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Impact of eosinophil-peroxidase (EPX) deficiency on eosinophil structure and function in mouse airways

Caroline M. Percopo[*], **Julia O. Krumholz**[*,§], **Elizabeth R. Fischer**[‡], **Laura S. Kraemer**[*,¶], **Michelle Ma**[*], **Karen Laky**[†], and **Helene F. Rosenberg**[*,‖]

[*]Inflammation Immunobiology Section, Laboratory of Allergic Diseases, NIAID, NIH, Bethesda, MD, 20892

[†]Food Allergy Research Unit, Laboratory of Allergic Diseases, NIAID, NIH, Bethesda, MD, 20892

[‡]Research Technologies Branch, Rocky Mountain Laboratories, NIAID, NIH, Hamilton, MT, 59840.

Abstract

Eosinophil-peroxidase (EPX) is a major constituent of the large cytoplasmic granules of both human and mouse eosinophilic leukocytes. Human EPX-deficiency is a rare, autosomal-recessive disorder limited to the eosinophil lineage. Our intent was to explore the impact of EPX gene-deletion on eosinophil content, structure and function. In response to repetitive intranasal challenge with a filtrate of the allergen, *Alternaria alternata*, we found significantly fewer eosinophils peripherally and in the respiratory tracts of *EPX*$^{-/-}$ mice compared to wild-type controls; furthermore, both the major population (Gr1$^{-/lo}$) and the smaller population of Gr1^{hi} eosinophils from *EPX*$^{-/-}$ mice displayed lower MFIs for Siglec F. Quantitative evaluation of transmission electron micrographs of lung eosinophils confirmed the relative reduction in granule outer matrix volume in cells from the *EPX*$^{-/-}$ mice, a finding analogous to that observed in human EPX deficiency. Despite the reduced size of the granule matrix, the cytokine content of eosinophils isolated from allergen-challenged *EPX*$^{-/-}$ and wild-type mice were largely comparable to one another, although the *EPX*$^{-/-}$ eosinophils contained reduced concentrations of IL-3. Other distinguishing features of lung eosinophils from allergen-challenged *EPX*$^{-/-}$ mice included a reduced fraction of surface TLR4-positive cells and reduced MFI for NOD1. Interestingly, the EPX gene-deletion had no impact on eosinophil-mediated clearance of gram-negative *Hemophilius influenzae* from the airways. As such, although no clinical findings have been

[‖]Correspondence to: Helene F. Rosenberg, MD, PhD, Building 50, Room 6241, 50 South Drive, NIAID, NIH, Bethesda, MD 20892; tel: +1-301-761-6682; fax: +1-301-480-8384; hrosenberg@niaid.nih.gov.

AUTHOR CONTRIBUTIONS

CMP performed most of the experimental work, analyzed data, and reviewed the manuscript.
JOK performed additional experimental work, researched information for the text, and reviewed the manuscript.
ERF performed transmission electron microscopy on isolated eosinophils and reviewed the manuscript.
LSK developed methods for identification of NOD1 by flow cytometry and reviewed the manuscript.
MM assisted with genotyping of *EPX*$^{-/-}$ mice and reviewed the manuscript.
KL provided critical assistance on the splenocyte experiments and reviewed the manuscript.
HFR designed the study, analyzed data, wrote the first and subsequent drafts of the manuscript.

[§]Current address: Boston University School of Medicine, Boston, MA, 02118
[¶]Current address: Uniformed Services University of Health Sciences, Bethesda, MD, 20814

CONFLICT OF INTEREST DISCLOSURE

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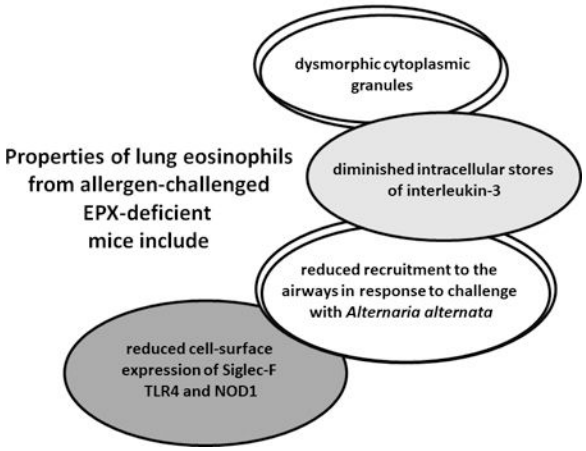
associated with human EPX-deficiency, our findings suggest that further evaluation for alterations in eosinophil structure and function may be warranted.

Graphical Abstract



Keywords

Inflammation; Allergy; Cytokine

INTRODUCTION

Eosinophils are immunomodulatory leukocytes with complex roles in health and disease that remain poorly understood [1, 2]. As but one example, eosinophils are recognized as primary inflammatory cells that are recruited to and activated in the respiratory tract as part of the pathophysiology of severe eosinophilic asthma [3]. However, from an evolutionary perspective, eosinophils would no longer exist within living vertebrate species if their sole function was to promote disorder and disease [1, 4]. In the absence of disease, eosinophils are prominent in the small intestines, where they are poised to interact directly with macrophages, dendritic cells, B and T lymphocytes, and to direct mucosal immunity at this interface [5, 6]. While eosinophils are required for full support of IgA-producing plasma cells in the gastrointestinal tract and have been implicated in promoting microbiome homeostasis and protection against gastrointestinal infection [7–10], eosinophil-deficient mice are not overly handicapped in the absence of extreme provocation [11]. An improved understanding of eosinophils is clearly needed, including their homeostatic as well as their dysregulated responses.

Eosinophil peroxidase (previously abbreviated EPO, now EPX) is a major cationic protein found in the cytoplasmic granules of both human and mouse eosinophils (reviewed in [12]). Archer and Hirsch [13] were among the first to characterize peroxidase activity in eosinophil granules; purification schemes for EPX from guinea pig and horse eosinophils followed shortly thereafter [14, 15]. Human and mouse EPX polypeptides were isolated, followed by cDNA and genomic sequences [16, 17]. EPX is highly conserved, with 90% amino acid sequence homology between human and mouse orthologs; both are also homologous to their

respective neutrophil myeloperoxidases. Mature EPX consists of two-polypeptides (55 kDa heavy and 15 kDa light chain) linked by disulfide bonds; active EPX generates hypohalous acids from ambient hydrogen peroxide generated by eosinophil NADPH oxidase [18, 19]. Among other functions, Klebanoff and colleagues [20, 21] found that EPX displayed prominent anti-bacterial activity against gram-negative bacteria in experiments carried out *ex vivo* in the presence of exogenous hydrogen peroxide and halide ions.

In 1968, Presentey [22] identified the first cases of human EPX deficiency, detected as part of routine lab evaluation of two siblings from a Yemenite Jewish family. Although unremarkable at the light microscopic level, structural alterations were noted under the electron microscope. Specifically, the volume of the eosinophil granule matrix (the outer of the two layers), the site that would ordinarily contain EPX, was reduced compared to the inner (Major Basic Protein (MBP)-1-containing) granule core. Further screening yielded additional cases within Yemenite, Iraqi and North African Jewish populations. No specific clinical findings were attributed to EPX deficiency and transmission of this trait was determined to be autosomal recessive [23]. Hereditary EPX deficiency was later reported in several families undergoing routine blood testing in Northern Italy; Romano and colleagues [24] identified a G to A transition within the coding sequences (exon 7) from one cohort that resulted in conversion of Arg^{286} to His. Affected individuals generated immunoreactive EPX that was spectroscopically abnormal and did not react appropriately with cytochemical dyes. Nakagawa and colleagues [25] described another cohort in which a G to A transition (exon 11) resulted in a conversion of Asp^{648} to Asn, a mutation that could alter the three-dimensional conformation and catalytic capacity of the EPX molecule.

