate lymphoid cells) and airway contraction. J Allergy Clin Immunol. 2013; 132(4):933–941. [PubMed: 23810766]
35. Gross O, et al. Inflammasome activators induce interleukin-1alpha secretion via distinct pathways with differential requirement for the protease function of caspase-1. Immunity. 2012; 36(3):388–400. [PubMed: 22444631]
36. Hayakawa M, et al. Mature interleukin-33 is produced by calpain-mediated cleavage in vivo. Biochem Biophys Res Commun. 2009; 387(1):218–222. [PubMed: 19596270]
37. Glading A, et al. Epidermal growth factor activates m-calpain (calpain II), at least in part, by extracellular signal-regulated kinase-mediated phosphorylation. Mol Cell Biol. 2004; 24(6):2499–2512. [PubMed: 14993287]
38. Giannoni E, Chiarugi P. Redox circuitries driving Src regulation. Antioxid Redox Signal. 2014; 20(13):2011–2025. [PubMed: 23901911]
39. Paulsen CE, et al. Peroxide-dependent sulfenylation of the EGFR catalytic site enhances kinase activity. Nat Chem Biol. 2012; 8(1):57–64. [PubMed: 22158416]
40. Christianson CA, et al. Persistence of asthma requires multiple feedback circuits involving type 2 innate lymphoid cells and IL-33. J Allergy Clin Immunol. 2015
41. Jackson DJ, et al. IL-33-dependent type 2 inflammation during rhinovirus-induced asthma exacerbations in vivo. Am J Respir Crit Care Med. 2014; 190(12):1373–1382. [PubMed: 25350863]
42. Shaw JL, et al. IL-33-responsive innate lymphoid cells are an important source of IL-13 in chronic rhinosinusitis with nasal polyps. Am J Respir Crit Care Med. 2013; 188(4):432–439. [PubMed: 23805875]
43. Snelgrove RJ, et al. Alternaria-derived serine protease activity drives IL-33 mediated asthma exacerbations. J Allergy Clin Immunol. 2014
44. Palm NW, Rosenstein RK, Medzhitov R. Allergic host defences. Nature. 2012; 484(7395):465–472. [PubMed: 22538607]
45. Niethammer P, Grabher C, Look AT, Mitchison TJ. A tissue-scale gradient of hydrogen peroxide mediates rapid wound detection in zebrafish. Nature. 2009; 459(7249):996–999. [PubMed: 19494811]
46. McSorley HJ, Blair NF, Smith KA, McKenzie AN, Maizels RM. Blockade of IL-33 release and suppression of type 2 innate lymphoid cell responses by helminth secreted products in airway allergy. Mucosal Immunol. 2014

