

Children with Neutrophil-Predominant Severe Asthma Have Proinflammatory Neutrophils With Enhanced Survival and Impaired Clearance



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What is already known about this topic? Eosinophils are the hallmark type 2 inflammatory cells targeted by emerging biologic therapies for severe asthma. However, neutrophils are also abundant in a subset of children with severe asthma, yet their associated functions are poorly understood.

What does this article add to our knowledge? Children with neutrophil-predominant severe asthma have proinflammatory neutrophils with altered function and enhanced survival as well as proinflammatory airway macrophages with impaired innate immune functions that may perpetuate airway inflammation and susceptibility to respiratory infection.

How does this study impact current management guidelines? Therapeutic strategies targeting neutrophils and neutrophil activation pathways may be beneficial in some children with severe asthma refractory to standard treatment approaches.

BACKGROUND: Airway neutrophils are abundant in some children with severe asthma, but their functions are poorly understood.

OBJECTIVE: To characterize that the inflammatory airway environment of children with neutrophil-predominant severe asthma promotes neutrophil survival and disrupts neutrophil-associated innate immune defenses.

METHODS: Sixty-seven children with severe asthma refractory to high-dose inhaled corticosteroid treatment undergoing bronchoscopy with bronchoalveolar lavage (BAL) for clinical indications were stratified into neutrophil “high” versus “low” groups on the basis of BAL differential counts. Neutrophil activation markers, functional assays, and phenotyping studies were performed, as well as airway macrophage functional assays. Results were compared with those from children with moderate asthma treated with inhaled corticosteroids.

RESULTS: Children with neutrophil-predominant severe asthma had increased markers of neutrophil activation/degranulation and a greater magnitude of airway proinflammatory

cytokine and chemokine release. Primary neutrophils exposed to BAL of these children exhibited greater phagocytic capability and greater neutrophil extracellular trap formation, but a more impaired respiratory burst. Despite greater abundance of airway TGF- β 1, the neutrophils were not more apoptotic. Instead, neutrophils had a highly proinflammatory phenotype associated with a number of surface markers that regulate neutrophil activation, recruitment/migration, and granule release. Airway macrophages from children with neutrophil-predominant severe asthma were also more proinflammatory with impaired phagocytosis and increased apoptosis.

CONCLUSIONS: Children with neutrophil-predominant severe asthma have proinflammatory neutrophils with enhanced survival. Airway macrophages are also proinflammatory and dysfunctional and may contribute to global innate immune impairment. Therapies that target neutrophils and related inflammation may be warranted in this subset of children. © 2018 American Academy of Allergy, Asthma & Immunology (J Allergy Clin Immunol Pract 2019;7:516-25)

Key words: Severe asthma; Asthma phenotype; Asthma endotype; Neutrophil activation; Neutrophil extracellular trap; Respiratory burst; Airway macrophage; Phagocytosis; Non-type 2 inflammation; Pediatric patients with asthma; Healthy adults

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INTRODUCTION

Patients with severe corticosteroid refractory asthma are a heterogeneous group necessitating a “personalized” versus a “one-size-fits-all” treatment approach.¹ Although eosinophils are the hallmark type 2 inflammatory cells and the target of a number of emerging biologic therapies for severe asthma, neutrophils are much less understood. In adults, abundant airway neutrophils have been identified in some patients with severe asthma,²⁻⁴ but

Abbreviations used

BAL- Bronchoalveolar lavage
CCL- chemokine (C-C motif) ligand
CXCL- chemokine (C-X-C motif) ligand
FVC- Forced vital capacity
ICS- Inhaled corticosteroid
NET- Neutrophil extracellular trap
PARP-1- poly [ADP-ribose] polymerase 1
PMA- phorbol 12-myristate 13-acetate
RFU- Relative fluorescent unit

their role is controversial,⁵ and others have argued that neutrophil elevations may simply result from intensive corticosteroid treatment, which can alter neutrophil survival.^{6,7} Studies in children are even more limited. In the few phenotypic⁸⁻¹⁰ and bronchoscopic^{11,12} pediatric studies that have been performed, most children with severe asthma had an eosinophil-predominant pattern of type 2 airway inflammation, raising questions about the relevance of neutrophils to pediatric disease.¹³

We have previously demonstrated that neutrophil elevations are present in the airways of a subpopulation of children with severe asthma and are associated with elevations in C-X-C motif chemokine ligand (CXCL) and C-C motif chemokine ligand (CCL) proteins responsible for neutrophil activation and chemotaxis, even in the absence of pathogenic bacteria or fungi.¹¹ Other recent studies outside our laboratory suggest that neutrophil elevations may be present in up to 30% of the entire population of children with severe asthma.^{14,15} Furthermore, others have noted increased expression of IL-17 receptor A, which promotes neutrophil maturation,¹⁶ and largely undetectable IL-4, IL-5, and IL-13 concentrations in the airways of children with severe asthma,¹² suggesting that the inflammation in these children is not isolated to eosinophils alone.

Despite the potential importance of neutrophils in subsets of children with severe asthma, studies of neutrophil biology are lacking in this population. Neutrophils play a fundamental role in the innate immune response through killing of invading microbes and are, by design, highly reactive cells that release proteolytic enzymes from their granules and secretory vesicles and kill pathogens using bursts of reactive oxygen species. However, unlike eosinophils or T cells, neutrophils are less sensitive to the effects of corticosteroids,^{17,18} and thus their presence in the airways in large numbers cannot be benign. We hypothesized that the inflammatory airway environment of children with neutrophil-predominant severe asthma refractory to high doses of inhaled corticosteroids (ICSs) promotes neutrophil survival and disrupts innate immune defenses. To this end, we characterized airway neutrophil activation markers and key functions of phagocytosis, neutrophil extracellular trap (NET) formation, respiratory burst, and apoptosis in primary neutrophils exposed to airway fluid from these children. Furthermore, because airway macrophages are the key instigator of host defense and can assume differing immune phenotypes on the basis of the airway cytokine milieu,¹⁹ a secondary objective was to characterize airway macrophage inflammatory patterns and functions in these same patients.

METHODS

Participants

Participants included children and adolescents aged 6 to 21 years with physician-diagnosed asthma and a history of 12% or more relative change in FEV₁ after bronchodilator administration undergoing bronchoscopy with bronchoalveolar lavage (BAL) for clinical indications. Children with severe asthma met published criteria for diagnosis²⁰ and were treated with high-dose inhaled fluticasone equivalent (>800 µg daily if ≥12 years or >400 µg daily if <12 years) plus an additional asthma controller medication (ie, long-acting β agonist or montelukast). These participants underwent bronchoscopy for wheezing unresponsive to recommended treatment. For the purpose of comparison, children and adolescents with moderate persistent asthma treated with ICSs for at least 6 consecutive months and undergoing bronchoscopy with BAL for clinical indications (suspected foreign body, n = 3; suspected aspiration, n = 5; abnormal chest radiograph, n = 5) were also recruited. Exclusion criteria for all participants with asthma included birth before 35 weeks of gestation, an asthma exacerbation treated with systemic corticosteroid within the previous 4 weeks, or a positive BAL bacterial, viral, or fungal culture. Informed consent and assent to share the BAL fluid between the clinical and research laboratories were obtained.

Clinical procedures

Spirometry was performed with a portable spirometer (KoKo® Legend, Ferraris, Louisville, Colo). The best of 3 forced vital capacity maneuvers was interpreted according to population reference standards.²¹ Exhaled nitric oxide was quantified using a commercial device (NIOX, Aerocrine, Solna, Sweden).²² Aeroallergen sensitization was defined as a positive skin prick test result or a specific aeroallergen IgE test (>0.35 kU/L). Venipuncture was performed at the time of bronchoscopy for total serum IgE, which was quantified by a local laboratory (Children's Healthcare of Atlanta, Atlanta, Ga). Bronchoscopy was performed as described previously using 0.9% sodium chloride warmed to room temperature.¹¹ BAL samples were subjected to standard culture and sensitivity testing for bacteria, viruses, and fungus (Children's Healthcare of Atlanta).