The EPX gene-deleted ($EPX^{-/-}$) mouse strain generated and characterized by Denzler and colleagues [26] represents a model of profound EPX deficiency. These mice generate no EPX due to disruption of the coding sequence at exons 6, 7 and 8. Analogous to the human clinical syndrome, eosinophils from $EPX^{-/-}$ mice appear normal at the light microscopic level and granules are intact, although granule size was visually reduced secondary to loss of volume in the granule matrix. Of interest, this observation is in profound contrast to that seen in mice devoid of the granule core protein MBP-1, in which granule biosynthesis is highly irregular and eosinophils are nearly unrecognizable at the light microscopic level [27]. Mice devoid of both EPX and MBP-1 are unable to generate eosinophils at all [28], results suggesting that EPX deficiency alone may have a larger impact on eosinophil development and homeostasis than has been previously considered.

Our intent in this study was to explore the overall impact of EPX gene-deletion on eosinophil contents, structure and function. Earlier work, including ours, suggested that EPX gene-deletion had no impact on eosinophil recruitment to the lungs and airways using standard intraperitoneal adjuvant-mediated sensitization and challenge strategies [26, 29]. In these studies, the relevant antigens, either ovalbumin [26] or extract of *Aspergillus fumigatus* ([29]; family Trichomaceae) were combined with an aluminum-containing adjuvant and administered as an intraperitoneal sensitization agent prior to secondary respiratory challenges. While highly effective at promoting eosinophil recruitment to the lungs and airways, the alum adjuvant-enhanced Th2 response [30] may mask subtle differences between the wild-type and the gene-deleted mouse strain.

Here, we examine eosinophils from *EPX*$^{-/-}$ mice utilizing a more physiologic, purely intranasal challenge strategy (i.e., no adjuvants or intraperitoneal sensitization) featuring antigens in a filtrate from *Alternaria alternata* (family Pleosporaceae), a saprophytic fungus and environmental aeroallergen that has been clinically associated with the development of allergic asthma [31]. We have also explored the interactions allergen-challenged wild-type and *EPX*$^{-/-}$ mice with *Haemophilus influenzae* a gram-negative pathogen and that has been implicated in airway disease [32–34].

MATERIALS AND METHODS

Mice.

Wild-type C57BL/6 mice (7 – 10 weeks old) were from Charles River Laboratories, Frederick, MD. Eosinophil-deficient Δ*dbl*GATA mice [35] on the C57BL/6 background are maintained in the 14BS vivarium on campus. *EPX*$^{-/-}$ mice on the C57BL/6 background originally described by Denzler and colleagues ([26]; back-crossed more than 20-generations on to the C57BL/6 background [28]) are also maintained in the 14BS vivarium. Studies were carried out on age and gender matched mice. The National Institute of Allergy and Infectious Diseases Division of Intramural Research Animal Care and Use Program, as part of the National Institutes of Health Intramural Research Program, approved all the experimental procedures as per protocol LAD 8E.

Challenge with *Alternaria alternata* and evaluation of cells and cytokines in the airways.

Mice under isoflurane anesthesia were inoculated intranasally via repetitive challenge with a reconstituted filtrate of *A. alternata* (Stallergenes-Greer, Lenoir, NC, USA; stock 10 mg/mL, diluted to 50 μg/mouse in 50 μL phosphate buffered saline (PBS) with 0.1% bovine serum albumin (BSA)) on days 0, 3 and 6 as shown in Fig. 1A. At day 10, mice were sacrificed and subjected to bronchoalveolar lavage (BAL) with PBS with 0.1% BSA. Cytospins were prepared and stained with modified Giemsa (Diff-Quik; ThermoScientific). Eosinophils as percent of total leukocytes was determined by visual inspection and scoring of minimum of 100 stained cells / mouse. Cytokine levels in BAL fluid were evaluated by DuoSet assay (R&D Systems).

Generation of bone-marrow derived eosinophils.

Bone marrow-derived eosinophils (bmEos) were prepared from unselected progenitors from both wild-type and *EPX*$^{-/-}$ mice using the method previously described [36] with minor changes as follows. Briefly, bone marrow progenitors were flushed from the tibiae and femurs of mice with sterile RPMI; red blood cells were lysed and remaining cells were suspended at a concentration of 10^6/mL in RPMI with 20% fetal calf serum, 25 mM HEPES, 100 IU/mL and 10 μg/mL Pen-Strep, 2 mM glutamine, non-essential amino acids, 1 mM sodium pyruvate, 50 μM beta-mercaptoethanol, 100 ng/mL recombinant murine (rm) stem cell factor (SCF) and rmFlt3-Ligand (Flt3L). On day 4, culture medium was replaced and included no SCF or FLt3-L but contained 10 ng/mL interleukin-5 (IL-5). Half of the media was replaced with fresh medium with 10 ng/mL IL-5 on days 6, 8, 10, 12, 14, 16, and 18; all non-adherent cells were moved to new flasks on days 8, 12, and 16. Cells were collected for analysis on day 20. In a recent study [37] we utilized bmEos from C57BL/6

mice from day 20 cultures which were effective at phagocytosing bacteria; viability of bmEos derived from C57BL/6 progenitors at day 20 in culture is > 90% as shown in Suppl. Fig. 1.

Flow cytometry, fluorescence-activated cell sorting (FACS) and evaluation of eosinophil contents.

For routine flow cytometry, single cell suspensions were prepared from whole lung tissue of mice as previously described [29]; upon thawing, cells were washed and stained for viability (live-dead), and eosinophils were identified as $CD45^+$ $CD11c^-MHCII^-Gr1^{-/lo}SiglecF^+$ cells as originally shown in Fig. 1 of reference 29. Cells in specific experiments as noted were permeabilized with buffer containing 0.1% saponin, 1mM $CaCl_2$, 1mM $MgSO_4$, 0.05% NaN_3, 0.1% BSA, 10 mM HEPES and 5% nonfat dry milk. All antibodies and isotype controls are included in Suppl. Table 1. When performing FACS, single cells were subjected to sorting immediately after isolation without any intervening freeze/thaw step. Eosinophils isolated by FACS were washed with PBS and re-suspended in lysis buffer (1% Igepal CA-630, 20 mM Tris-HCl (pH 8.0), 137 mM NaCl, 10% glycerol, 2 mM EDTA with protease inhibitors (Protease Inhibitor Cocktail Tablets, Roche) at 10^7 cells/mL, also as described previously [29]. Lysates were rocked gently for 30 min at 4°C. Supernatants were clarified by centrifugation (14,000g x 5 min) and final protein concentration was determined by BCA assay (Pierce). Relative cytokine levels (from wild-type *vs.* *EPX*$^{-/-}$ mice) were determined using 300 μg total protein (5.3 x 10^6 eosinophils) from each sample to probe a Cytokine profiler (ARY006; R&D Systems) as per manufacturer's instructions. Relative expression was evaluated by scanning on a Licor Odyssey CLX. Scans were analyzed using Image Studio Ver 4.0 Software. Absolute levels of specific cytokines of interest were determined by Quantikine and DuoSet ELISA assays (R&D Systems).