47. Fink K, et al. IFNbeta/TNFalpha synergism induces a non-canonical STAT2/IRF9-dependent pathway triggering a novel DUOX2 NADPH oxidase-mediated airway antiviral response. Cell Res. 2013; 23(5):673–690. [PubMed: 23545780]
48. Saglani S, et al. IL-33 promotes airway remodeling in pediatric patients with severe steroid-resistant asthma. J Allergy Clin Immunol. 2013; 132(3):676–685. e613. [PubMed: 23759184]
49. Sonnenberg GF, Artis D. Innate lymphoid cells in the initiation, regulation and resolution of inflammation. Nat Med. 2015; 21(7):698–708. [PubMed: 26121198]
50. Enoksson M, et al. Mast cells as sensors of cell injury through IL-33 recognition. J Immunol. 2011; 186(4):2523–2528. [PubMed: 21239713]
51. Voraphani N, et al. An airway epithelial iNOS-DUOX2-thyroid peroxidase metabolome drives Th1/Th2 nitrative stress in human severe asthma. Mucosal Immunol. 2014; 7(5):1175–1185. [PubMed: 24518246]
52. Ryu JH, et al. Distinct TLR-mediated pathways regulate house dust mite-induced allergic disease in the upper and lower airways. J Allergy Clin Immunol. 2013; 131(2):549–561. [PubMed: 23036747]
53. Cho DY, et al. Expression of dual oxidases and secreted cytokines in chronic rhinosinusitis. Int Forum Allergy Rhinol. 2013; 3(5):376–383. [PubMed: 23281318]
54. Salimi M, et al. A role for IL-25 and IL-33-driven type-2 innate lymphoid cells in atopic dermatitis. J Exp Med. 2013; 210(13):2939–2950. [PubMed: 24323357]
55. Hirakawa S, Saito R, Ohara H, Okuyama R, Aiba S. Dual oxidase 1 induced by Th2 cytokines promotes STAT6 phosphorylation via oxidative inactivation of protein tyrosine phosphatase 1B in human epidermal keratinocytes. J Immunol. 2011; 186(8):4762–4770. [PubMed: 21411736]
56. Franco SJ, Huttenlocher A. Regulating cell migration: calpains make the cut. J Cell Sci. 2005; 118(Pt 17):3829–3838. [PubMed: 16129881]
57. Evans JS, Turner MD. Emerging functions of the calpain superfamily of cysteine proteases in neuroendocrine secretory pathways. J Neurochem. 2007; 103(3):849–859. [PubMed: 17666040]
58. Hristova M, et al. Identification of DUOX1-dependent redox signaling through protein S-glutathionylation in airway epithelial cells. Redox Biol. 2014; 2:436–446. [PubMed: 24624333]

Key Messages

- Allergen-induced epithelial secretion of IL-33 is mediated by redox-dependent activation of EGFR-dependent signaling due to 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.
- Elevated expression and activation of DUOX1 may be an important feature of enhanced IL-33 secretion in severe and persistent asthma.