Laboratory procedures

Laboratory procedures are detailed in this article's [Online Repository](http://www.jaci-inpractice.org) at www.jaci-inpractice.org. BAL was centrifuged at 1200 rpm for 7 minutes at 4°C within 1 hour of collection and the cell pellet was resuspended in 10 mL of 1:1 Dulbecco modified Eagle medium/Ham's F-12 solution containing 2% FBS, L-glutamine, 15 mmol/L 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid, penicillin (10,000 U), streptomycin (10,000 mg/mL), amphotericin (25 mg/mL), and gentamicin (4 µg/mL). Total cell counts were performed with a hemocytometer, and cellular differentials were determined from 300 consecutive cells after Kwik-Diff staining (Thermo Shandon Ltd, Runcorn, UK).

Enzyme-linked immunosorbent assays

IL-1β, IL-6, IL-7, IL-8 (CXCL8), IL-10, IL-12p70, TNF-α, CXCL1 (growth-related oncogene), CXCL10 (human interferon-inducible protein 10), CCL2 (monocyte chemoattractant protein 1), CCL3 (macrophage inflammatory protein 1α), CCL4 (macrophage inflammatory protein 1β), and CCL22 (macrophage-derived chemokine) were quantified with bead-based multiplex assays (HSCYTO-60SK and MPXHCYTO-60K, Millipore, Billerica,

Mass). IL-33 (R&D Systems, Minneapolis, Minn), TGF- β 1 (Promega, Madison, Wis), neutrophil elastase (Abcam, Cambridge, Mass), myeloperoxidase (R&D Systems), and lactoferrin (Abcam) ELISAs were performed according to manufacturer instructions. Concentrations were determined with a BioTek Synergy HT 96-well plate reader (BioTek, Winooski, Vt) using manufacturer-recommended wavelengths and standard curve fit.

Cell culture experiments

HL-60 cells (Sigma-Aldrich, St Louis, Mo) were cultured and differentiated as described in this article's [Online Repository](http://www.jaci-inpractice.org) at www.jaci-inpractice.org. Primary human neutrophils were isolated from healthy donor blood using the EasySep Direct Human Neutrophil Isolation Kit (Stemcell Technologies, Vancouver, BC, Canada) as per the manufacturer's instructions. Neutrophil purity was confirmed with Kwik-Diff (Thermo Shandon Ltd) staining. Cells were cultured at 37°C, 5% CO₂ in Roswell Park Memorial Institute 1640 medium with L-glutamine supplemented with 10% heat-inactivated FCS, 1% penicillin/streptomycin, 50 mg/mL gentamicin, and 5 ng/mL recombinant human TGF- β 1 (BioLegend, San Diego, Calif) for 16 hours. Recombinant human GM-CSF (Sigma-Aldrich) was added at 100 ng/mL as indicated for 30 minutes before harvesting.

Neutrophil experiments

Neutrophil phagocytosis was assessed after overnight culture in equal parts media and BAL supernatant, followed by addition of phorbol 12-myristate 13-acetate (PMA) and pHrodo *Staphylococcus aureus* BioParticles (Molecular Probes, Eugene, Ore). Kinetic measurements of NET release were taken as per established protocol²³ and apoptosis was assessed with phycoerythrin-conjugated cleaved caspase-3 antibody (Cell Signaling Technology, Danvers, Mass) quantified by flow cytometry (FACSCalibur, BD Biosciences, Franklin Lakes, NJ). Respiratory burst was assessed with dihydrorhodamine 123 after PMA stimulation using flow cytometry (FACSCalibur). Neutrophil surface staining was performed with CD66b-PerCP-Cy5.5, CD62L-BV421, CD11b-AF488, CD11c-PECy7, and CD16-APCCy7 (BioLegend); cells were permeabilized and then stained intracellularly with CCL2-AF647 (BioLegend) and analyzed with a CytoFLEX flow cytometer (Beckman-Coulter, Brea, Calif). Expression data were analyzed using FlowJo version 10 software (FlowJo LLC, Ashland, Ore).

Airway macrophage experiments

Airway macrophages (100,000 cells) were isolated from the BAL cell suspension and incubated with and without 1 mg/mL lipopolysaccharide as previously described.²⁴ Protein expression of TGF- β 1 and poly [ADP-ribose] polymerase 1 (PARP-1) cleavage was analyzed by fluorescent microscopy in cytopins using anti-TGF- β 1 and PARP-1 antibodies at a 1:100 dilution (sc-31609 and sc-8007, Santa Cruz Biotechnology, Inc, Santa Cruz, Calif). Phagocytosis of fluorescein isothiocyanate-conjugated, inactivated *S aureus* (Invitrogen, Carlsbad, Calif) was assessed with an Olympus confocal microscope (model BX51, Olympus America, Inc, Melville, NY) with quantitative digital fluorescence imaging software (Olympus FluoView 300, version 4.3, Olympus America, Inc). The phagocytic index of *S aureus* (relative fluorescent units [RFUs] $\times$ percentage of positive cells) was determined for each phagocytic cell at 50% of the cell depth as described previously.²⁴

Statistical analyses

Statistical analyses were performed with IBM SPSS statistics software, version 24 (SPSS, Chicago, Ill). Skewed variables were

logarithmically transformed before analysis. Chi-square tests and *t* tests were used to compare baseline clinical features between groups. Experimental differences were assessed by ANOVA with Tukey post hoc tests. A 2-tailed probability of .05 or less was the threshold of significance for all comparisons.

RESULTS

Seventy-one children with severe asthma were approached for the study. Four children had positive bacterial BAL cultures (*Haemophilus influenzae* [n = 2], *Streptococcus pneumoniae* [n = 2]) and were excluded from further analysis, resulting in a final sample of 67 children. BAL neutrophil percentages varied widely among children with severe asthma, with a median of 5.0% (minimum, 0.66%; maximum, 19.80%). In contrast, BAL neutrophil percentages in children with moderate asthma treated with ICSs were all less than 5%. Children with severe asthma were therefore stratified into neutrophil "high" versus "low" groups using arbitrary cutoff points of less than 5.0% and 5.0% or more as shown in [Figure 1, A](#).

Participant characteristics are presented in [Table I](#). Previous health care utilization, medication requirements, and exhaled nitric concentrations were significantly greater and lung function values were significantly lower in children with severe versus moderate asthma. There were no differences in clinical characteristics between the 2 severe groups, aside from lower monocyte/macrophage percentages and higher neutrophil percentages (by definition).

Neutrophil activation

Neutrophil activation, reflected by release of myeloperoxidase, elastase, and lactoferrin protein in the BAL fluid, was significantly greater in children with neutrophil high versus neutrophil low severe asthma ([Figure 1, B-D](#)) and was accompanied by increased concentrations of IL-1 β , IL-6, IL-33, CXCL1, CXCL8, CXCL10, CCL2, CCL3, CCL4, and CCL22 protein ([Table II](#)). IL-7, IL-12, and TNF- α were also elevated in the BAL fluid of children with neutrophil high severe asthma but were similar to concentrations in the neutrophil low severe asthma group ([Table II](#)). Normalization of the neutrophil activation markers and cytokine and chemokine concentrations to BAL protein levels did not significantly alter these results (see [Table E1](#) in this article's [Online Repository](http://www.jaci-inpractice.org) at www.jaci-inpractice.org).

Neutrophil functional assays

Neutrophil functions were assessed in primary neutrophils exposed to BAL fluid from each patient group. Neutrophil elastase concentrations in the media after culture were similar to those noted in the BAL fluid from each patient group (see [Figure E1](#) in this article's [Online Repository](http://www.jaci-inpractice.org) at www.jaci-inpractice.org). Neutrophil phagocytic function was enhanced in neutrophils exposed to BAL fluid from children with neutrophil high severe asthma compared with the other groups ([Figure 2, A](#)). NET formation (ie, extracellular DNA release) was also significantly higher in neutrophils exposed to BAL fluid from children with neutrophil high severe asthma at baseline ([Figure 2, B](#)), 60 minutes, and 80 minutes (see [Figure E2](#) in this article's [Online Repository](http://www.jaci-inpractice.org) at www.jaci-inpractice.org). In contrast, the neutrophil oxidative burst reflected by dihydrorhodamine 123 expression in response to PMA stimulation was significantly less than that observed in neutrophils exposed to BAL fluid from children with moderate asthma treated with ICSs