Transmission electron microscopy and image evaluation.

Eosinophils were isolated from mouse lung tissue by FACS as described above. Specimens were fixed with 2.5% glutaraldehyde in 0.1 M phosphate buffer. Samples were post-fixed 1h with 0.5% osmium tetroxide/0.8 % potassium ferricyanide, 1 hour with 1% tannic acid and overnight with 1% uranyl acetate at 4°C. Samples were dehydrated with a graded ethanol series and embedded in Spurr's resin. Thin sections were cut with a Leica UCT ultramicrotome (Vienna, Austria) prior to viewing at 120 kV on a FEI Tecnai BT Spirit transmission electron microscope (Hillsboro, OR). Digital images were acquired with an AMT digital camera system (AMT, Chazy, NY). Digital images were evaluated with Image J 1.49v available online at NIAID. Relative area was calculated as area of the granule core as a fraction of the area of an entire granule within eosinophils from both wild-type and *EPX*$^{-/-}$ mice.

Splenocyte isolation and challenge with PMA and ionomycin.

Spleens were isolated from wild-type and *EPX*$^{-/-}$ mice challenged via intraperitoneal inoculation with antigen, either 50 μg ovalbumin (Sigma) or 20 μg *A. fumigatus* extract (Hollister-Stier) complexed with Imject Alum (Pierce). Splenocytes were generated from spleens minced in Hanks Balanced Salt Solution with 1% fetal bovine serum and 10 mM HEPES and then passed through a 100 micron cell strainer. Splenocytes were pulled into a

10 ml syringe and passed through a 21-gauge needle, followed by centrifugation at 300 x g for 5 minutes. Cell pellet was resuspended in 1 ml of HBSS, 1% FBS, 10 mM HEPES and red blood cells were lysed with distilled water followed by 10X PBS. Cells were enumerated using trypan blue dye exclusion and resuspended at 5 x 10^6 cells per ml of RPMI 1640 plus 10% fetal bovine serum (Atlanta Biologics S11150), Penicillin 10 units/mL and Streptomycin 10 μg/mL (Life Technologies) and challenged with combined phorbol 12-myristate 13-acetate; (PMA; Sigma) at 20 ng/mL) and ionomycin (Sigma) at 1 μg/mL for 3 hours. IL-3 released into the supernatants was evaluated by ELISA using the Quantikine R & D kits as per manufacturer's instructions.

Culture, quantification and *in vivo* challenge with *Hemophilus influenzae*.

The non-typeable *Hemophilus influenzae* (NTHi) reference strain NCTC 4560 was obtained from American Type Culture Collection (Manassas, VA; Cat. No. 19418). Colonies from frozen stocks maintained at −80°C were generated by streaking onto sBHI agar plates (Brain Heart Infusion agar with 1 mg / mL nicotinamide adenine dinucleotide (NAD; Sigma) and 10 μg/mL hemin (MP Biomedicals, LLC) after overnight growth at 37°C at 5% CO_2 [38]. Bacteria were inoculated into 30 mL of sBHI broth with NAD and hemin as above and grown to an OD_{600} of 0.45, determined experimentally to generate 2 x 10^9 colony forming units (CFU)/mL. Bacteria were collected by centrifugation (4100 x g) and resuspended at 10^7/mL in pbs with 0.1% bsa. Wild-type and *EPX*$^{-/-}$ mice challenged with *A. alternata* on days 0, 3, and 6 as described above were subjected to further intranasal challenge on day 10 with 5 x 10^5 CFU *H. influenzae* in 50 μL prepared as above. At t = 2 hrs, mice were sacrificed and BAL fluid was collected. CFUs per mL BAL fluid were determined after overnight growth on sBHI agar plates at 37°C in 5% CO_2 as described above.

Statistical analysis.

All quantitative findings were from replicate datasets. Flow plots shown are representative of typical results. Data were analyzed via appropriate algorithms (Mann-Whitney u-test, Student's t-test, ANOVA) utilizing GraphPad PRISM. Error bars shown represent standard deviation (SD) unless stated otherwise in the Figure Legend.

RESULTS AND DISCUSSION

In this study, wild-type and *EPX*$^{-/-}$ mice were challenged intranasally with a filtrate of *Alternaria alternata* (50 μg per mouse) on days 0, 3 and 6 as shown in Fig. 1A. We and others [39, 40] have found that this protocol, which uses no adjuvant and no distinct sensitization phase, results in eosinophil recruitment to the lungs and airways of wild-type mice.

Morphologic characterization of eosinophils from allergen-challenged *EPX*$^{-/-}$ mice.

As noted in earlier publications, eosinophils from antigen-challenged *EPX*$^{-/-}$ mice appear normal at the light microscopic level [26, 29]. Eosinophils from the airways of *A. alternata*-challenged *EPX*$^{-/-}$ mice stained with modified Giemsa have ring-shaped, polymorphic nuclei with red-staining, microgranular cytoplasm, and are visually indistinguishable from those from the airways of *A. alternata*-challenged wild-type mice [Figs. 1B and 1C].

Electron micrographs of peritoneal eosinophils isolated from *EPX*$^{-/-}$ mice suggested that the granule matrix, the site of EPX localization, was reduced in volume [26], analogous to what has been described for human eosinophil-deficiency [23, 24]. Transmission electron micrographs of eosinophils from the lungs of wild-type and *EPX*$^{-/-}$ mice challenged with a filtrate from *A. alternata* culture as in Fig. 1A are shown in Figs. 1D – 1G. As indicated by the arrows in Figs. 1D and 1F, among the most prominent differences are the characteristics of the large cytoplasmic granules. In the eosinophils from wild-type mice (Fig. 1D and 1E), the granule in cross-section maintains a substantial outer matrix surrounding the central core. By contrast, the granule matrix in eosinophils from *EPX*$^{-/-}$ strain includes only a narrow margin of outer matrix around the granule core (Figs. 1F and 1G). We examined this observation quantitatively by measuring the area of granule core relative to the total granule area. We confirmed that, in eosinophil granules from *EPX*$^{-/-}$ mice, the core represents a larger fraction of the total granule area than in wild-type mice (0.47 ± 0.13 *vs.* 0.27 ± 0.05, $^{**}p < 0.02$), representing a significant difference between these strains [Fig. 1H].

Biochemical characterization of eosinophils from allergen-challenged *EPX*$^{-/-}$ mice.

In addition to EPX, eosinophils store numerous pre-formed cytokine mediators. These cytokines are mobilized in response to biochemical and inflammatory stimuli and contribute to their immunomodulatory potential [41 – 43].

Initial screening of the eosinophils isolated from the lungs of *A. alternata*-challenged wild-type and *EPX*$^{-/-}$ mice by cytokine profiling suggested that the contents of eosinophils from the two strains were largely comparable to one another (data not shown). As shown in Fig. 2A, lysates were prepared from eosinophils isolated by FACS which were then evaluated by ELISA. This analysis revealed that eosinophils from the lungs of *A. alternata*-challenged *EPX*$^{-/-}$ mice and wild-type mice contained largely comparable levels of IL-1Ra/IF3, CXCL12 and IL-1β; however, eosinophils from wild-type mice contained relatively more immunoreactive IL-3 than eosinophils isolated from the lungs of *A. alternata*-challenged *EPX*$^{-/-}$ mice (ratio 5.3 ± 2.2; $^{**}p < 0.01$ [Fig. 2A]). No IFN-γ, GM-CSF, IL-13 or IL17 was detected in eosinophil lysates from either strain (data not shown).