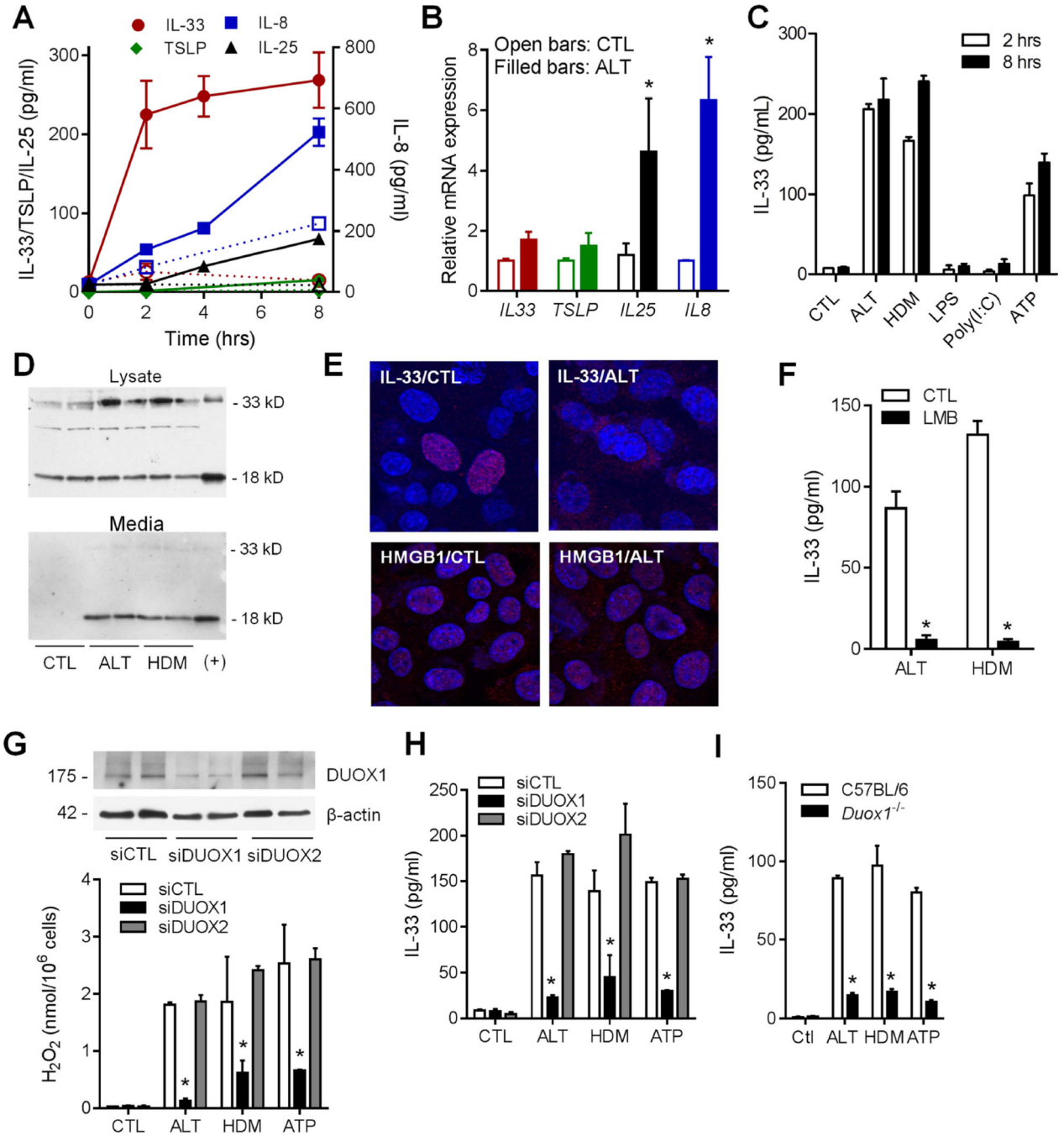

Figure 1.

Allergen-induced airway epithelial secretion of IL-33 is mediated by activation of DUOX1. (*A,B*) Cytokine/chemokine production and cytokine mRNA expression (8 hrs) after NHBE cell stimulation with ALT (50 μg/ml). Open symbols represent PBS controls. (*C*) IL-33 release into NHBE cell supernatants after 2- or 8-hr stimulation with ALT (50 μg/ml), HDM (50 μg/ml), LPS (1 μg/ml), poly(I:C) (100 ng/ml) or ATP (100 μM). (*D*) Western blot analysis of cell lysates and culture supernatants of stimulated NHBE cells (8 hrs) for full-length (33 kD) or mature (18 kD) IL-33. (+): positive IL-33 (18 kD) control. (*E*)

Immunofluorescence analysis of IL-33 or HMGB1 in HBE1 cells after 1-hr stimulation with ALT. Nuclei are counterstained in blue. (*F*) Effect of 15-min pre-incubation with leptomycin B (LMB; 100 nM) on allergen-induced IL-33 secretion (2 hrs) from HBE1 cells. (*G,H*) Effects of siRNA-mediated silencing of DUOX1 or DUOX2 on NHBE cell DUOX protein, and allergen-induced H_2O_2 production (15 min) and IL-33 secretion (2 hrs). (*H*) IL-33 secretion after allergen stimulation (2-hr) of MTE cells from wild-type C57BL/6 or *Duox1*$^{-/-}$ mice. Mean ± S.E from 4–6 replicates from 2–3 experiments. **P*<0.05.