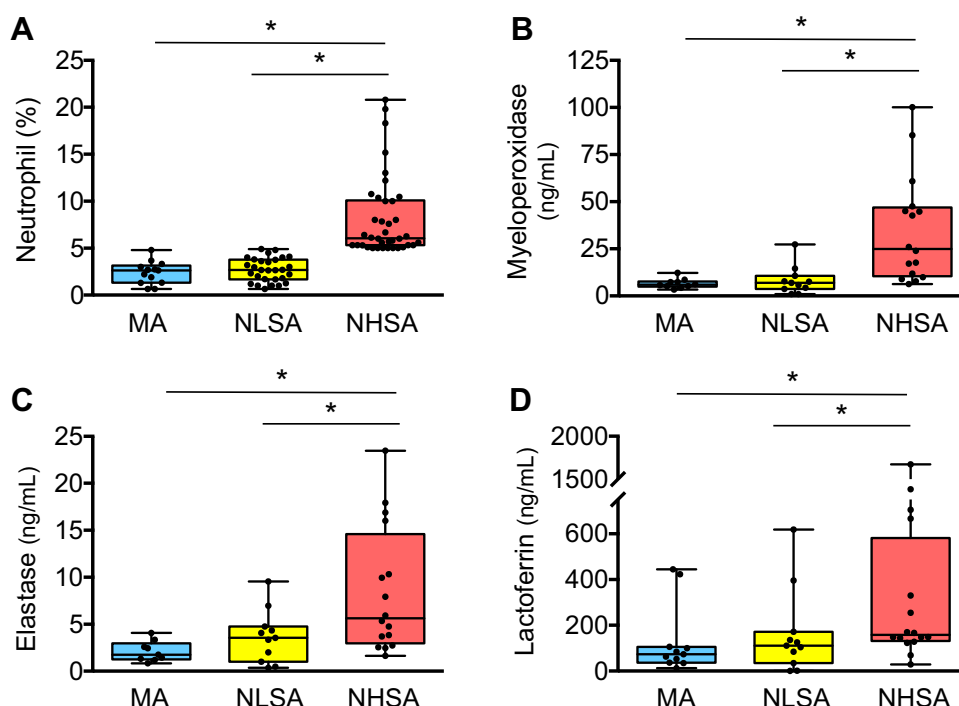


FIGURE 1. (A) Airway neutrophil percentages and concentrations of (B) myeloperoxidase, (C) elastase, and (D) lactoferrin in BAL fluid from children with moderate asthma treated with ICSs (MA), children with neutrophil “low” severe asthma (NLSA), and children with neutrophil “high” severe asthma (NHA). Whiskers represent minimum and maximum values; individual participants are shown as dots. * $P < .05$.

(Figure 2, C). Neutrophil apoptosis, evidenced by cleavage of caspase-3, was not significantly different (Figure 2, D).

Neutrophil phenotyping studies

In a cell culture model system, TGF- β 1 inhibited apoptosis in HL-60–derived neutrophils in a time-dependent manner (see Figure E3, A and B, in this article’s Online Repository at www.jaci-inpractice.org). Prolonged (ie, overnight) TGF- β 1 exposure also enhanced phagocytosis and increased NET formation in HL-60–derived neutrophils treated with dexamethasone (see Figure E3, C and D). In primary neutrophils primed with GM-CSF, prolonged TGF- β 1 exposure also reduced expression of the L-selectin, also known as CD62L (see Figure E4, A, in this article’s Online Repository at www.jaci-inpractice.org), reduced intracellular CCL2 (see Figure E4, B), increased expression of the integrins CD11b and CD11c (see Figure E4, C and D), and increased expression of the phagocytosis receptor CD16 (see Figure E4, E). No differences in CD66b expression were noted (see Figure E4, F).

Children with neutrophil high severe asthma had higher concentrations of free TGF- β 1 in the BAL fluid than did children with neutrophil low severe asthma (Figure 3, A). But despite increased abundance of BAL TGF- β 1, primary neutrophils exposed to BAL fluid from these children exhibited a more proinflammatory phenotype characterized by increased CD62L, increased CCL2, increased CD11b, decreased CD11c, increased CD16, and increased CD66b expression (Figure 3, B–G).

Children with neutrophil high severe asthma also had higher TGF- β 1 protein expression in airway macrophages (Figure 4, A) as well as increased macrophage concentrations of IL-10, CXCL1,

CXCL8, CXCL10, and CCL2 (Figure 4, B–F). Airway macrophage phagocytic function was also significantly impaired at baseline in children with neutrophil high severe asthma and did not improve with lipopolysaccharide stimulation (Figure 4, G). Moreover, airway macrophage apoptosis, assessed by cleavage of PARP-1, was also increased in children with neutrophil high severe asthma and increased further in response to lipopolysaccharide (Figure 4, F).

DISCUSSION

The role of airway neutrophils in children with severe asthma is not understood, and the existence of a unique “endotype” of neutrophilic severe asthma in school-aged children has been debated.¹³ We hypothesized that abundant airway neutrophils in children with severe asthma are not benign and that the airway environment of children with neutrophil-predominant severe asthma promotes neutrophil survival and disrupts innate immune defenses. Our findings suggest that airway neutrophils in children with neutrophil-predominant severe asthma are highly dysfunctional and characterized by greater release of proinflammatory mediators and granules, greater phagocytic capability and NET formation, but more impaired respiratory burst. Despite a highly immunosuppressive airway TGF- β 1 environment, neutrophils exposed to the airway environment of these children were not more apoptotic and displayed a number of surface markers associated with activation, including increased expression of CD62L, an adhesion molecule that promotes endothelial attachment for the purpose of migration, and increased expression of CCL2, which mediates neutrophil recruitment. Although airway

TABLE I. Characteristics of participants

Feature	Moderate asthma treated with ICSs (n = 13)	Neutrophil "low" severe asthma (n = 29)	Neutrophil "high" severe asthma (n = 38)
Age (y)	18.0 ± 2.2	14.7 ± 2.4	13.0 ± 1.8
Sex: male	6 (46.2)	11 (37.9)*	21 (55.3)*
Race			
White	8 (61.5)	17 (58.6)	24 (63.2)
Black	5 (38.5)	12 (41.4)	14 (36.8)
Hispanic ethnicity	1 (7.7)	2 (6.9)	2 (7.9)
Obese (body mass index ≥95 percentile)	6 (46.2)	9 (31.0)	14 (36.8)
Aeroallergen sensitization	4 (30.8)	18 (62.1)	22 (57.9)
Emergency department visit for asthma (previous year)	1 (7.7)	14 (48.3)*	21 (55.3)*
Hospitalization for asthma (previous year)	0	13 (44.8)*	17 (44.7)*
Intubation for asthma (ever in lifetime)	0	2 (6.9)	5 (13.2)
Daily medication use			
ICS monotherapy	7 (53.8)	0*	0*
ICS + LABA or LTRA	6 (46.2)	29 (100)*	38 (100)*
Daily ICS dose (fluticasone equivalent)	300.3 ± 49.6	749.8 ± 66.9*	705.2 ± 56.9*
Serum IgE (kU/L)†	109.5 ± 46.5	294.3 ± 105.4	419.9 ± 148.2
Exhaled nitric oxide (ppb)†	28.2 ± 12.7	42.0 ± 9.2	51.9 ± 9.4*
Lung function values			
FVC (% predicted)	95.7 ± 6.2	89.8 ± 3.7	90.9 ± 3.1
FEV ₁ (% predicted)	92.0 ± 5.4	80.3 ± 4.8	83.6 ± 3.5
FEV ₁ /FVC	0.84 ± 0.02	0.81 ± 0.02	0.81 ± 0.02
FEF ₂₅₋₇₅ (% predicted)	86.3 ± 8.7	74.3 ± 5.9	77.5 ± 6.8
FEV ₁ bronchodilator reversibility (relative % change from baseline)§	5.0 ± 1.2	15.0 ± 3.2*	16.3 ± 3.0*
Airway lavage characteristics			
Protein (μg/mL)	144.4 ± 27.2	182.88 ± 18.2	192.9 ± 17.9
Cell count (×10 ⁶ cells)	3.99 ± 0.69	3.67 ± 0.50	3.93 ± 0.48
Macrophages/monocytes (%)	91.2 ± 0.8	91.0 ± 0.8	85.9 ± 1.0*‡
Neutrophils (%)	2.4 ± 0.3	2.7 ± 0.2	8.0 ± 0.7*‡
Eosinophils (%)	1.4 ± 0.5	1.4 ± 0.3	1.5 ± 0.5
Lymphocytes (%)	4.3 ± 0.5	4.5 ± 0.5	4.5 ± 0.5

FEF₂₅₋₇₅, Forced expiratory flow at 25%-75% of vital capacity; LABA, long-acting β agonist; LTRA, leukotriene receptor antagonist.