As reported by Doyle and colleagues [28], the *EPX*$^{-/-}$ mice used in this study were backcrossed to the C57BL/6 wild-type parent strain for > 20 generations; this is a process designed to eliminate any unanticipated mutations in gene-deleted mouse strains. Taken further, we show that splenocytes isolated from *EPX*$^{-/-}$ and wild-type mice that were activated *ex vivo* are capable of synthesizing and releasing equivalent amounts of immunoreactive IL-3 [Fig. 2B]. As such, we found no evidence for overall instability or diminished capacity at the IL-3 locus.

To date, there are only a few published reports that profile the cytokine contents of mouse eosinophils in a systematic fashion [29, 44], although there have been several studies of this nature performed on human eosinophils [42, 45]. As is clear from these studies, and the comparative analysis presented by Lee and colleagues [44], the cytokine contents of human and mouse eosinophils are far from identical, although both do contain IL-3 [29, 46], as discussed further below. To the best of our knowledge, cytokine biosynthesis and trafficking

has not been examined in any of the known human EPX deficiency cohorts [22 – 25]. The results of this work suggest that this may be an interesting area for future exploration.

EPX$^{-/-}$ mice respond to *A. alternata* challenge with diminished numbers of eosinophils in the lungs and reduced expression of Siglec F.

We examined eosinophils ($CD45^+CD11c^-MHCII^-$ $Gr1^{-/lo}$ $SiglecF^+$) in bone marrow, spleen and lungs of wild-type and *EPX*$^{-/-}$ mice subjected to repetitive challenge with *A. alternata* as in Fig. 1A. As shown in Fig. 3A, significantly fewer eosinophils were detected in the bone marrow, lungs and in the spleens of *EPX*$^{-/-}$ mice compared to wild-type controls. Furthermore, while median fluorescence intensity (MFI) for Siglec F, the glycan-binding, immunoglobulin-like lectin and major marker for the eosinophil lineage in mice (reviewed in [47]) is highest on eosinophils isolated from the lungs compared to those from bone marrow and spleen, it is significantly lower among lung eosinophils from *EPX*$^{-/-}$ mice compared to wild-type [Fig. 3B]. As described previously for wild-type mice [29], a small subset of $SiglecF^+Gr1^{hi}$ eosinophils was also identified in the lungs of *EPX*$^{-/-}$ mice, [Fig. 3C]; this subset from lungs of *A. alternata*-challenged *EPX*$^{-/-}$ mice also displayed a reduced MFI for Siglec F compared to those from *A. alternata*-challenged wild-type controls [Fig. 3D].

While the full implications of these findings remain unclear, as noted above, IL-3 has been detected in human eosinophils [46], contributes to normal eosinophil hematopoiesis and is a pro-survival factor [48]. Of note, Yamamoto and colleagues [49] have shown that human eosinophils can be sustained by autocrine release of the pro-survival cytokine, GM-CSF, although this has not been demonstrated directly for murine counterparts. Nonetheless, we consider the possibility that the reduced numbers of eosinophils detected in *EPX*$^{-/-}$ mice (see Fig 3A) may relate to diminished intracellular content of IL-3, a hypothesis that awaits further evaluation.

Siglec F is a cell surface glycoprotein and marker of committed eosinophils [47]. Allergen-challenged *Siglec-F*$^{-/-}$ mice feature increased eosinophil numbers in the airways, a result that suggests that eosinophil numbers may be modulated *in vivo* by physiologic apoptosis via ligand-receptor interactions [50]. While an imperfect measure of receptor numbers, eosinophils in the lungs of *EPX*$^{-/-}$ mice display a reduced MFI for Siglec F; as such, it is unlikely that the reduced number of eosinophils in the lungs (Fig. 3A) relates to enhanced Siglec F-mediated apoptosis. Of interest, McMillan and colleagues [51] reported that Siglec-F-dependent regulation of eosinophil numbers in the lung was dependent on the distinct attributes of the experimental model used for study. As the aforementioned studies both utilized ovalbumin sensitization and challenge models, the role of Siglec F in modulating eosinophil apoptosis *in vivo* might merit further evaluation in models featuring mice challenged with *A. alternata*.

We also examined expression of maturation markers; sub-populations of $SiglecF^+Gr1^{-/lo}$ eosinophils from the airways of wild-type and *EPX*$^{-/-}$ mice were also $CCR3^+$ (33 ± 10%) or $CD34^+$ (11 ± 7%); no CD125 was detected [Suppl. Fig. 2]. CCR3 (CD193) is a receptor for the critical eosinophil chemoattractants, CCL11 and CCL24 [52]. In a previous study, we found that anti-CCR3 detected only a fraction (26 ± 6%) of the Siglec F^+ cells in hypereosinophilic mouse bone marrow [53]; the results presented here suggest that the same

may be true for CD11c^{-}Siglec F^{+} eosinophils recruited to lungs of allergen-challenged mice. Of note, the anti-Gr1 antibody featured here detects the two distinct and cross-reacting cell surface antigens, Ly6G and Ly6C [54]; we found previously that the SiglecF^{+}Gr1^{hi} population is uniquely Ly6G^{+} [29].

Differential expression of the pattern recognition receptor, TLR4, on eosinophils from allergen-challenged wild-type and *EPX*$^{-/-}$ mice.

Although pattern recognition receptors (PRRs) have been defined on human eosinophils [55], comparatively less is known regarding their expression and function among populations of eosinophils in the mouse [56]. The role of PRRs in modulating eosinophil-mediated interactions with bacteria, including phagocytosis and generation of extracellular traps is likewise a subject that remains to be explored [6, 10].

The PRR, Toll-like receptor 4 (TLR4), is expressed widely among myeloid cells and responds to gram-negative bacterial lipopolysaccharides (LPS) and endogenous proinflammatory ligands [57]. We find here that only a small fraction of eosinophils isolated from bone marrow, spleen or lungs of *A. alternata*-challenged mice are surface TLR4^{+} (see Suppl. Fig. 3 for gating strategy). Nonetheless, there are more TLR4^{+} eosinophils in the lungs and spleens of *A. alternata*-challenged wild-type than in *EPX*$^{-/-}$ mice [Fig. 4A], although the MFI was higher among eosinophils isolated from the spleens of mice from the *EPX*$^{-/-}$ strain [Fig. 4B]. It is intriguing to consider these findings in light of those shown in Fig. 4C, in which we demonstrated that virtually all eosinophils contain intracellular stores of TLR4 (ie., all are TLR4^{+} when the cells are fully permeabilized). Taken together, these findings suggest that the absence of EPX may have a broader impact on eosinophil cell structure and protein trafficking than is currently appreciated.

While TLR-mediated signaling has been implicated in modulating allergic inflammation in mouse models [58–61], expression of TLR4 on mature eosinophils *in vivo* and the functional consequences of this finding remain incompletely explored. Of note, O'Flaherty and colleagues [62] have shown that bone marrow-derived eosinophils (mature eosinophils generated in cytokine-enriched culture from unselected progenitors [36]) from wild-type but not those from *TLR4*$^{-/-}$ mice, stimulated for 4h with LPS will recruit NK cells upon introduction into the peritoneal cavity of naïve mice. This observation is worthy of further consideration, notably given recent observations featuring eosinophils and reports documenting their interactions with gram-negative bacteria *in vivo* [6]; see further below.