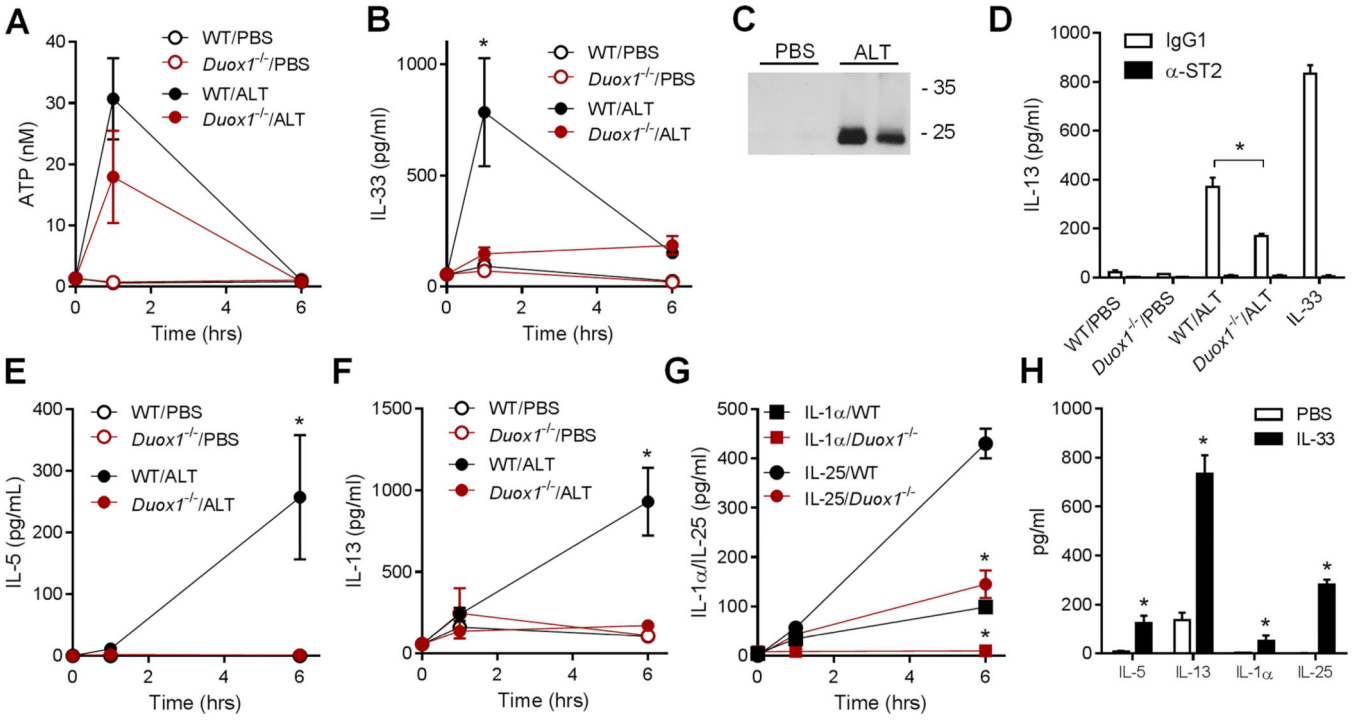

Figure 2.

DUOX1 contributes to allergen-induced IL-33 secretion and type 2 responses *in vivo*. (*A-C*) Analysis of ATP or IL-33 in BAL from WT or *Duox1*$^{-/-}$ mice after i.t. instillation of ALT (50 μg). (*D*) Analysis of IL-13 production from BMDM after 72-hr exposure to BAL fluids from PBS- or ALT-challenge mice (1 hr) or IL-33 (10 ng/ml) in the presence of α-ST-2 blocking antibody or control IgG (1 μg/ml). (*E-G*) Analysis of BAL IL-5, IL-13, IL-25 or IL-1α, after ALT challenge of WT or *Duox1*$^{-/-}$ mice. (*H*) Analysis of BAL cytokines after i.t. instillation of recombinant IL-33 (1 μg; 6 hrs). Mean ± S.E. from 4–8 replicates from 2–3 experiments. **P*<0.05.