Data represent the mean ± SEM or the number of participants (%).

**P* < .05 vs moderate asthma treated with ICSs.

†Log-transformed for significance testing.

‡*P* < .05 vs neutrophil low severe asthma.

§Defined as (post value in liters – baseline value in liters)/baseline value in liters.

macrophages provide some defense against activated neutrophils, airway macrophages from children with neutrophil-predominant severe asthma were also highly proinflammatory with disrupted innate immune defenses (ie, impaired phagocytosis and increased apoptosis) and may perpetuate a sustained environment of neutrophil-mediated inflammation rendering children more susceptible to lower respiratory tract infections.

Severe asthma is clearly heterogeneous, with a number of different endotypes that have been classically defined by neutrophil and eosinophil percentages in the airways.²⁵ In adults, airway neutrophil numbers tend to increase in difficult-to-treat populations^{2,26-28} in association with increased IL-17 levels²⁹ and other inflammatory cytokines and chemokines.³⁰⁻³² Airway neutrophil numbers may also increase further during acute exacerbations.³³ However, little is known about neutrophil functions in patients with severe asthma. A few studies suggest that systemic neutrophils may be more activated in patients with noneosinophilic asthma, with greater CXCL8 release³⁴ and greater NET formation,³⁵ which may be augmented after migration to the

airways. However, other studies of patients with asthma suggest that the process of neutrophil activation may occur in the airways themselves and lead to an activated and degranulated phenotype³⁶ with increased NET formation as assessed by antimicrobial proteins.³⁷ Furthermore, although circulating neutrophils are conventionally thought to have a 6- to 8-hour limited life span,³⁸ more recent work suggests that neutrophils can survive up to several days and return to the systemic circulation by reverse migration.^{39,40} Indeed, Uddin et al⁴¹ similarly noted delayed airway neutrophil apoptosis in patients with severe asthma, with the greatest neutrophil survival in patients with the greatest airway neutrophil counts.

Recent work suggests that neutrophils, like macrophages, are not terminally differentiated and have functional plasticity with cytokine-driven polarization.⁴² In tumors, neutrophils can acquire an antitumorigenic N1 phenotype characterized by increased cytotoxic activity with more superoxide and hydrogen peroxide release and promotion of CD8⁺ T-cell recruitment and activation through increased proinflammatory cytokine and chemokine

TABLE II. Concentrations of cytokines and chemokines in the BAL fluid

Analyte (pg/mL)	Minimal detectable concentration	Moderate asthma treated with ICSs (n = 13)	Neutrophil "low" severe asthma (n = 29)	Neutrophil "high" severe asthma (n = 38)
IL-1 β	0.06	0.14 $\pm$ 0.02	0.15 $\pm$ 0.02	0.26 $\pm$ 0.05*†
IL-6	0.10	7.17 $\pm$ 2.57	7.22 $\pm$ 1.23	26.44 $\pm$ 6.12*†
IL-7	0.12	2.18 $\pm$ 0.65	2.46 $\pm$ 0.58	2.47 $\pm$ 0.38
IL-12	0.11	0.29 $\pm$ 0.12	0.32 $\pm$ 0.08	0.33 $\pm$ 0.04
IL-33	0.07	1.96 $\pm$ 0.73	8.52 $\pm$ 4.05	21.01 $\pm$ 4.39*†
TNF- α	0.05	0.33 $\pm$ 0.07	0.91 $\pm$ 0.41	1.53 $\pm$ 0.69
CXCL1	10.10	587.85 $\pm$ 229.11	2133.19 $\pm$ 364.66*	2790.68 $\pm$ 263.42*†
CXCL8	0.11	42.19 $\pm$ 18.64	40.33 $\pm$ 6.80	314.51 $\pm$ 125.61*†
CXCL10	1.20	173.67 $\pm$ 38.20	444.55 $\pm$ 63.87*	1067.77 $\pm$ 224.52*†
CCL2	0.90	26.06 $\pm$ 10.49	35.61 $\pm$ 4.95	55.59 $\pm$ 8.79*†
CCL3	3.50	4.44 $\pm$ 0.63	6.20 $\pm$ 0.69	15.30 $\pm$ 4.66*†
CCL4	4.50	11.02 $\pm$ 3.87	22.93 $\pm$ 4.10*	51.81 $\pm$ 14.16*†
CCL22	3.70	63.03 $\pm$ 19.69	114.59 $\pm$ 20.25*	165.45 $\pm$ 35.33*

Note. Data represent the mean $\pm$ SEM.

* P < .05 vs moderate asthma treated with ICSs.

† P < .05 vs neutrophil low severe asthma.

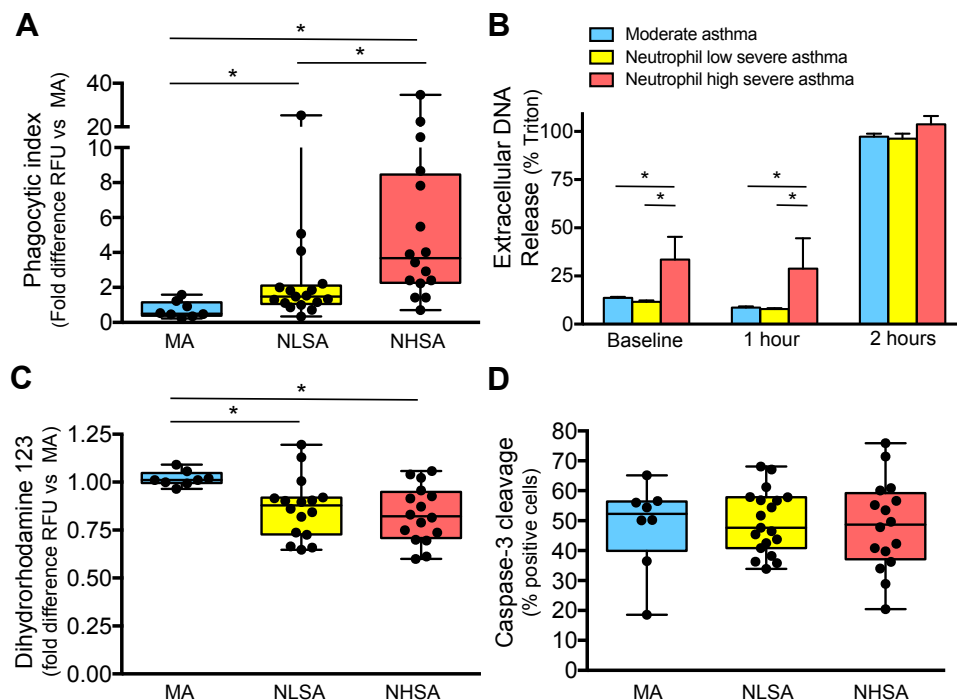


FIGURE 2. (A) Neutrophil phagocytic index, (B) extracellular DNA release (mean $\pm$ SEM), (C) oxidative burst, and (D) apoptosis in children with moderate asthma treated with ICSs (MA), children with neutrophil "low" severe asthma (NLSA), and children with neutrophil "high" severe asthma (NHSA). Whiskers represent minimum and maximum values; dots reflect individual participants. SEM, Standard error of the mean. * P < .05.

release. These same neutrophils can be modulated toward a pro-tumorigenic N2 phenotype by TGF- β 1, which inhibits neutrophil degranulation and reactive mediator release and promotes neutrophil accumulation in the tumor.^{43,44} Although global classification of patient neutrophil populations as either N1 or N2 is an oversimplification, our results suggest that the neutrophils present in airways of children with neutrophil-predominant severe

asthma are largely proinflammatory despite a highly immunosuppressive TGF- β 1-rich environment, evidenced by a number of N1-like features that may provide an opportunity for future therapeutic intervention. Indeed, although a hyperinflammatory response may result in damage to the host epithelium and endothelium (and increased mucosal permeability and leakage), modulation of the N1 neutrophil phenotype could reduce some

