

ASTHMA

Neutrophil cytoplasts induce T_H17 differentiation and skew inflammation toward neutrophilia in severe asthma

Nandini Krishnamoorthy^{1*}, David N. Douda^{1*†}, Thayse R. Brüggemann¹, Isabell Ricklefs¹, Melody G. Duvall^{1‡}, Raja-Elie E. Abdunour¹, Kimberly Martinod^{2§}, Luciana Tavares¹, Xiao Wang³, Manuela Cernadas^{1‡}, Elliot Israel¹, David T. Mauger⁴, Eugene R. Bleecker⁵, Mario Castro⁶, Serpil C. Erzurum⁷, Benjamin M. Gaston⁸, Nizar N. Jarjour⁹, Sally Wenzel¹⁰, Eleanor Dunican^{11||}, John V. Fahy¹¹, Daniel Irimia³, Denisa D. Wagner², Bruce D. Levy^{1¶}, National Heart, Lung, and Blood Institute Severe Asthma Research Program-3 Investigators

Copyright © 2018
The Authors, some
rights reserved;
exclusive licensee
American Association
for the Advancement
of Science. No claim
to original U.S.
Government Works

Severe asthma is a debilitating and treatment refractory disease. As many as half of these patients have complex neutrophil-predominant lung inflammation that is distinct from milder asthma with type 2 eosinophilic inflammation. New insights into severe asthma pathogenesis are needed. Concomitant exposure of mice to an aeroallergen and endotoxin during sensitization resulted in complex neutrophilic immune responses to allergen alone during later airway challenge. Unlike allergen alone, sensitization with allergen and endotoxin led to NETosis. In addition to neutrophil extracellular traps (NETs), enucleated neutrophil cytoplasts were evident in the lungs. Surprisingly, allergen-driven airway neutrophilia was decreased in peptidyl arginine deiminase 4-deficient mice with defective NETosis but not by deoxyribonuclease treatment, implicating the cytoplasts for the non-type 2 immune responses to allergen. Neutrophil cytoplasts were also present in mediastinal lymph nodes, and the cytoplasts activated lung dendritic cells in vitro to trigger antigen-specific interleukin-17 (IL-17) production from naïve CD4⁺ T cells. Bronchoalveolar lavage fluid from patients with severe asthma and high neutrophil counts had detectable NETs and cytoplasts that were positively correlated with IL-17 levels. Together, these translational findings have identified neutrophil cytoplast formation in asthmatic lung inflammation and linked the cytoplasts to T helper 17-mediated neutrophilic inflammation in severe asthma.

INTRODUCTION

Asthma is a common inflammatory disorder of the airways with several underlying endotypes and excess morbidity (1–4). About 10% of patients have severe disease, which responds poorly to corticosteroids and can be associated with neutrophil predominant inflammation and high interleukin-17 (IL-17) levels (5–8).

In response to inflammatory stimuli, neutrophils form neutrophil extracellular traps (NETs). NETosis releases DNA-containing NETs from cells that may result in the formation of enucleated cell bodies called cytoplasts (9). When nuclei are removed ex vivo, neutrophil cytoplasts remain viable and demonstrate some residual cell functions (10–12). Extracellular DNA in NETs has vital roles in host defense (9, 13), yet these structures are also associated with organ injury and inflammation (14, 15). Roles for cytoplasts and NETs in asthma pathogenesis remain to be determined.

Here, in a preclinical model of allergic lung inflammation that incorporates both type 2 and non-type 2 immune responses, we identified neutrophil-derived cytoplasts in lungs and mediastinal lymph nodes. Cytoplasts activated lung dendritic cells (DCs) to differentiate naïve CD4⁺ T cells to antigen-specific T helper 17 (T_H17) effectors. Some bronchoalveolar lavage fluids (BALFs) collected from severe asthmatic patients had NETs and cytoplasts that were associated with increased IL-17 and BAL neutrophils. These findings have identified NETosis-derived cytoplasts in vivo and a pivotal mechanism for the cytoplasts to bridge innate and adaptive immune responses with relevance to non-type 2 inflammation in severe asthma (SA) pathogenesis.

RESULTS

Neutrophilic inflammation in a murine model of house dust mite extract and endotoxin

Common indoor and outdoor environmental exposures can influence airway inflammation in asthma (16). The presence of endotoxin during allergen sensitization can lead to neutrophilic airway