Differential expression of NOD1 on wild-type and *EPX*$^{-/-}$ mice.

NOD1 is an intracellular PRR of the NOD-Like Receptor (NLR) family that responds to γ-D-glutamyl-*meso* diaminopimelic acid component of peptidoglycan which comprises the gram-negative bacterial cell wall [63]. Interestingly, expression of NOD1 varies widely depending on source of eosinophils; 60 to 70% of the eosinophils from spleens of *A. alternata*-challenged mice express NOD1 (and 80 – 85% of the spleen eosinophils from *EPX*$^{-/-}$ mice), while eosinophils isolated from the lungs are primarily NOD1-negative [Fig. 5C]. Similar to our observations with TLR4 and Siglec F, MFI is highest among lung eosinophils; a somewhat lower MFI was observed among lung eosinophils from *EPX*$^{-/-}$ mice

[Fig. 5D]. This pattern – a high fraction of NOD1$^+$ eosinophils in the spleen and lower fraction of NOD1$^+$ eosinophils in the lung – was also observed among eosinophils isolated from *IL-5* transgenic mice [Suppl. Fig. 4]. NOD1 receptors on spleen eosinophils are functional and respond in a dose-dependent fashion to biochemical ligand, iE-DAP (Kraemer and Rosenberg, unpublished data). TLR4$^+$ and NOD1$^+$ eosinophils in similar proportions were also detected on bone marrow-derived eosinophils [36] from both wild-type and *EPX*$^{-/-}$ mice [Suppl. Fig. 5].

NOD1 was detected in human peripheral blood eosinophils, and can be activated by the canonical ligand, iE-DAP to release the proinflammatory cytokine, CXCL8, and to up-regulate expression of CD11b, CD18, and ICAM-1 [64, 65]. To the best of our knowledge, this is the first comprehensive evaluation of NOD1 expression in mouse eosinophils. Apart from the differential expression observed between wild-type and *EPX*$^{-/-}$ mice, it is also interesting to note that the fraction of eosinophils that are NOD1$^+$ differ depending on localization. This remarkable degree of eosinophil heterogeneity – for example, the fraction of NOD1$^+$ eosinophils is 15 – 20 times higher in the spleen than in the lung – is not yet understood mechanistically. Likewise, it is not yet clear how the structural anomaly related to EPX-deficiency alters expression of NOD1 in its intracellular locale, other than as noted above.

However, within the broader scope of issues related to eosinophil heterogeneity, it is interesting to consider the differential expression of pattern recognition receptors targeting gram-negative bacteria, ie., TLR4 and NOD1, in eosinophils, particularly in the lung. As noted above and in the paragraph to follow, until relatively recently [6, 9, 10, 56, 66], eosinophils were not perceived as having specific, physiologically meaningful interactions with bacteria.

A. alternata-challenged *EPX*$^{-/-}$ mice were no less effective at clearing gram-negative *Haemophilus influenzae* from the airways than wild-type counterparts.

Studies by Yousefi and colleagues [66] demonstrated a role for eosinophils in promoting clearance in a gram-negative bacterial sepsis model via a mechanism involving both EPX and MBP-1 in eosinophil extracellular traps (EETs). EPX itself can generate anti-bacterial reactive oxygen metabolites [21, 67] and has been implicated directly in specific nitrosylation and carbamoylation reactions *in vivo* [68, 69]. Furthermore, as shown here, *EPX*$^{-/-}$ mice display diminished recruitment of eosinophils to the lungs, and, of the eosinophils that are recruited, a smaller proportion are TLR4$^+$. Given these findings, we asked whether eosinophils, as a component of *A. alternata*-mediated inflammation, might be effective in clearing gram-negative non-typeable *Haemophilus influenzae* (NTHi) from the airways, and whether EPX-deficiency might have an impact on this activity; this was addressed via the protocol shown in Fig. 5A. As in whole lungs [Fig. 2A], fewer eosinophils were detected in the airways of *EPX*$^{-/-}$ mice on day 10 after challenge with *A. alternata* on days 0, 3 and 6, and none were detected in the airways of the eosinophil-deficient *ΔdblGATA* strain [Fig. 5B]. *A. alternata*-challenged mice of all three strains were inoculated intranasally with 5 x 10^5 colony forming units of NTHi and clearance from the airways was evaluated at 2 hrs thereafter. As shown in Fig. 5C, fewer colonies were detected overall (~3

– 4-times) in the presence of eosinophilic inflammation (wild-type or *EPX*$^{-/-}$ vs. *ΔdblGATA*, *$p < 0.05$). However, clearance was no more effective from the airways of *A. alternata*-challenged wild-type mice than from their *A. alternata*-challenged *EPX*$^{-/-}$ counterparts (n.s., no significant difference). As such, the contributions of TLR4, NOD1, or for that matter, EPX, to the bacterial clearance in this *in vivo* remain unclear.

In summary, we compared the responses of wild-type and *EPX*$^{-/-}$ mice to the physiologic aeroallergen, *A. alternata*, which elicits eosinophil recruitment to the lungs and airways following a brief period of repetitive challenge. We find that eosinophils are reduced in number both peripherally and in the lungs and airways in response to allergen challenge in *EPX*$^{-/-}$ mice although eosinophil-mediated clearance of gram-negative *H. influenzae* is not attenuated. While eosinophils from allergen-challenged *EPX*$^{-/-}$ mice are visually indistinguishable from those from the wild-type under routine microscopy, they have profound granule abnormalities, display differential expression of Siglec F and maintain reduced quantities of the pro-survival cytokine IL-3. Although no clinical findings have been associated with the cases of human EPX-deficiency that have been reported thus far [22 – 25], our findings suggest that further evaluation for subtle alterations in eosinophil content, structure and function may be warranted.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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ABBREVIATIONS

EPX	Eosinophil peroxidase
FACS	Fluorescence-activated cell sorting
MBP-1	Major basic protein-1
MFI	Mean fluorescence intensity
NOD1	Nucleotide-binding oligomerization domain-containing protein 1
TLR4	Toll-like receptor 4