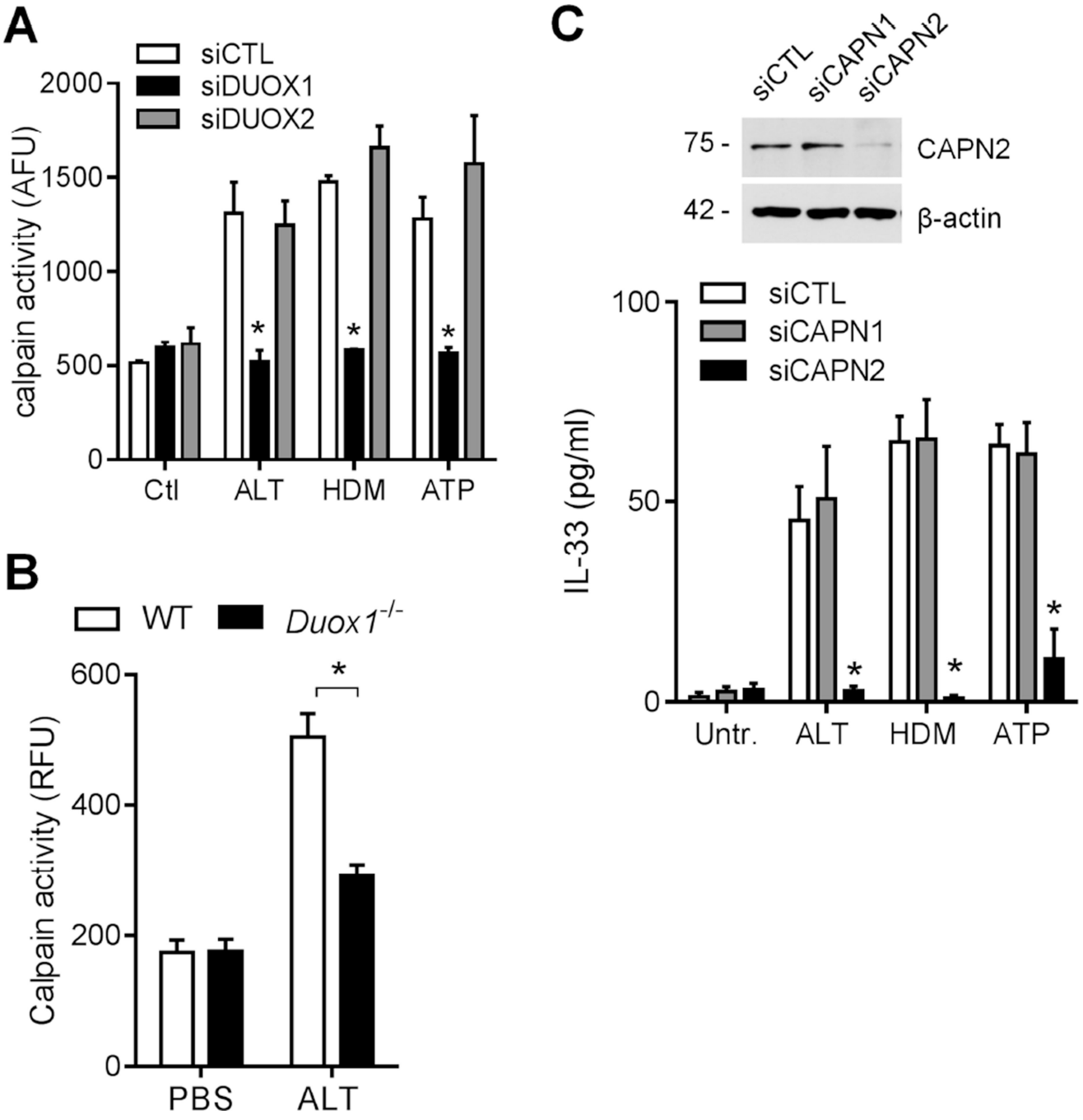

Figure 3.

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 min) activation of calpain in NHBE cells. (*B*) PBS or ALT were instilled i.t. into mice (1 hr) 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 hrs). Mean ± S.E from 4–6 replicates from 2–3 experiments. **P*<0.05.