¹Pulmonary and Critical Care Medicine Division, Department of Medicine, Brigham and Women's Hospital and Harvard Medical School, Boston, MA 02115, USA. ²Program in Cellular and Molecular Medicine, Division of Hematology and Oncology, Boston Children's Hospital, Boston, MA 02115, USA. ³BioMEMS Resource Center, Massachusetts General Hospital, Harvard Medical School, MA 02129, USA. ⁴Division of Statistics and Bioinformatics, Department of Public Health Sciences, Pennsylvania State University, Hershey, PA 17033, USA. ⁵Center for Genomics and Personalized Medicine Research, School of Medicine, Wake Forest University, Winston-Salem, NC 27157, USA. ⁶Division of Pulmonary and Critical Care Medicine, Departments of Medicine and Pediatrics, Washington University, St. Louis, MO 63110, USA. ⁷Department of Pathobiology, Cleveland Clinic, Cleveland, OH 44195, USA. ⁸Department of Pediatrics, Rainbow Babies and Children's Hospital, Case Western Reserve University, Cleveland, OH 44106, USA. ⁹Section of Pulmonary and Critical Care Medicine, University of Wisconsin School of Medicine, Madison, WI 53792, USA. ¹⁰Pulmonary, Allergy, and Critical Care Medicine Division, Department of Medicine, University of Pittsburgh School of Medicine, Pittsburgh, PA 15213, USA. ¹¹Division of Pulmonary and Critical Care Medicine, Department of Medicine and the Cardiovascular Research Institute, University of California, San Francisco, San Francisco, CA 94143, USA.

*These authors contributed equally to the work.

†Present address: Zymeworks, Inc., Vancouver, Canada.

‡Present address: Boston Children's Hospital, Boston, MA 02115 USA.

§Present address: Laboratory for Thrombosis Research, KU Leuven Campus Kulak Kortrijk, Kortrijk, Belgium.

||Present address: School of Medicine, St Vincent's Hospital, University College Dublin, Dublin, Ireland.

¶Corresponding author. Email: blevy@bwh.harvard.edu

inflammation in mice (17, 18). We established a protocol in which mice were exposed to the common indoor allergen house dust mite (HDM) alone or in combination with lipopolysaccharide (LPS) for 3 days to sensitize the animals, followed by 4 days of rest and then 8 days of intranasal HDM challenge (see Materials and Methods and Fig. 1A). Although there was no significant difference after HDM challenge in BALF total cell counts between the two different sensitization approaches (Fig. 1B), leukocyte differential cell counts showed significantly higher neutrophil and lower eosinophil numbers in mice sensitized with HDM and LPS (HDM/LPS) compared with HDM and vehicle (HDM/Veh; Fig. 1C and fig. S1A). Despite 4 days of rest between sensitization with HDM/

LPS and airway challenge with HDM alone, BALF neutrophilic inflammation continued to be more prominent than eosinophilia, a response that was distinct from mice sensitized with HDM/Veh. Effector CD4⁺ T cells were next characterized in the mediastinal lung-draining lymph nodes (MLNs). At 4 days after sensitization (Fig. 1A, day 7), there was a significantly higher number of T_H17 cells in HDM/LPS-sensitized mice compared with HDM/Veh (Fig. 1, D and E, and fig. S1B).

Exposure to LPS and allergen promoted NETosis

Instillation of LPS in mouse airways can induce NETosis (14, 19, 20), so BALF were obtained 1 day after sensitization to address this