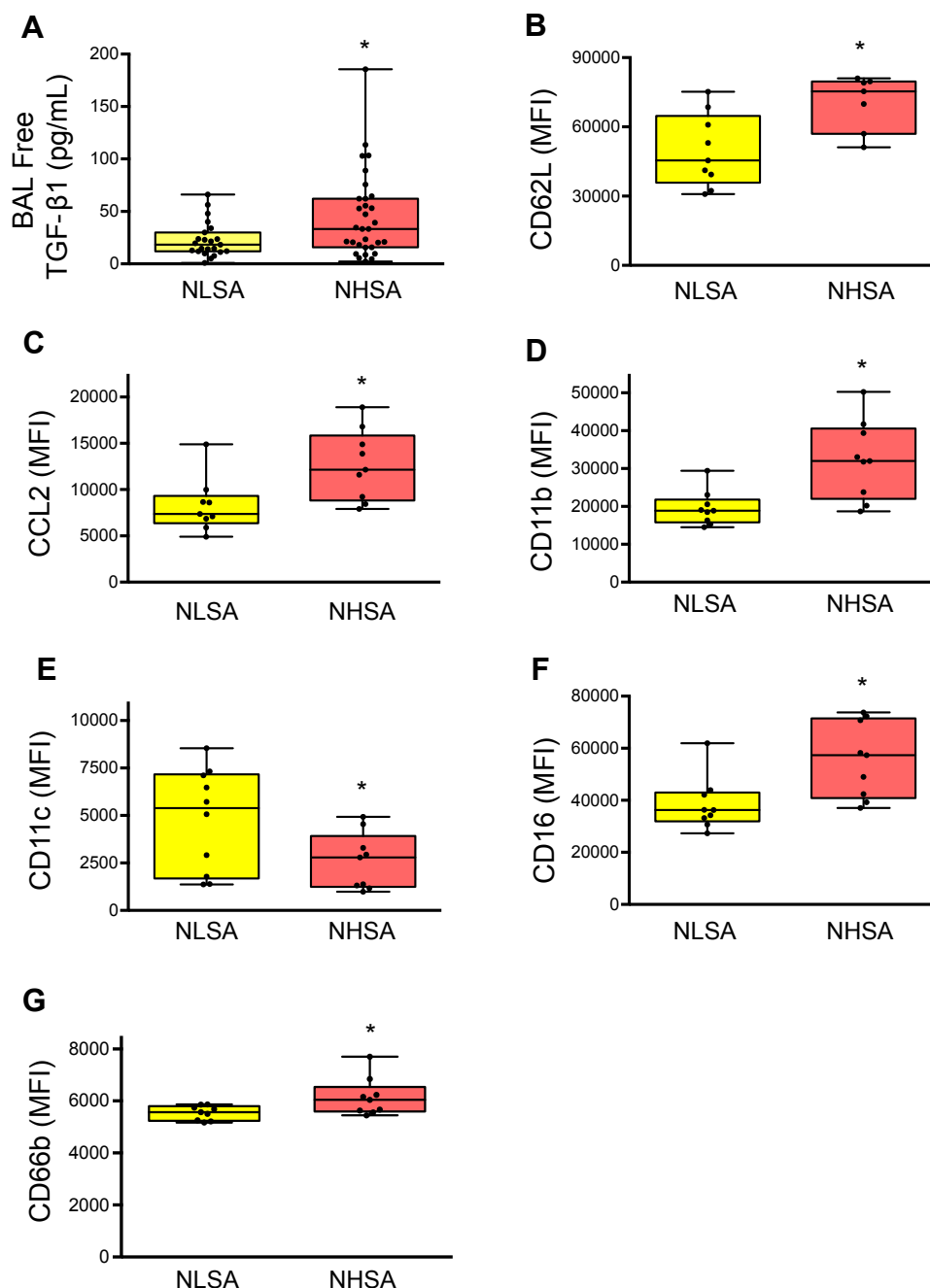


FIGURE 3. (A) TGF- β 1 concentrations in the BAL fluid and mean fluorescent intensity (MFI) of (B) CD62L, (C) CCL2, (D) CD11b, (E) CD11c, (F) CD16, and (G) CD66 in primary neutrophils exposed to BAL fluid from children with neutrophil "low" severe asthma (NLSA) and neutrophil "high" severe asthma (NHSA). Whiskers represent minimum and maximum values. * $P < .05$.

collateral damage from reactive oxygen species and granulocyte/protease release without compromising antimicrobial functions.⁴⁵ In contrast, a predominance of N2 neutrophils may compromise antimicrobial functions and result in bacterial co-infections.⁴⁵

To our knowledge, this is the first study to comprehensively examine neutrophil predominance, neutrophil-associated markers of inflammation, neutrophil functions, and associated airway macrophage functions in children with severe asthma. However, there are limitations. First, severe asthma by definition necessitates

treatment with high doses of ICSs²⁰; therefore, it is unclear how intensive corticosteroid treatment may have had an impact on our results. Nonetheless, we still observed significant differences in neutrophil and macrophage functions between children with neutrophil high and low severe asthma despite equivalent ICS treatment. Furthermore, unlike eosinophils or T cells, neutrophils are relatively insensitive to corticosteroids.^{17,18} Second, we were unable to perform neutrophil functional experiments on freshly isolated airway neutrophils given the relatively low neutrophil

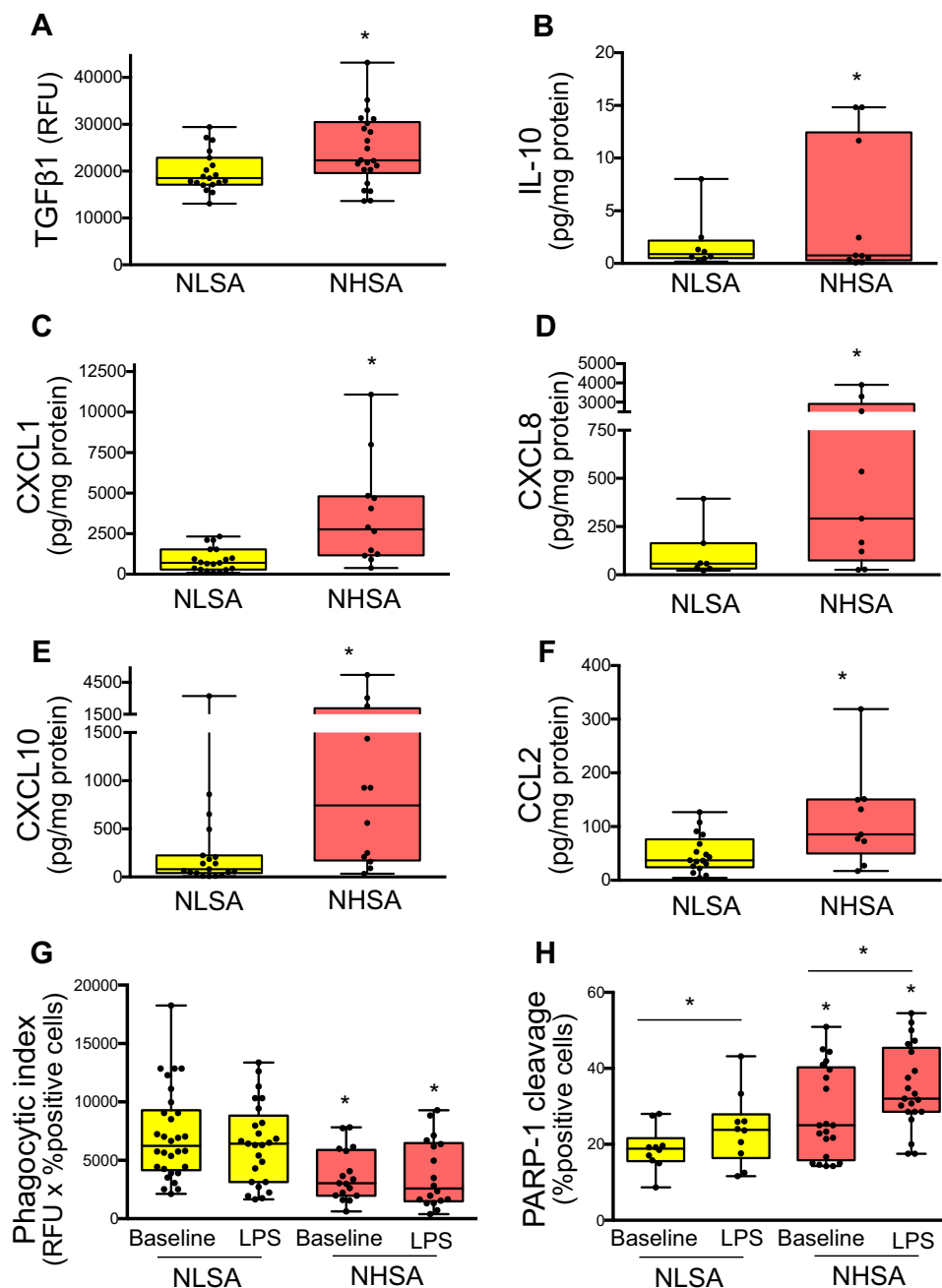


FIGURE 4. (A) Airway macrophage TGF- β 1 protein expression. Protein-normalized concentrations of (B) IL-10, (C) CXCL1, (D) CXCL8, (E) CXCL10, and (F) CCL2. (G) Phagocytosis and (H) apoptosis in children with neutrophil "low" severe asthma (NLSA) and neutrophil "high" severe asthma (NHSA). Whiskers represent minimum and maximum values. * $P < .05$. LPS, Lipopolysaccharide.