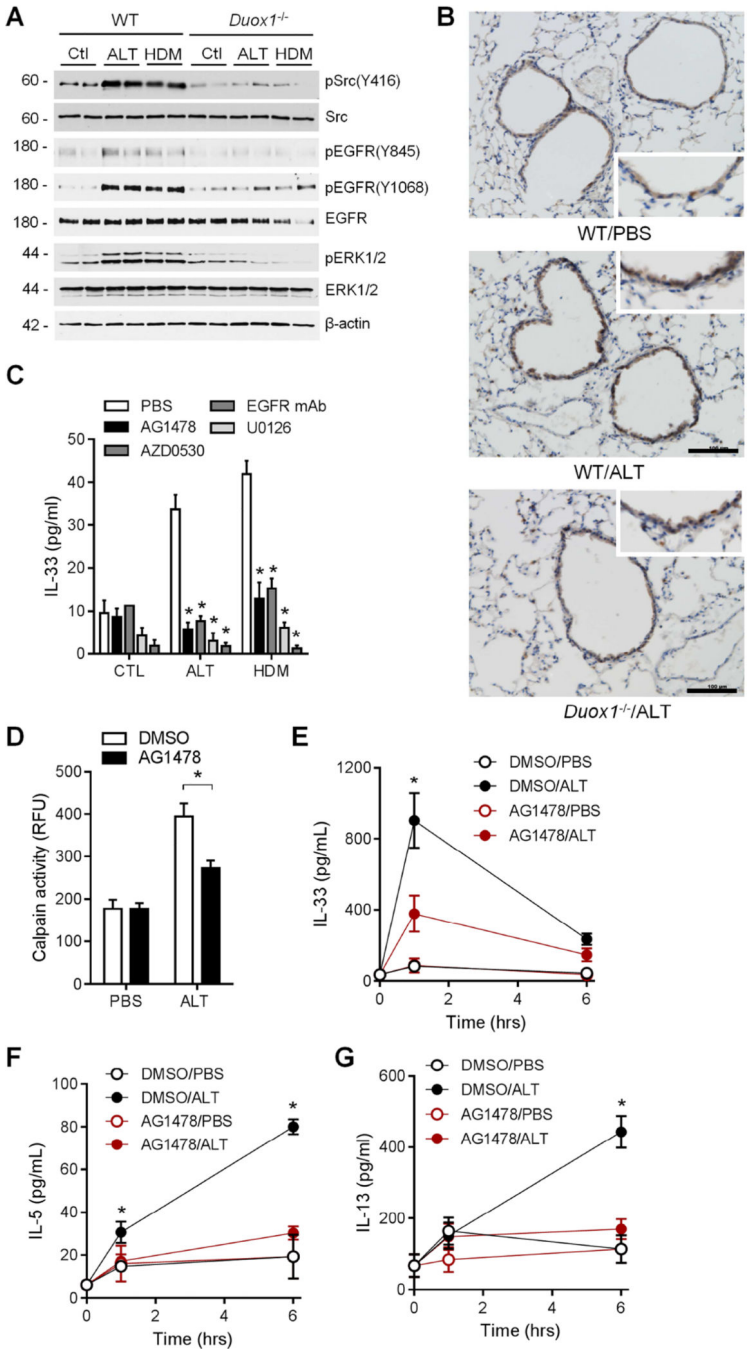

Figure 4.

DUOX1-dependent IL-33 secretion is mediated by EGFR signaling. (*A*) Analysis for phosphorylation of Src, EGFR, and ERK1/2 after 15-min stimulation of MTE cells from WT or *Duox1*$^{-/-}$ mice with ALT 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-hr instillation of PBS or ALT (representative of 4 animals). Bar: 100 μm. (*C*) Analysis of allergen-induced IL-33 secretion (2 hrs) from MTE cells after pre-incubation with AG1478 (1 μM), α-EGFR mAb (4 μg/ml), U0126 (10 μM) or

ADZ0530 (1 μM). (*D-G*) C57BL/6J mice were administered AG1478 (i.n.) prior to ALT challenge, and calpain activity was analyzed in lung tissue homogenates (1 hr) and BAL was analyzed for IL-33, IL-5, or IL-13. Mean ± S.E. of 4–6 replicates from 2–3 experiments. **P*<0.05.