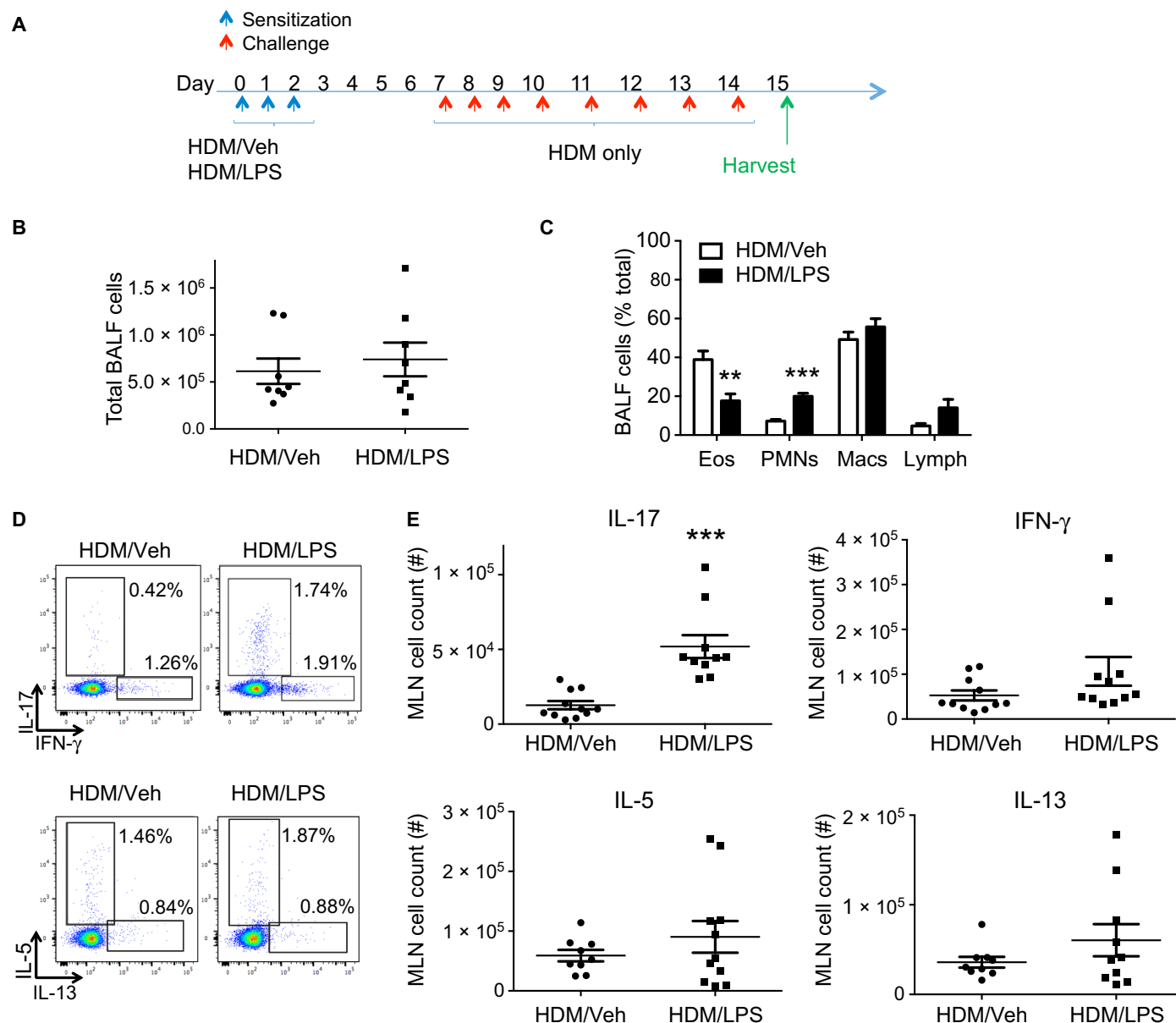


Fig. 1. Neutrophilic inflammation in a murine model of HDM and LPS. (A) Schematic diagram showing the allergen sensitization and challenge protocol. After sensitization with HDM/Veh or HDM/LPS (protocol day 0 to 2) followed by HDM challenge (intranasally, protocol day 7 to 14), the inflammatory response was assessed 24 hours later on protocol day 15 ($n = 8$ mice). (B) BALF total cell count (C) and leukocyte differential (% total leukocytes) were determined. Eos, eosinophils; Macs, macrophages; PMNs, polymorphonuclear neutrophils. (D and E) MLNs were collected on protocol day 7, and dissociated cells were restimulated with PMA and ionomycin. (D) Representative flow cytometry plot and (E) MLN total cell count of CD4⁺ T cells expressing IL-17, IFN-γ, IL-5, and IL-13 ($n = 10$ mice). ** $P < 0.01$ and *** $P < 0.001$ by Student's t test.