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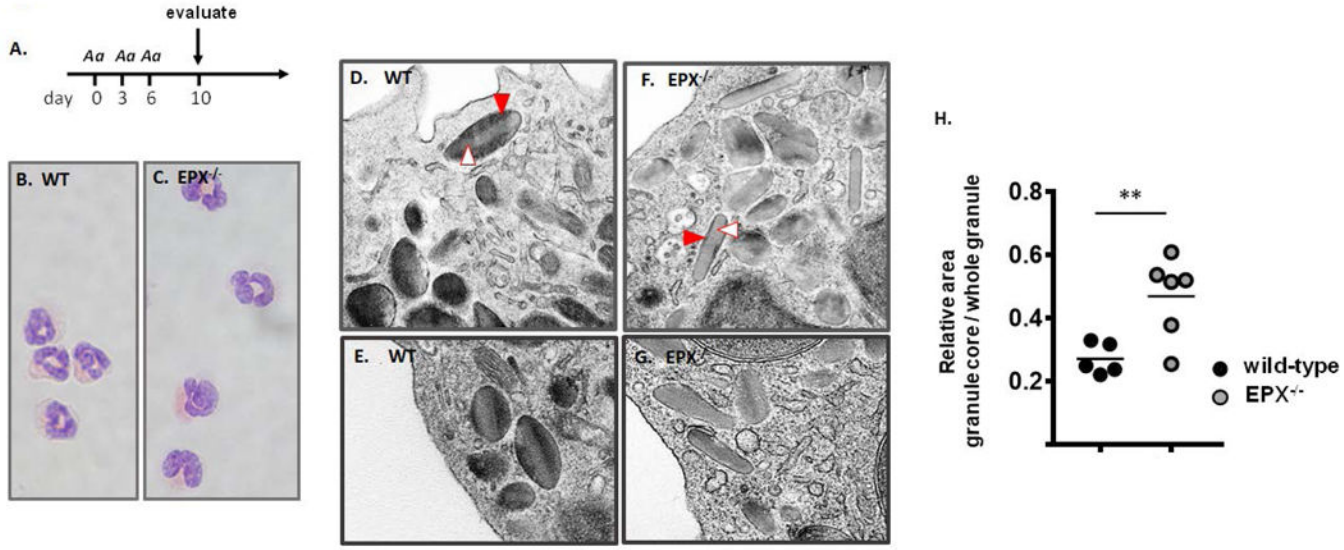

Fig. 1. Eosinophils recruited to the airways of *EPX*$^{-/-}$ mice display dysmorphic cytoplasmic granules.

A. Basic protocol: mice are inoculated intranasally with a filtrate of the fungus, *Alternaria alternata* (*Aa*) on days 0, 3 and 6 followed by evaluation at day 10 as indicated. **B.** Modified Giemsa-stained cytospin preparations of eosinophils from the airways of wild-type (WT) and **C.** *EPX*$^{-/-}$ mice subjected to repetitive challenge with *Aa* as per Fig. 1A. **D. - G.** Transmission electron micrographs (original magnification, 49,000x) of eosinophils from WT and *EPX*$^{-/-}$ mice as described in B. and C., respectively; red-filled arrowheads denote granule matrix, red-outlined arrowheads denote the internal granule core in panels D. and F. **H**. Relative area; ratio of area of granule core / area of whole granule as determined using ImageJ 1.49v; $^{**}p < 0.02$ Mann-Whitney u-test.

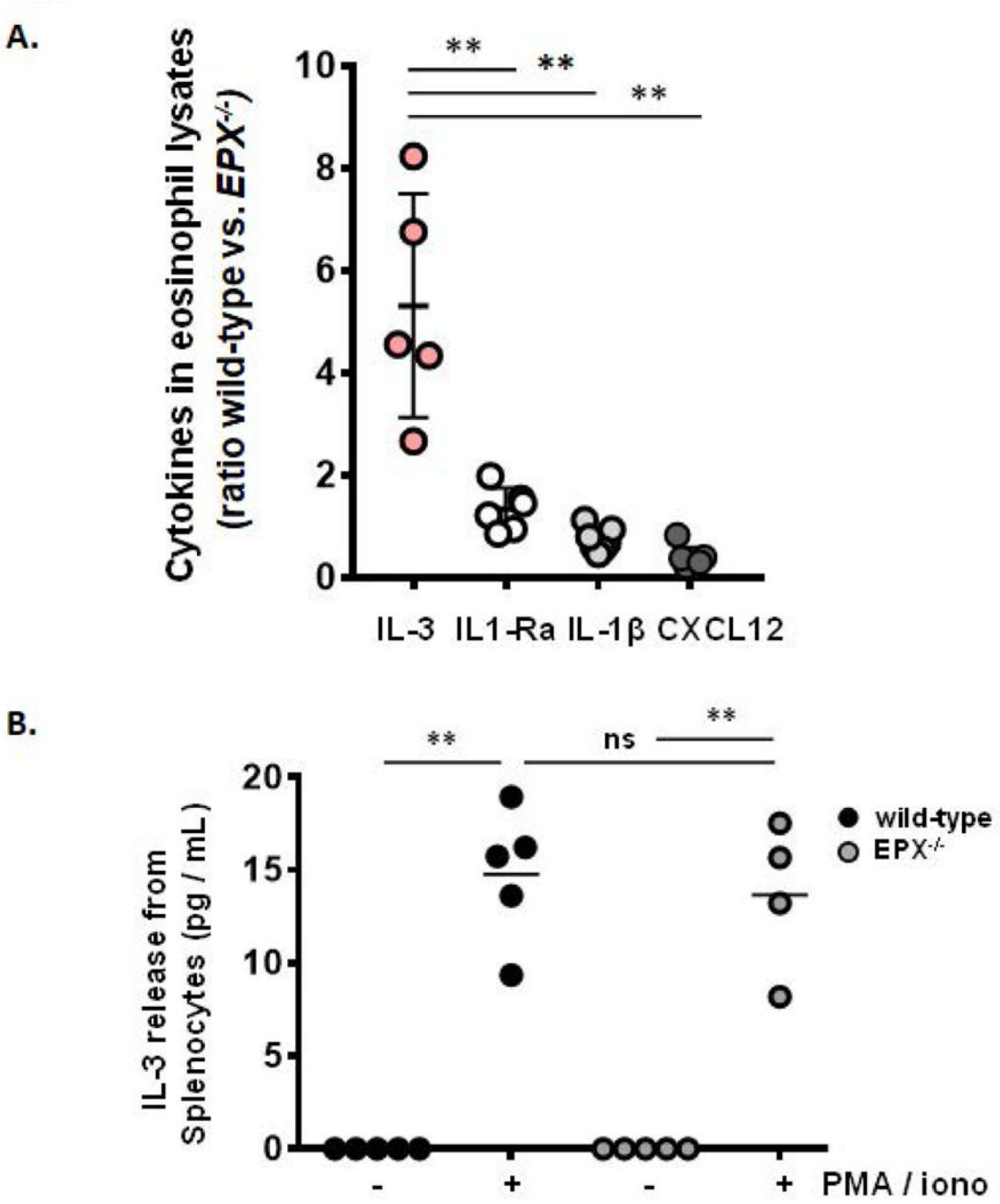

Fig. 2. Levels of immunoreactive IL-3 are diminished in eosinophils recruited to the airways of *EPX*$^{-/-}$ mice.

A. Cytokines detected in eosinophils; shown are ratios of wild-type *vs. EPX*$^{-/-}$ of pg/mg in lysates (10^7 eosinophils / mL) as determined by ELISA. Eosinophils were isolated by FACS from whole lungs of wild-type and *EPX*$^{-/-}$ mice subjected to repetitive challenge with *A. alternata* as in Fig. 1A; isolation strategy is as described in the Methods and as shown in Fig. 1 of reference 29; 5 mice per group, $^{**}p < 0.01$ by 1-way ANOVA. **B.** Immunoreactive IL-3 released from splenocytes isolated from wild-type and *EPX*$^{-/-}$ mice (5 x 10^6 cells / mL) with

and without challenge with PMA/ionomycin, n = 4 – 5 mice per group, ns, not significant, $^{**}p < 0.01$ 1-way ANOVA.