numbers (in comparison with macrophages) and the need to share samples with the clinical laboratory. We therefore do not expect that all our primary neutrophil surface markers will replicate perfectly in an airway environment, because these could change during the process of recruitment and migration.⁴⁶ Indeed, a previous study of induced sputum from patients with asthma noted that airway surface marker expression was not specific for asthmatic airway inflammation or asthma disease activity, and was instead related to tissue migration.⁴⁷ Likewise, CD62L, which was

higher in children with neutrophil high severe asthma in the present study, is typically shed during migration and is lower in airway versus systemic compartments.³⁶ However, other surface markers such as CD11b (which is associated with neutrophil granules and secretory vesicles) were also more highly expressed in children with neutrophil high severe asthma in the present study and would likely replicate in airway analyses, given our other observations of greater neutrophil-associated granule release (ie, lactoferrin, myeloperoxidase, and elastase) in the BAL fluid of these

same children. Finally, our sample sizes were relatively small and limited to children undergoing bronchoscopy with BAL for clinical purposes; this prohibited inclusion of healthy children.

CONCLUSIONS

The results of the study demonstrate that neutrophil abundance in children with severe asthma is not benign. Instead, neutrophils are highly activated and display a number of functional alterations including greater inflammatory mediator and granule release and greater NET formation without an accompanying increase in apoptosis. Children with neutrophil high severe asthma therefore exhibit a paradox of immune activation with dysfunctional respiratory burst capacity and prolonged survival despite an immunosuppressive environment. Macrophage defenses were also paradoxically impaired in these same children. Together, these findings may ultimately promote an environment of sustained airway inflammation and structural damage in children with neutrophil high severe asthma refractory to treatment with high doses of ICSs. Novel therapeutic strategies targeting neutrophil activation such as prostaglandin E3/E4 inhibitors, protein kinase inhibitors, and other emerging treatment strategies may be beneficial in these patients.

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ONLINE REPOSITORY

METHODS

Bronchoscopy and BAL processing

Pediatric flexible bronchoscopy was performed under general anesthesia using a laryngeal mask airway and flexible bronchoscope (Olympus BF-3C160 or BF-P160, Olympus America, Inc, Melville, NY). BAL fluid was collected from the right middle lobe with three 1-mL/kg (50 mL maximum) saline lavages (0.9% sodium chloride warmed to room temperature) using hand pressure on a syringe. BAL fluid was collected and pooled under continuous low-pressure suction. The BAL fluid was pooled and divided between the research and clinical laboratories. The samples submitted to the clinical laboratories were subjected to standard culture and sensitivity testing for bacteria, viruses, and fungus.

BAL was centrifuged at 1200 rpm for 7 minutes at 4°C within 1 hour of collection. The supernatant was divided into 250-μL aliquots and frozen at -80°C before analysis. The cell pellet was resuspended in 10 mL of 1:1 Dulbecco modified Eagle medium/Ham's F-12 solution containing 2% FBS, L-glutamine, 15 mmol/L 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid, penicillin (10,000 U), streptomycin (10,000 mg/mL), amphotericin (25 mg/mL), and gentamicin (4 μg/mL). Total cell counts were performed with a hemocytometer, and cellular differentials were determined from 300 consecutive cells after Kwik-Diff staining (Thermo Shandon Ltd, Runcorn, UK).

Enzyme-linked immunosorbent assays

IL-1β, IL-6, IL-7, IL-8 (CXCL8), IL-8, IL-12p70, TNF-α, CXCL1 (growth-related oncogene), CXCL10 (human interferon-inducible protein 10), CCL2 (monocyte chemoattractant protein 1), CCL3 (macrophage inflammatory protein 1α), CCL4 (macrophage inflammatory protein 1β), and CCL22 (macrophage-derived chemokine) were quantified using a bead-based multiplex cytokine assay (HSCYTO-60SK and MPXHCYTO-60K, Millipore, Billerica, Mass). Analytes were selected on the basis of their relevance to neutrophil biology. For the assays, duplicate samples and serial dilutions of the cytokine standards were added to 96-well plates and incubated overnight. Data were analyzed using the Bio-Plex system (Bio-Rad Laboratories, Hercules, Calif) with gates of 4,335 and 10,000. Sample concentrations were determined using a 5-point logistic curve fitting algorithm (Bio-Plex Manager 3.0 software, Bio-Rad Laboratories).

Human myeloperoxidase (R&D Systems, Minneapolis, Minn), lactoferrin (Abcam, Cambridge, Mass), neutrophil elastase (Abcam), IL-33 (R&D Systems), and TGF-β1 (Promega, Madison, Wis) ELISAs were performed as indicated following standard manufacturer instructions. Antibody concentrations were determined with a BioTek (Winooski, Vt) Synergy HT 96-well plate reader using manufacturer-recommended wavelengths and standard curve fit.

Cell culture

HL-60 cells (Sigma-Aldrich, St Louis, Mo) were cultured at 37°C, 5% CO₂ in RPMI-1640 media with L-glutamine (Gibco, Life Technologies, Carlsbad, Calif) supplemented with 20% heat-inactivated FCS (Atlanta Biologicals, Flowery Branch, Ga), 1% penicillin/streptomycin (Cellgro, Corning Life Sciences, Corning, NY), and 50 mg/mL gentamicin (Cellgro, Corning Life

Sciences). Cells were differentiated to demonstrate neutrophil-like properties by the addition of 1 μM retinoic acid (Sigma-Aldrich) and 1.25% dimethyl sulfoxide (Sigma-Aldrich) into the base media for 3 to 5 days.^{E1} Neutrophil differentiation of cells was confirmed by both Kwik-Diff (Thermo Shandon Ltd, Cheshire, Wash) staining of slide-mounted samples and increased CD11b expression using CD11b-AF488 antibody (BioLegend, San Diego, Calif) as determined by flow cytometry (FACSCalibur, BD Biosciences, Franklin Lakes, NJ). Unstained and phycoerythrin-conjugated isotype (IgG) (BioLegend) stained cells were used as controls. Data were analyzed using FlowJo version 10 software (FlowJo LLC, Ashland, Ore).

Isolation and culture of primary human neutrophils

Primary human neutrophils were isolated from donor blood collected in K2-EDTA tubes with the EasySep Direct Human Neutrophil Isolation Kit (Stemcell Technologies, Vancouver, BC, Canada) as per manufacturer's instructions. Briefly, plasma was separated from the blood by centrifugation and cells were washed with Ca²⁺- and Mg²⁺-free PBS containing 1 mM EDTA. A magnetic bead and antibody cocktail were then added to the cells and unwanted cells depleted from the sample by repeated magnetic separation. Neutrophil purity was confirmed using Kwik-Diff (Thermo Shandon Ltd) staining. Cells were cultured at 37°C, 5% CO₂ in RPMI-1640 medium with L-glutamine supplemented with 10% heat-inactivated FCS, 1% penicillin/streptomycin, 50 mg/mL gentamicin, and 5 ng/mL recombinant human TGF-β1 (BioLegend), as noted for 16 hours. Recombinant human GM-CSF (Sigma-Aldrich) was added at 100 ng/mL as indicated for 30 minutes before harvesting.