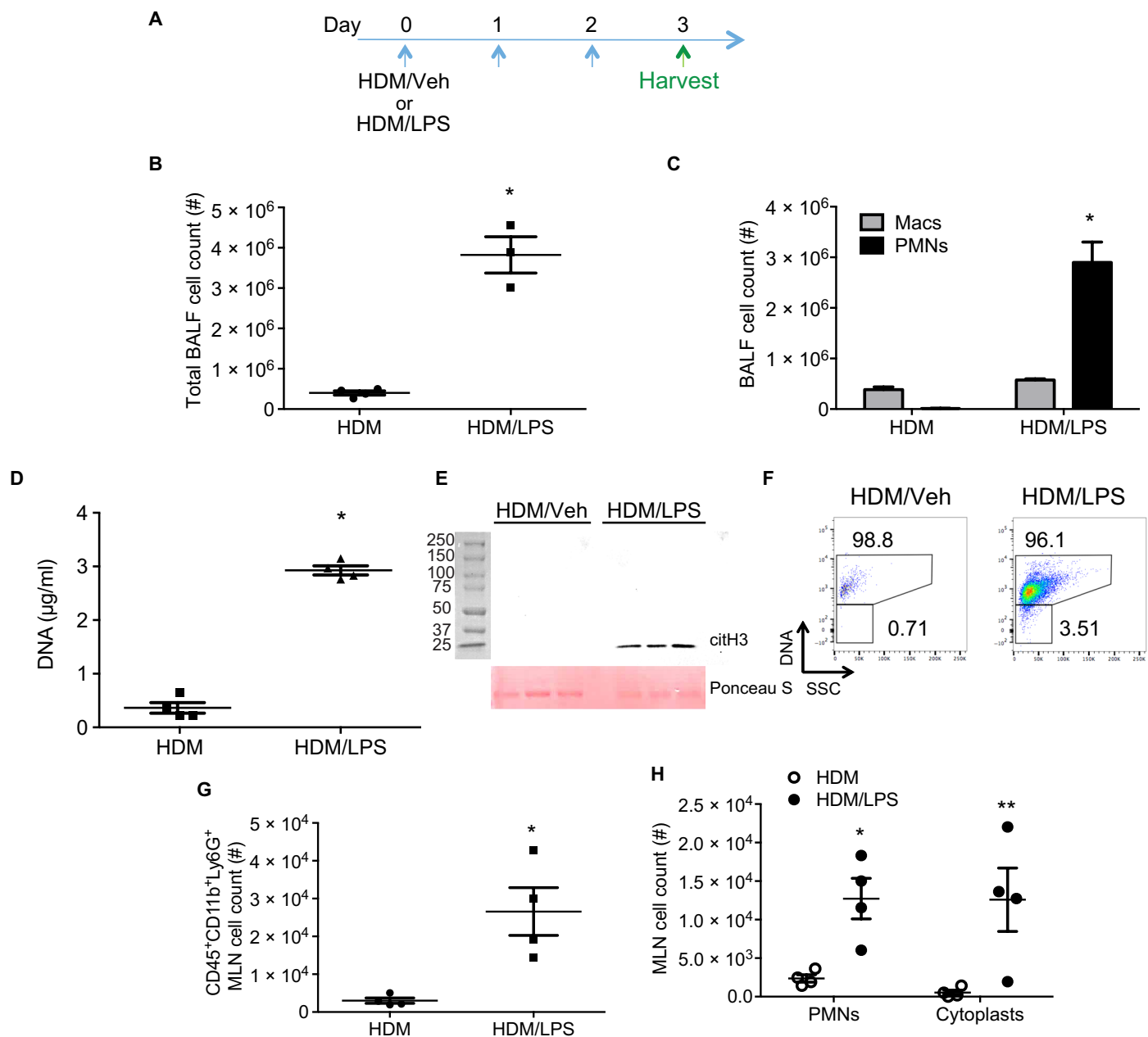


Fig. 2. LPS promotes NETosis during allergen sensitization. (A) BALFs and MLNs were harvested on protocol day 3 after allergen sensitization with HDM/Veh or HDM/LPS. (B) BALF total cell count and (C) leukocyte differential count on protocol day 3 from HDM/Veh- or HDM/LPS-sensitized mice. Data are representative of two independent experiments with $n > 3$. (D) PicoGreen assay showing NET-associated DNA present in BALF ($n = 4$ mice). (E) Western blot showing citH3 in BALFs (top) and Ponceau S stain as loading control (bottom). (F) Representative flow cytometry plot showing the presence of DNA-positive neutrophils (PMNs) and DNA-negative cytoplasts in BALFs from HDM/Veh (left) and HDM/LPS (right) mice ($n = 8$). SSC, side scatter. (G) MLN total cell count for CD45⁺CD11b⁺Ly6G⁺ cells and (H) DNA-positive PMN and DNA-negative cytoplast count ($n = 4$ mice). * $P < 0.05$ and ** $P < 0.01$ by two-tailed unpaired Student's t test, where indicated, or Mann-Whitney U test.

possibility (Fig. 2A, day 3). Significantly higher numbers of BALF cells were present with HDM/LPS sensitization compared with HDM/Veh (Fig. 2B) with predominant BALF neutrophilia (Fig. 2C and fig. S1C). Notably, some of the recruited neutrophils underwent NETosis in the lung, as evidenced by increased BALF DNA (Fig. 2D) and hypercitrullinated histone H3 [citH3 (Fig. 2E)]. Cytoplasts are largely devoid of DNA, so the presence of DNA in cells was tracked by

flow cytometry using a cell-permeable DNA dye. Analyses of BALFs revealed the presence of CD45⁺CD11b⁺Ly6G⁺DNA⁺ neutrophils and CD45⁺CD11b⁺Ly6G⁺DNA⁻ cytoplasts from HDM/LPS-exposed mice immediately after sensitization (Fig. 2F and gating strategy in fig. S2). MLNs at protocol day 3 also had CD45⁺CD11b⁺Ly6G⁺DNA⁻ cytoplasts (Fig. 2, G and H, and fig. S1D). Together, these data indicate that concomitant allergen and LPS exposure but not