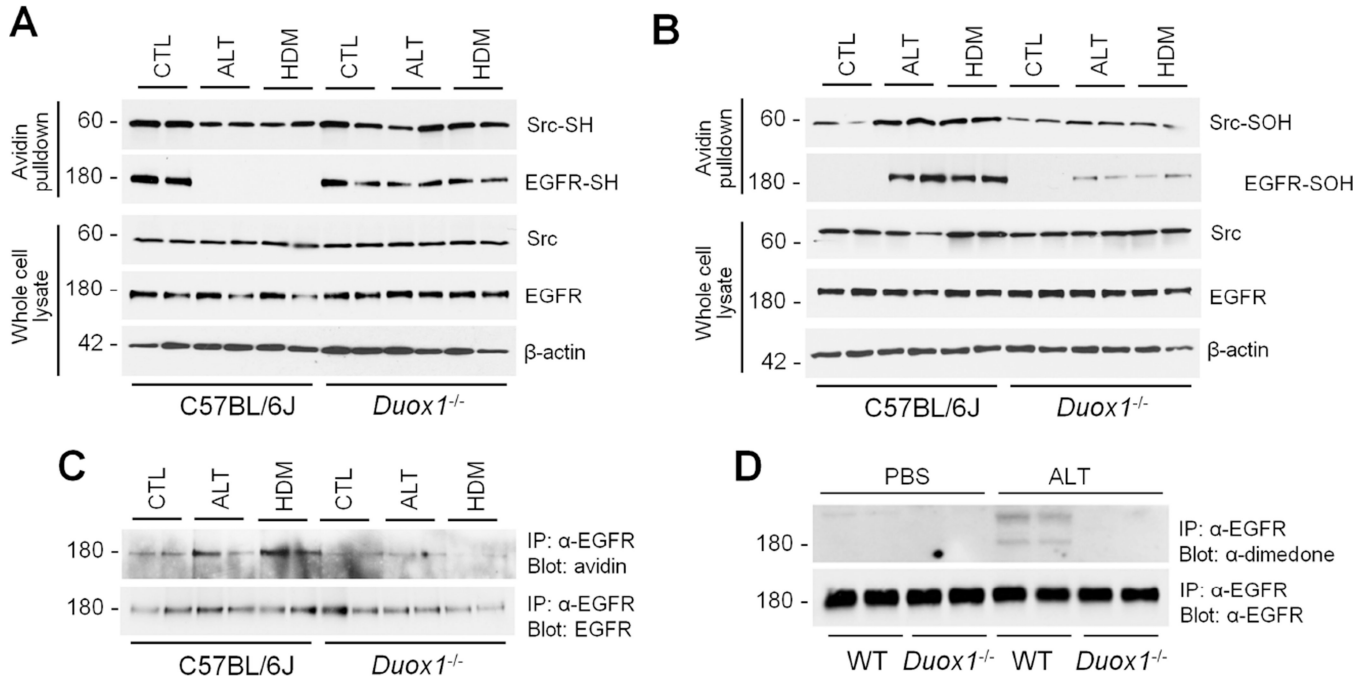

Figure 5.

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 min) 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 following 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 (*B*) for biotin incorporation by streptavidin blot. (*D*) Identification of EGFR-SOH in lung tissues from allergen-challenged WT mice or *Duox1*$^{-/-}$ mice by trapping with dimedone, EGFR immunoprecipation and Western blot with α-dimedone or α-EGFR. Representative of 2–3 experiments.