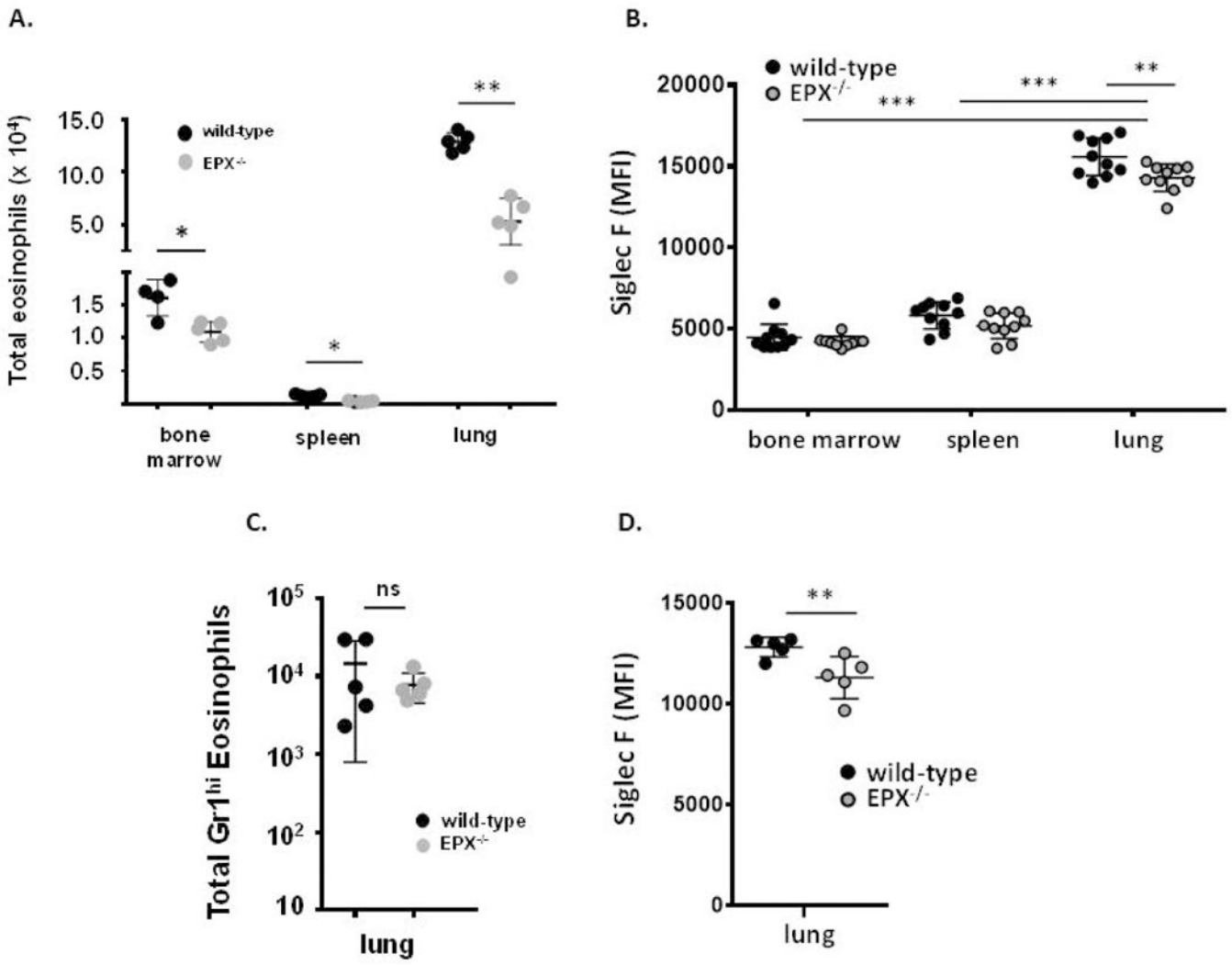

Fig. 3. Eosinophil recruitment to the respiratory tract and expression of Siglec F are diminished in *Aa*-challenged *EPX*$^{-/-}$ mice.

A. Eosinophils identified by flow cytometry (CD11c^{-}MHCII^{-}SiglecF^{+}Gr1$^{-/lo}$; see Fig. 1 in reference 29) from bone marrow, spleen and lungs of wild-type and *EPX*$^{-/-}$ mice subjected to repetitive *Aa*-challenge as in Fig. 1A; n = 4 – 5 mice per group, *$p < 0.05$, **$p < 0.01$. **B**. Siglec F expression (median fluorescence intensity, MFI) on eosinophils in A., n = 10 mice per group **$p < 0.01$ **C.** Siglec F^{+}Gr^{hi} eosinophils identified in lungs of wild-type and *EPX*$^{-/-}$ mice subjected to repetitive *Aa*-challenge as in Fig. 1A, n = 5 mice per group, ns, not significant. **D.** Siglec F expression (MFI) on eosinophils in C., n = 5 mice per group, **$p <$ 0.01.