allergen alone triggered NETosis in the lung with both DNA and cytoplasts present in vivo. In addition to the lung, cytoplasts were in MLNs where allergen exposure skewed effector T cells toward T_H17 differentiation.

To determine the role of IL-17 after HDM/LPS exposure, we gave mice an anti-IL-17 antibody or control antibody (80 µg per mouse, intraperitoneal route) before and during the sensitization phase (fig. S3A, protocol days -1 and 1). After challenge with HDM, the mice sensitized in the presence of the anti-IL-17 antibody had reduced BALF total cell numbers with a significant decrease in BALF neutrophils and concomitant increase in eosinophils (fig. S3B). Exposure to the anti-IL-17 antibody did not have a significant effect on lung cytoplast numbers after sensitization (fig. S3C). Together, these findings indicate that IL-17 production is downstream of NETosis but pivotal to lung neutrophil accumulation with later allergen challenge.

Deoxyribonuclease instillation altered levels of NETs but not of neutrophilia

To address the potential impact of NET DNA, we instilled deoxyribonuclease (DNase) during sensitization to disrupt the NETs in HDM/LPS-exposed mice. DNase led to a significant decrease in NETs compared with phosphate-buffered saline (PBS) control (Fig. 3, A and B). With DNase, there was a modest decrease in BALF cells after HDM challenge (day 15; Fig. 3C) without significant changes in the percent or number of BALF neutrophils (Fig. 3, D and E). Macrophages and eosinophils were reduced in part, suggesting a potential role for NETs in the recruitment of these cells

(Fig. 3, D and E). Protease-free DNase (21) gave similar partial decrements in BALF macrophages without significant decreases in BALF neutrophils (fig. S4), consistent with a role for the cytoplasts rather than the NETs in neutrophilia after allergen challenge.