Neutrophil NETosis assay

Kinetic measurements of NET release were taken as per established protocol.^{E2} Day 4 neutrophil-differentiated HL-60 cells and primary neutrophils were suspended at a concentration of 2 × 10⁶ cells/mL in HBSS (Sigma-Aldrich) supplemented with 5 mM glucose (Sigma-Aldrich). A 10-μM solution of Sytox Orange (Thermo Fisher, Waltham, Mass) was added to the cells and 50 μL of cell suspension per well was aliquoted into a clear, flat-bottomed, black 96-well plate (Corning Life Sciences) and equilibrated at 37°C for 10 minutes. Noted treatment (BAL plus 200 nM PMA) of 50 μL was added in triplicate to the cells including a 0.4% Triton-X (Sigma-Aldrich) treatment (0.2% final concentration) as a positive control. Kinetic measurements were taken every 2 minutes for 4 hours on a BioTek Synergy HT 96-well plate reader at an excitation of 530 nm and an emission of 590 nm. Data were expressed as a percentage of maximum release (ie, percentage of positive control).

Neutrophil-cleaved caspase-3 assay

Day 3 neutrophil-differentiated HL-60 cells and primary neutrophils were cultured overnight at a density of 2 × 10⁶ cells/mL in equal parts media and BAL or 0.9% saline, and 200 nM PMA (Sigma-Aldrich) was added to the cells 30 minutes before harvesting, as noted. Cells were harvested by centrifugation and washed in PBS containing 2.5 mM EDTA, and media saved at -80°C until used in macrophage experiments or ELISAs. Cells were fixed in 4% formaldehyde for 10 minutes at 37°C, chilled for 1 minute on ice, and washed again in PBS-EDTA buffer and permeabilized with 90% ice-cold methanol. Cells were washed and blocked in PBS containing 5 mg/mL BSA (Fisher Scientific, Hampton, NH) before being stained with

phycoerythrin-conjugated cleaved caspase-3 antibody (Cell Signaling Technology, Danvers, Mass) at manufacturer-recommended dilutions. Expression levels were determined on a Becton-Dickinson FACSCalibur (BD Biosciences). Unstained and phycoerythrin-conjugated isotype (IgG) (Cell Signaling Technology) stained cells were used as controls. Data were analyzed using FlowJo version 10 software (FlowJo LLC).

Neutrophil phagocytosis assay

Day 3 neutrophil-differentiated HL-60 cells and primary neutrophils were cultured overnight at a density of 2×10^6 cells/mL in equal parts media and BAL or 0.9% saline, and 200 nM PMA (Sigma-Aldrich) was added to the cells for 30 minutes before the addition of pHrodo *S aureus* BioParticles (Molecular Probes, Eugene, Ore) at a ratio of 2.5 particles/cell for 2 hours at 37°C. Cells and particles were harvested by centrifugation. Cells were washed with PBS containing 2.5 mM EDTA and stained with NucBlue Live Cell Stain (Molecular Probes) as per vendor's instructions. Cells were then fixed using Lyse/Fix (BD Biosciences). Particle ingestion was measured using the ImageStream X Mark II Imaging Flow Cytometer (Millipore, Billerica, Mass) and data analyzed using the associated IDEAS 6.2 software (Millipore).

Neutrophil respiratory burst assay

Isolated primary neutrophils were treated with patient BAL or 0.9% saline control and incubated at 37°C with dihydrorodamine 123 (Molecular Probes) at a concentration of 375 ng/mL for 15 minutes. Respiratory burst was then induced by the addition of 200 nM PMA to samples as indicated for another 15 minutes at 37°C. Cells were washed with PBS-EDTA buffer and fixed using Lyse/Fix (BD Biosciences), and oxidative burst was measured using a BD FACSCalibur (BD Biosciences) and FlowJo version 10 software (FlowJo LLC).

Neutrophil flow cytometry panels

Primary neutrophils were cultured overnight at a density of 2×10^6 cells/mL in equal parts media and BAL or 0.9% saline, and 200 nM PMA (Sigma-Aldrich) was added to the cells for 30 minutes before harvesting. Cells were harvested and media stored at -80°C . Cells were washed with PBS containing 2.5 mM EDTA, treated with Fc Block (BioLegend) and Live/Dead Aqua (Molecular Probes), and then surface-stained using CD66b-PerCPCy5.5, CD62L-BV421, CD11b-AF488, CD11c-PECy7, and CD16-APCCy7 (BioLegend). Cells were then permeabilized using Perm/Wash Buffer I (BD Biosciences) and stained intracellularly with CCL2-AF647 (BioLegend). After staining was complete, all cells were fixed with Lyse/Fix (BD Biosciences) and expression data collected using the CytoFLEX flow cytometer (Beckman-Coulter, Brea, Calif). Data were analyzed using FlowJo version 10 software (FlowJo LLC).

Airway macrophage experiments

Airway macrophages (100,000 cells) were isolated from the cell suspension and incubated with and without 1 mg/mL LPS as previously described.^{E3} Basal protein expression of TGF- β 1 and PARP-1 cleavage was analyzed by fluorescent microscopy in cytopins using anti-TGF- β 1 and antimouse PARP-1 antibodies at a 1:100 dilution (sc-31609 and sc-8007, Santa Cruz Biotechnology, Inc, Santa Cruz, Calif). Bacterial phagocytosis of fluorescein isothiocyanate-conjugated, inactivated *S aureus* (Invitrogen, Carlsbad, Calif) was assessed with an Olympus confocal microscope (model BX51, Olympus America, Inc) with quantitative digital fluorescence imaging software (Olympus FluoView 300, version 4.3, Olympus America, Inc). The RFUs and phagocytic index of *S aureus* were determined for each phagocytic cell at 50% of the cell depth.

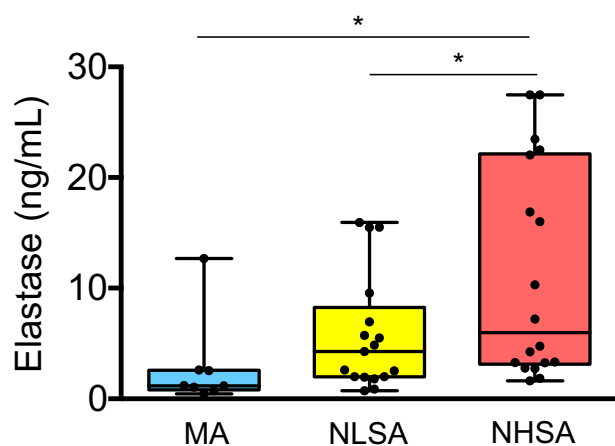


FIGURE E1. Media elastase concentrations after overnight exposure to BAL fluid from children with moderate asthma treated with ICSs (MA), children with neutrophil “low” severe asthma (NLSA), and children with neutrophil “high” severe asthma (NHSA). Whiskers represent minimum and maximum values; dots reflect individual participants. * $P < .05$.

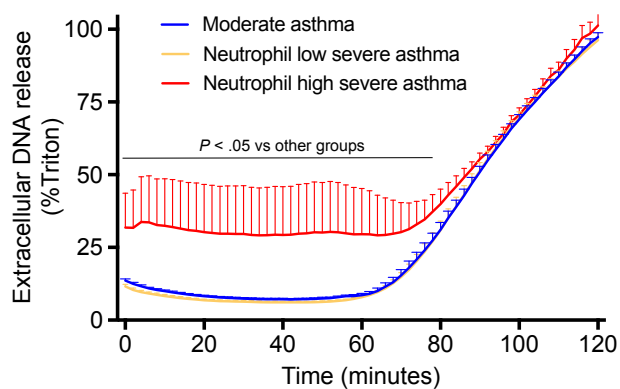


FIGURE E2. Time course of extracellular DNA release (mean $\pm$ SEM).