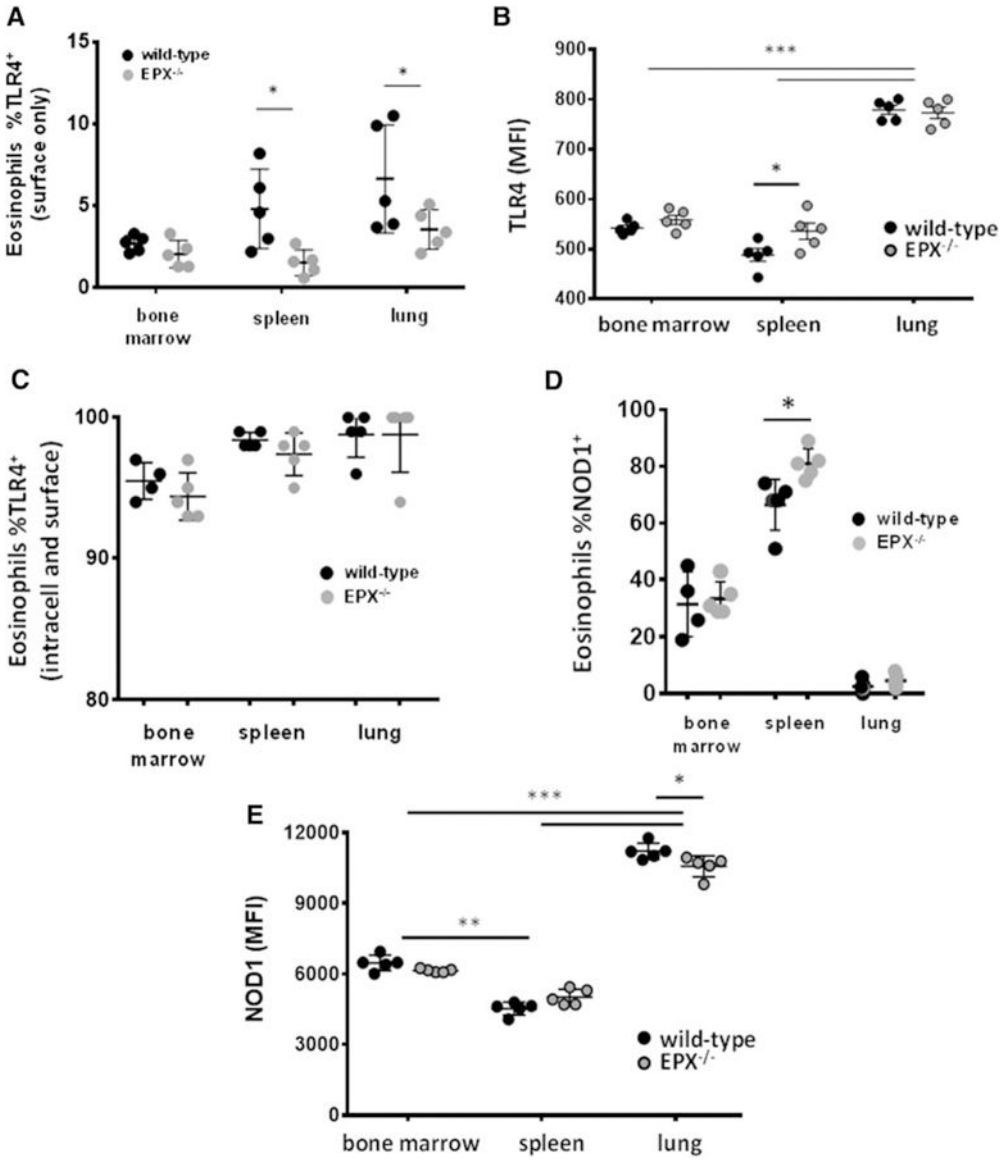

Fig. 4. Pattern recognition receptors TLR4 on and NOD1 in eosinophils isolated from *Aa*-challenged wild-type and *EPX*$^{-/-}$ mice.

A. TLR4 was detected on the surface of a fraction of eosinophils from bone marrow, spleen and lung (fewer than 10% overall; see Suppl. Fig. 3 for gating strategy). A higher percentage of spleen and lung eosinophils from wild-type mice express TLR4 than do their *EPX*$^{-/-}$ counterparts; n = 5 mice per group, *$p < 0.05$. **B.** Highest MFI was detected among eosinophils isolated from the lung, although differential expression (wild-type vs. *EPX*$^{-/-}$) was detected in spleen only; n = 5 mice per group, *$p < 0.05$). **C.** TLR4 was detected on virtually all eosinophils in bone marrow, spleen and lung after permeabilization; n = 4 – 5 mice per group, see Methods. **D.** NOD1 was detected in (permeabilized) eosinophils from bone marrow and spleen (the latter, on a larger percentage of *EPX*$^{-/-}$ than wild type mice), and in smaller fraction of eosinophils recruited to the lung; n = 5 mice per group; *$p < 0.05$. **E.** Similar to findings documenting TLR4, the highest MFI was found among eosinophils in

the lung; the MFI for NOD1 was greater among the wild type eosinophils, n = 5 mice per group, $*p < 0.05$.

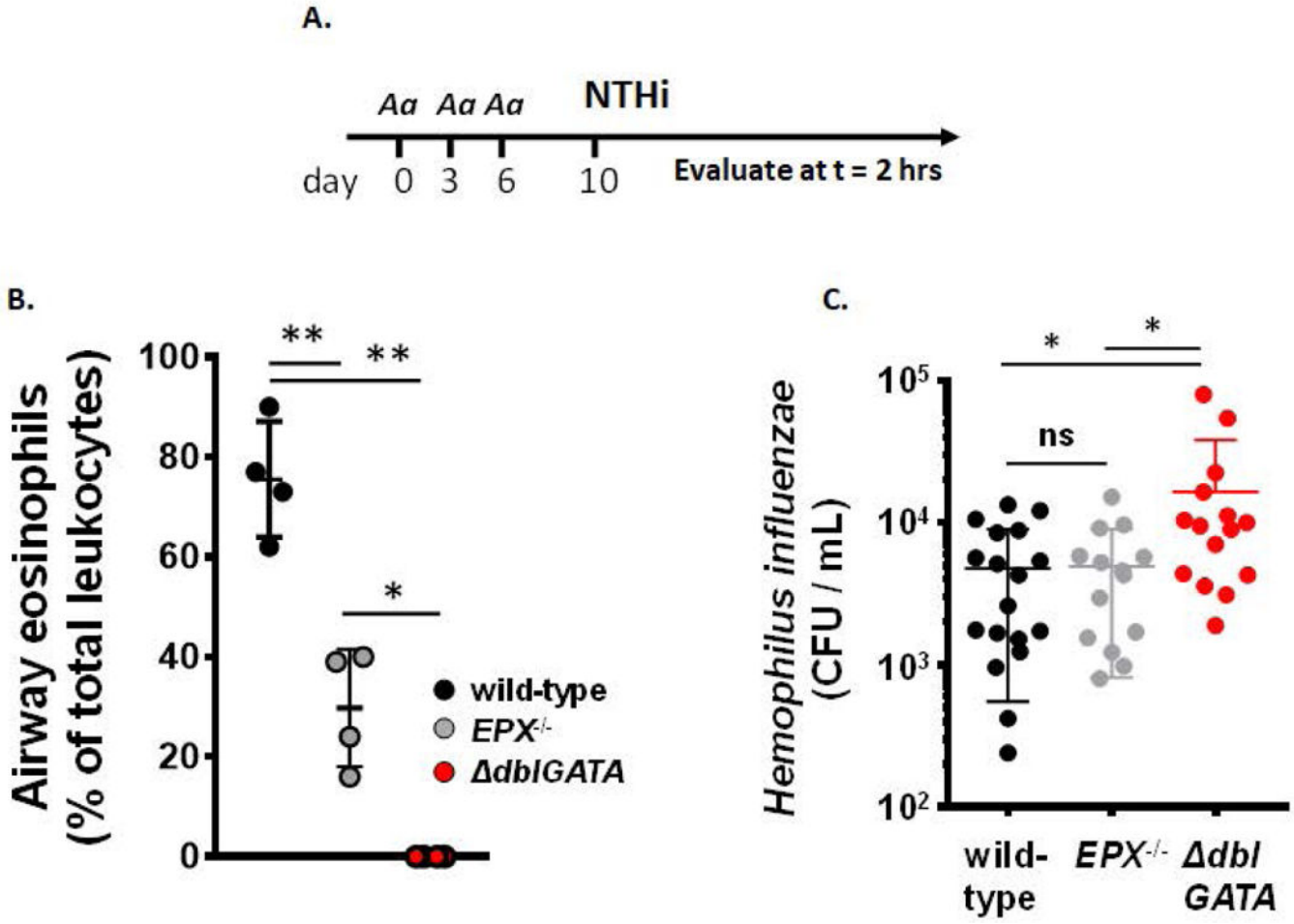

Fig. 5. *A. alternata*-challenged *EPX*$^{-/-}$ mice were just as effective as wild-type mice at clearing gram-negative *Haemophilus influenzae* from the airways.

A. Basic protocol. Mice inoculated intranasally with a filtrate of the fungus, *Alternaria alternata* (*Aa*) on days 0, 3 and 6 as in Fig. 1A were challenged on day 10 with live *H. influenzae* (NTHi; 5 x 10^5 colony forming units (CFU) in logarithmic-phase growth in 50 μL volume). CFU / mL in BAL fluid was determined at t = 2 hrs thereafter. **B.** Eosinophils detected in the airways (% of total leukocytes) of wild-type, *EPX*$^{-/-}$, and *Δdbl*GATA eosinophil-deficient mice after *Aa*-challenge was determined as in Fig. 1A; n = 3 – 4 mice per group, $^{**}p < 0.01$, $^{*}p < 0.05$. **C.** CFU / mL BAL fluid lung at t = 2 hrs after inoculation with 5 x 10^5 CFU NTHi as indicated in Fig 5A; n = 14 – 18 mice per group, bars indicate standard error of mean (SEM); $^{*}p < 0.05$, ns, no significant difference.