Neutrophil cytoplasts retained functional properties

To investigate whether the cellular remnants of NETosis have a functional role in responses to the environmental stimuli (i.e., HDM and LPS), we first sorted cells from murine lungs 3 days after HDM/LPS administration and then evaluated cellular responses. Neutrophils and enucleated cytoplasts were identified, and the majority of both cell types excluded trypan blue dye (>95%). Neutrophils (average diameter, 6.9 µm) were significantly larger than the cytoplasts (average diameter, 3.3 µm) that had expelled their DNA (Fig. 4, A and B). Notably, the cytoplasts were significantly larger than microvesicles (~200 nm), exosomes (~100 nm), and cellular debris. To assess chemotaxis, we placed the sorted cells in a microfluidic device with a gradient established to leukotriene B₄ (LTB₄; 100 nM). The cytoplasts displayed chemokinesis but, in contrast to neutrophils, did not exhibit chemotaxis to LTB₄ (see movies S1 and S2 and Fig. 4, C to F). The cytoplasts moved more slowly than the neutrophils (Fig. 4G) and displayed random movement despite the LTB₄ chemotactic gradient (Fig. 4H).

The cytoplast chemokinesis suggested an intact cytoskeleton, so phagocytosis was next assessed. After sorting, the cells were incubated with pHrodo-tagged *Escherichia coli* particles that fluoresce after engulfment upon pH change in acidic phagolysosomes (see Materials and Methods). Before incubation, the absence of nuclei in

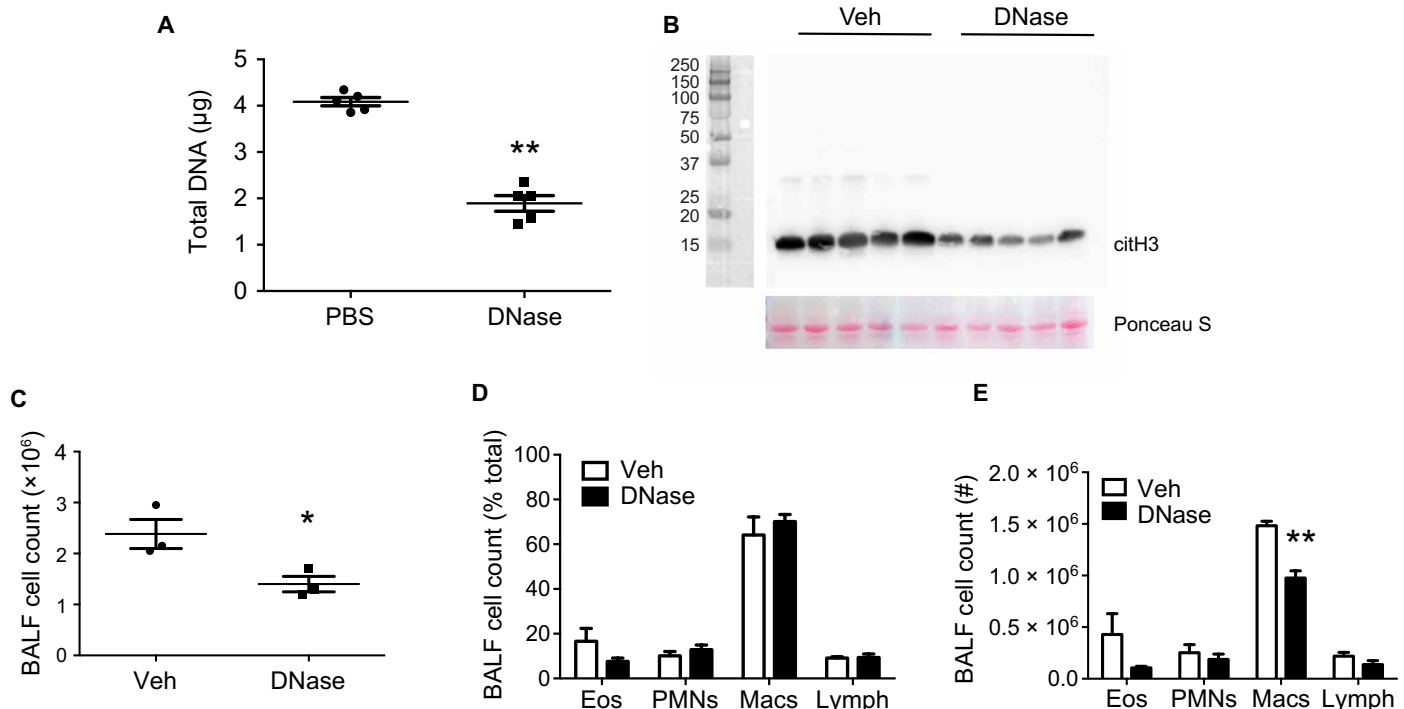


Fig. 3. DNase instillation altered levels of NETs but not of neutrophilia. Mice were subjected to DNase (intranasally, 6 hours after sensitization with HDM/LPS) or PBS control, and tissues were harvested at the end of the sensitization period (day 3). (A) PicoGreen assays showing the amount of DNA in BALFs ($n = 5$ mice). (B) Western blot showing citH3 (top) and Ponceau S stain as a loading control (bottom; $n = 5$ mice). (C to E) To assess inflammatory responses, BAL was performed at the end of the allergen (HDM) challenge on protocol day 15. (C) BALF total cell count and (D and E) leukocyte differential (% total leukocytes and cell count) were enumerated ($n = 3$ mice). * $P < 0.05$ and ** $P < 0.01$ by Student's t test.