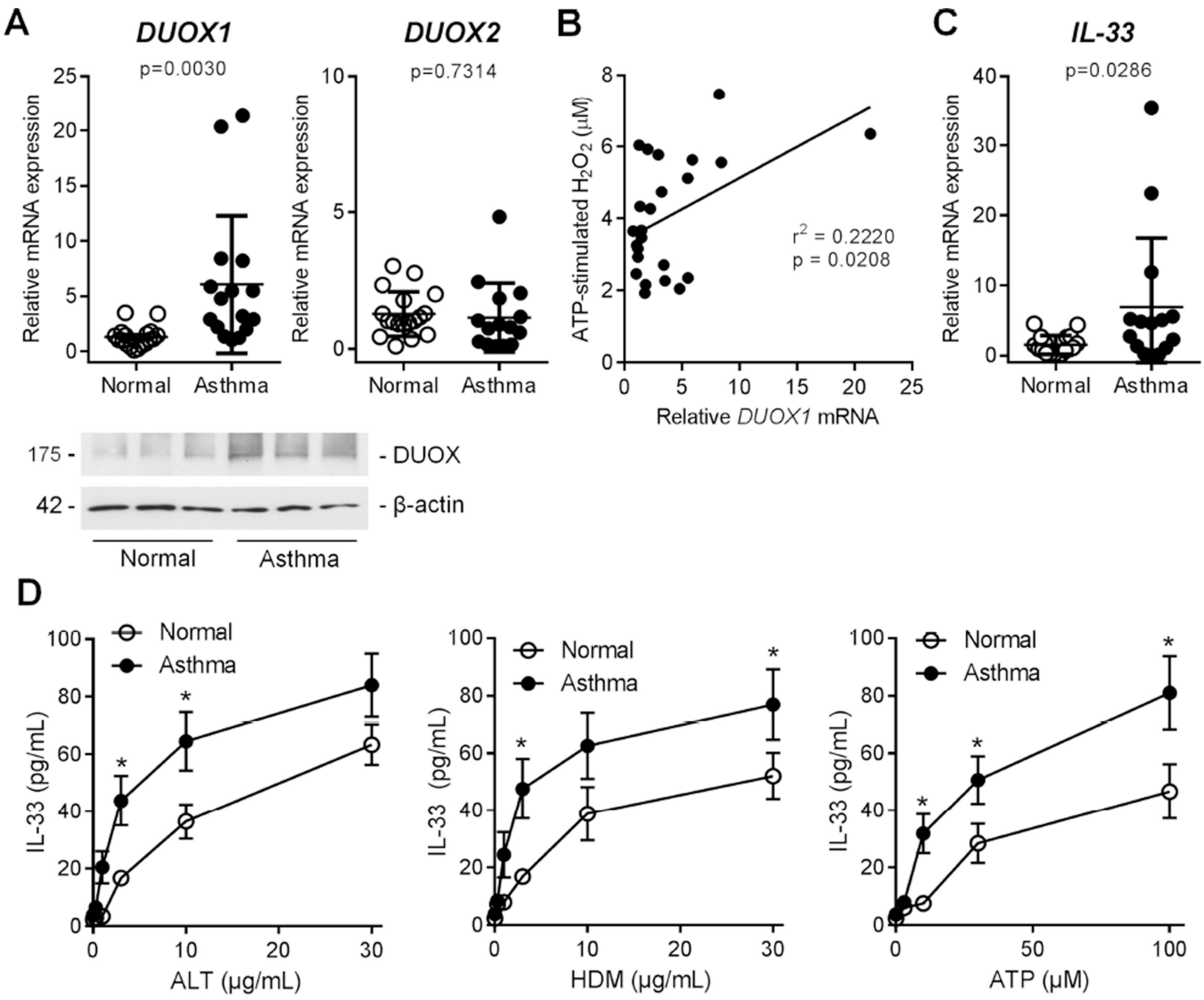

Figure 6.

DUOX1 expression and allergen responses are enhanced in HNE cells from asthmatic subjects. (*A*) Analysis DUOX1 and DUOX2 mRNA or DUOX protein in HNE cells from normal (n=18) or asthmatic (n=17) subjects. (*B*) Correlation between DUOX1 mRNA expression and ATP-stimulated (100 μM) production of H_2O_2 in HNE cells from normal subjects and subjects with asthma. (*C*) RT-PCR analysis of IL-33 mRNA in HNE cells from normal and asthmatic subjects. (*D*) Quantitation of allergen-induced IL-33 release (2 hrs) by HNE cells from normal (n=10) or asthmatic (n=11) subjects. Mean ± S.E. **P*<0.05.