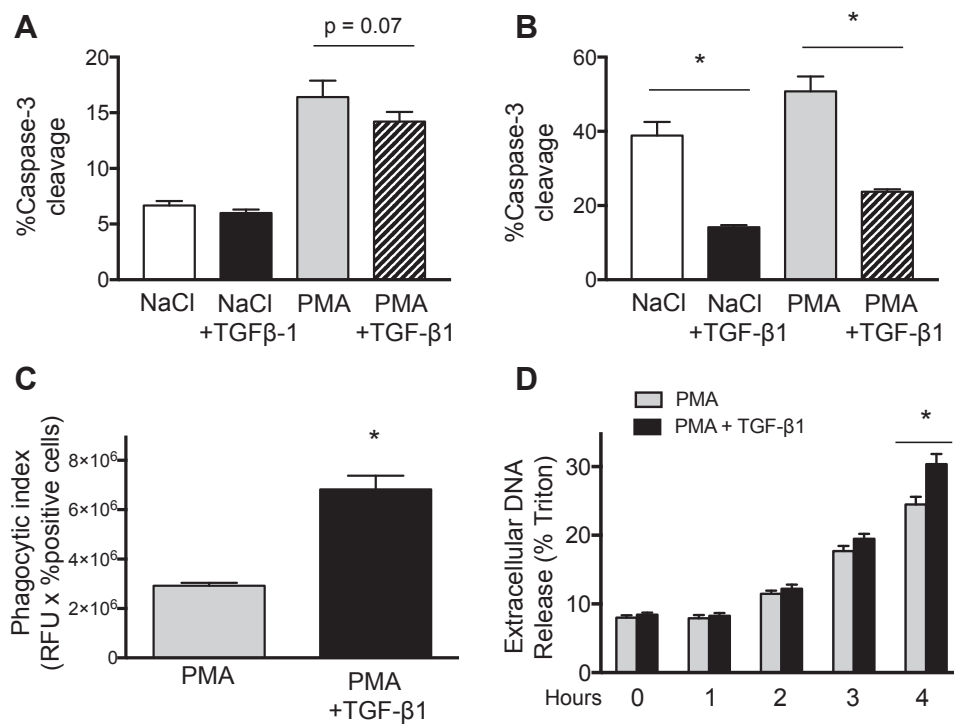


FIGURE E3. (A) HL-60 neutrophil apoptosis (mean $\pm$ SEM) after stimulation with PMA and exposure to 5 ng/mL TGF- β 1 for 2 h ($n = 3$) and (B) overnight ($n = 6$), and (C) phagocytosis and (D) extracellular DNA release after overnight TGF- β 1 exposure (5 ng/mL). * $P < .05$.

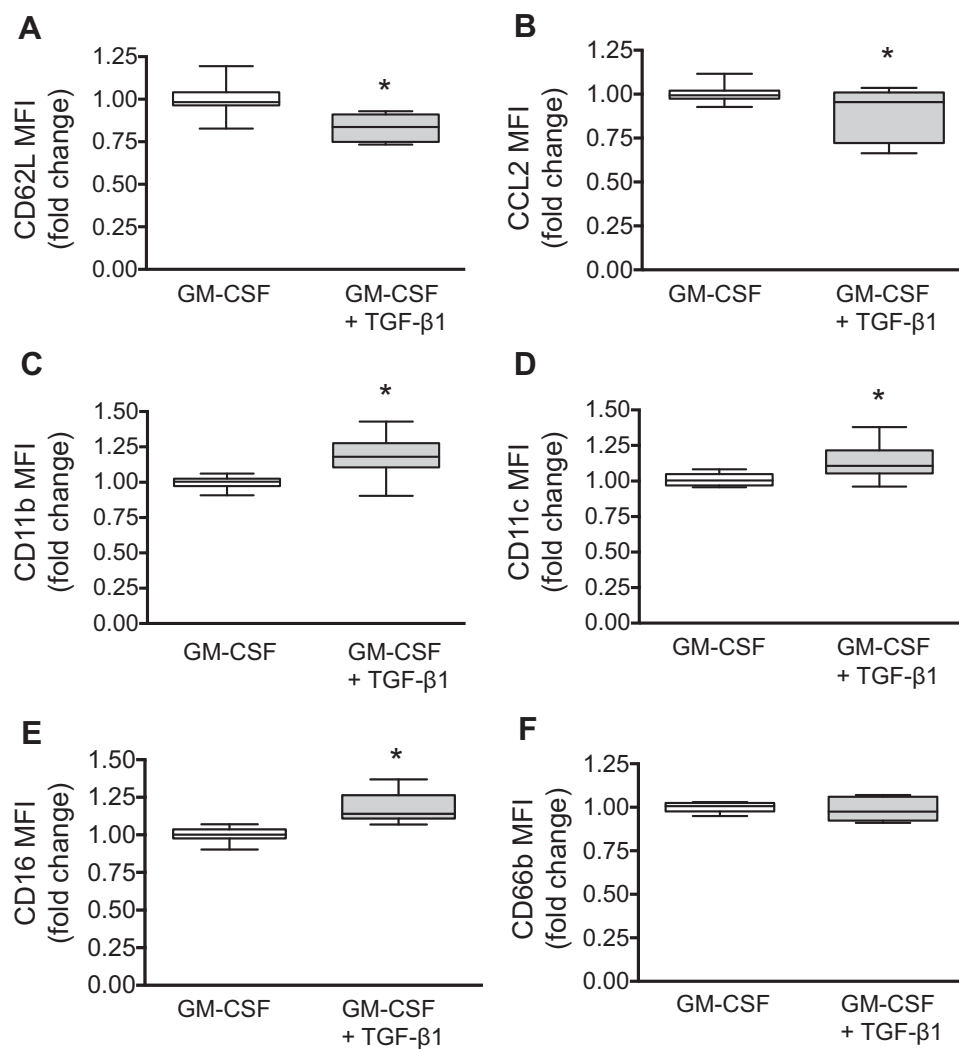


FIGURE E4. Fold-change (mean $\pm$ SEM) in mean fluorescent intensity (MFI) of (A) CD62L, (B) CCL2, (C) CD11b, (D) CD11c, (E) CD16, and (F) CD66b in primary neutrophils stimulated with GM-CSF and exposed to 5 ng/mL TGF- β 1 overnight. Whiskers reflect the 5th and 95th percentiles. * $P < .05$.

TABLE E1. Concentrations (mean $\pm$ SEM) of neutrophil activation markers, cytokines, and chemokines in the BAL fluid normalized to total protein concentration

Analyte	Moderate asthma treated with ICSs (N = 13)	Neutrophil "low" severe asthma (N = 29)	Neutrophil "high" severe asthma (N = 38)
Activation markers (ng/g protein)			
Myeloperoxidase	0.09 $\pm$ 0.02	0.07 $\pm$ 0.02	0.19 $\pm$ 0.04*†
Elastase	0.02 $\pm$ 0.01	0.03 $\pm$ 0.01	0.05 $\pm$ 0.01*†
Lactoferrin	0.52 $\pm$ 0.11	0.98 $\pm$ 0.32	1.83 $\pm$ 0.48*†
Cytokines/chemokines (pg/g protein)			
IL-1 β	0.80 $\pm$ 0.16	0.83 $\pm$ 0.14	1.41 $\pm$ 0.23*†
IL-6	24.98 $\pm$ 3.86	38.37 $\pm$ 6.20	168.48 $\pm$ 40.49*†
IL-7	10.75 $\pm$ 2.73	15.10 $\pm$ 3.62	14.17 $\pm$ 1.64
IL-12	1.63 $\pm$ 0.41	2.65 $\pm$ 0.89	2.16 $\pm$ 0.29
IL-33	21.20 $\pm$ 5.90	58.52 $\pm$ 21.08	109.09 $\pm$ 21.78*†
TNF- α	2.18 $\pm$ 0.41	5.42 $\pm$ 2.02	10.42 $\pm$ 6.08
CXCL1	6,366.9 $\pm$ 1,905.4	12,849.2 $\pm$ 1,902.8	18,280.7 $\pm$ 1,786.9*†
CXCL8	215.19 $\pm$ 58.09	189.15 $\pm$ 31.32	1,739.18 $\pm$ 671.64*†
CXCL10	1,793.9 $\pm$ 342.7	3,041.8 $\pm$ 562.6	7,091.6 $\pm$ 1,621.1*†
CCL2	287.35 $\pm$ 112.78	265.60 $\pm$ 49.31	349.26 $\pm$ 61.58*†
CCL3	45.41 $\pm$ 5.11	41.99 $\pm$ 4.68	109.37 $\pm$ 27.73*†
CCL4	120.64 $\pm$ 40.32	145.71 $\pm$ 21.92	325.43 $\pm$ 80.59*†
CCL22	557.92 $\pm$ 119.33	894.85 $\pm$ 304.33	1,031.40 $\pm$ 251.05*

* P < .05 vs moderate asthma treated with ICSs.

† P < .05 vs neutrophil low severe asthma.

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