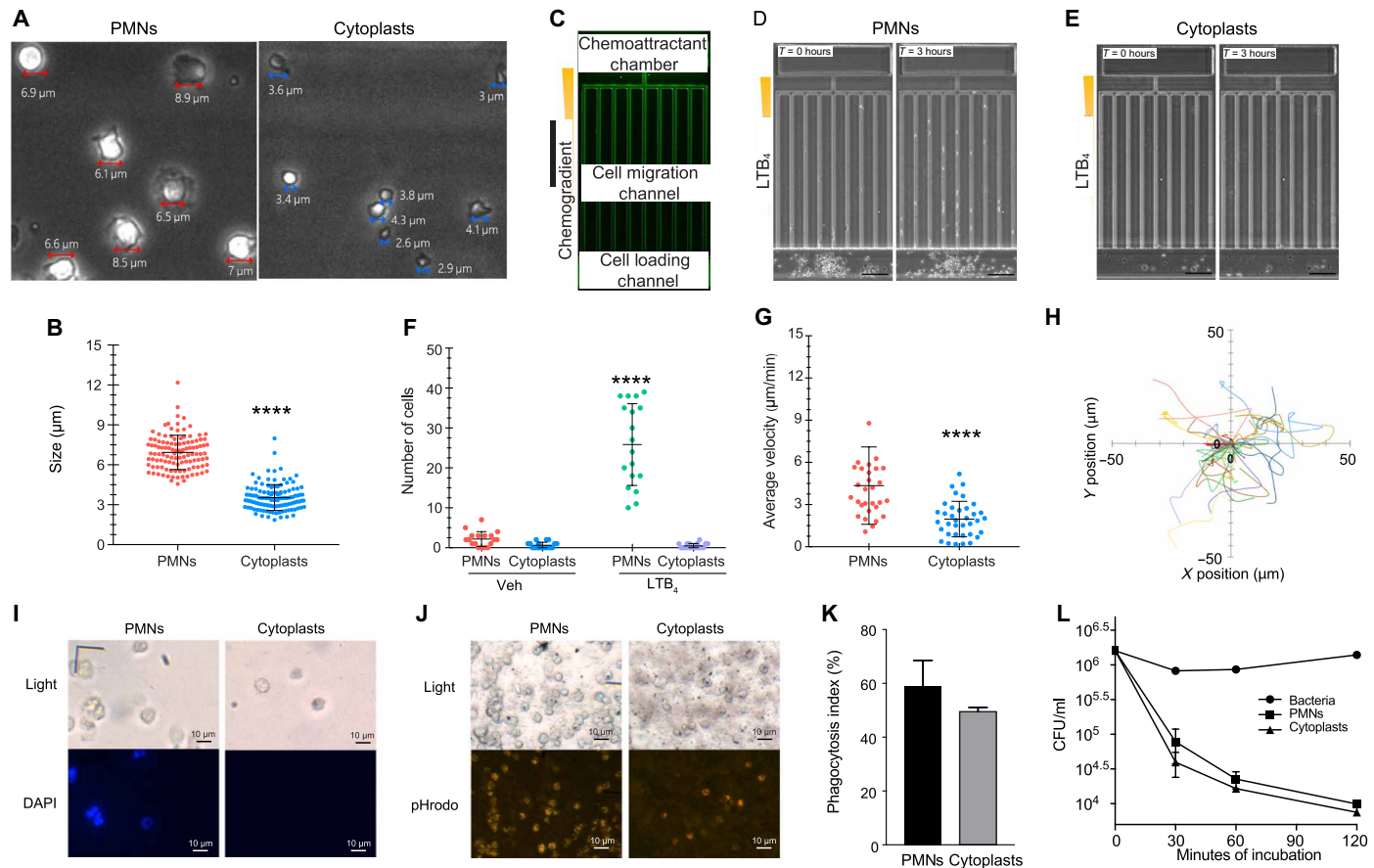


Fig. 4. Enucleated cytoplasts formed after NETosis are intact and retain functional responses. Neutrophils (PMNs) and neutrophil cytoplasts were flow-sorted from HDM/LPS-treated mouse lungs (protocol day 3). (A and B) The morphology and size of the sorted PMNs and cytoplasts were determined by phase-contrast microscopy. **** $P < 0.0001$ by two-tailed Student's t test. (C to E) Chemotaxis to LTB₄ was assessed using a microfluidic device with sorted cells (see Materials and Methods). (C) Fluorescent microscopic image showing one chemotaxis unit in the microfluidic device. (D) PMNs or (E) cytoplasts were loaded into the microfluidic chamber with an LTB₄ (100 nM) gradient and time lapse, and phase-contrast microscopic imaging was performed for 6 hours (representative images). (F) Measurements of the number of cells that entered the migration microchannels per unit at various conditions. **** $P < 0.0001$ using one-way ANOVA. (G) Measurements of chemotaxis velocity of PMNs in the migration channels and chemokinesis velocity of cytoplasts in the cell loading channel. **** $P < 0.0001$ using one-way ANOVA. (H) Individual trajectories of cytoplast chemokinesis in the cell loading channel. (I to K) Phagocytosis by PMNs and cytoplasts was determined using pHrodo-coupled *E. coli* particles. (I) Absence of nuclei in cytoplasts confirmed by DAPI staining. (J) Phagocytosis of pHrodo-coupled *E. coli* particles leading to fluorescent color change in PMNs and cytoplasts. (K) Phagocytosis index (% total) was determined. This experiment was performed three times. Values represent the mean, and error bars represent the SEM. (L) The killing capacity of sorted neutrophils and cytoplasts toward *S. pneumoniae* (serotype 1) was determined at different time points indicated. This experiment was performed two times. Values represent the mean between duplicate controls, and error bars represent the SEM used in a representative experiment.

the cytoplasts was confirmed by 4',6-diamidino-2-phenylindole (DAPI) stain (Fig. 4I). After 1 hour, phagocytosis for both the cytoplasts and neutrophils was evident (Fig. 4J). The phagocytosis index was similar for each cell type (Fig. 4K). To assess their capacity for killing a lung relevant pathogen, we incubated sorted cytoplasts and neutrophils with *Streptococcus pneumoniae* (serotype 1). Notably, the cytoplasts displayed killing of the bacteria to a comparable extent as autologous neutrophils (Fig. 4L). Together, these data indicate that the cytoplasts display several preserved functional responses of their parent neutrophils.

Deficiency in PAD4 resulted in reduced cytoplast generation and IL-17 levels

Because DNase did not affect neutrophil responses to allergen challenge and cytoplasts retained select cellular functions, we next

turned to peptidyl arginine deiminase 4-deficient (PAD4 KO) mice with defective NETosis (22). PAD4 hypercitrullinates histones and is involved in NETosis (22, 23). After sensitization with HDM/LPS, PAD4 KO mice had reduced numbers of BALF total cells (day 30; Fig. 5A) and neutrophils (Fig. 5B). Western blots of lung homogenates showed decreased citH3 in PAD4 KO mice during HDM/LPS sensitization (Fig. 5, C and D). By flow cytometry criteria (see Materials and Methods), cytoplast numbers were also much decreased in the PAD4 KO mice (Fig. 5, E and F), suggesting decreased NETosis in PAD4 KO mice in response to HDM/LPS. In addition, about 7% of wild-type (WT) neutrophils were citH3⁺ by flow cytometry, indicative of an association with NETs (fig. S5). The numbers of citH3⁺ neutrophils in PAD4 KO mice were substantially decreased, and neutrophil cytoplasts were citH3⁻ (fig. S5). Upon HDM challenge, BALF total cell counts in PAD4 KO were significantly lower than

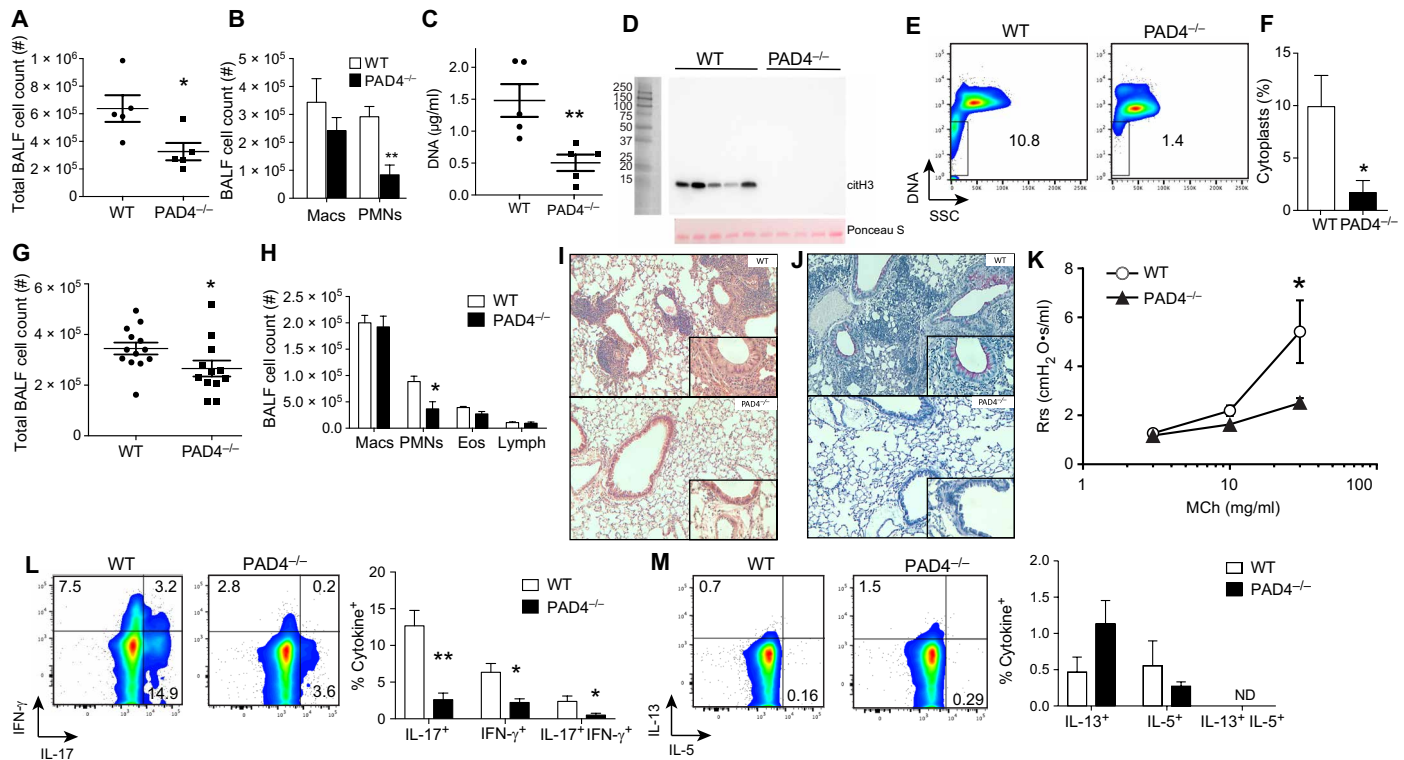


Fig. 5. Deficiency in PAD4 results in decreased cytoplasts, neutrophils, and IL-17. WT and PAD4^{-/-} mice were sensitized with HDM/LPS, and BALFs were collected on day 3. (A) BALF total cell count and (B) leukocyte differential were enumerated ($n = 5$ mice). (C) NETosis was monitored by BALF DNA levels on day 3 ($n = 5$ mice) and (D) Western blot for citH3 (top). Ponceau S stain was used as a loading control (bottom). (E) Representative flow cytometry plot showing DNA-positive PMNs and DNA-negative cytoplasts in BALFs collected on protocol day 3 after HDM/LPS sensitization from WT and PAD4^{-/-} mice. (F) Percent cytoplasts were enumerated by flow cytometry criteria. (G to K) In addition to the postsensitization period, the resulting antigen-driven lung inflammation in WT and PAD4^{-/-} mice was determined on protocol day 15 after HDM/LPS sensitization, followed by HDM challenge. (G) BALF total cell count and (H) BALF differential count were measured. Lung histology between WT and PAD4^{-/-} mice was evaluated by (I) hematoxylin and eosin staining. (J) PAS staining. (K) Resistance of the respiratory system (Rrs) was measured in anesthetized mice that were mechanically ventilated in the presence of ascending doses of inhaled methacholine (MCh). (L) IL-17 and IFN- γ . (M) IL-13 and IL-5. Values represent the mean, and error bars represent SEM [for (M), error bars represent SD]. * $P < 0.05$ and ** $P < 0.01$ using a Student's t test.

WT mice (Fig. 5G), and BALF neutrophil counts were significantly decreased (Fig. 5H). Changes in BALF eosinophils were not significant (Fig. 5H). After HDM/LPS sensitization and HDM challenge, lung histology was notable for decreased inflammation in PAD4 KO mice relative to WT mice (Fig. 5I). The PAD4 KO mice also had decreased airway mucous cell metaplasia [by periodic acid–Schiff (PAS) stain; Fig. 5J] and decreased methacholine (MCh)–induced airway responsiveness compared with WT mice (Fig. 5K). Cytokine production from lung cells after HDM challenge from the two groups of mice was compared. PAD4 KO mice displayed lower levels of cellular IL-17 and interferon- γ (IFN- γ ; Fig. 5L). Type 2 cytokines were not significantly changed in the PAD4 KO mice (Fig. 5M). Together, these data indicate that cytoplasts produced by NETosis during HDM/LPS sensitization were related to an adaptive inflammatory response characterized, in part, by neutrophilic lung inflammation and IL-17 and IFN- γ production in response to the HDM challenge.

Airway cytoplasts elicited IL-17 production by antigen-specific T lymphocytes

If NETosis-derived cytoplasts could direct neutrophil-enriched adaptive inflammatory responses, then cytoplasts and neutrophils should have distinct effects on allergen-initiated inflammation.

To test this hypothesis, we isolated lung DCs from HDM/LPS- and HDM/Veh-sensitized mice for incubation with cytoplasts or neutrophils before naïve T cells from DO11.10 mice in the presence of ovalbumin peptide (Fig. 6A). In this in vitro reporter assay of cell–cell interactions for antigen presentation and DC initiation of adaptive T cell effector responses, there was a higher abundance of antigen-specific CD4⁺ T_H17 cells with cytoplasts compared with neutrophils (Fig. 6B). The cytoplast-mediated skewing to T_H17 was dose-dependent for the two DC/cytoplast cell ratios tested (1:0.5 and 1:2) and greater than that observed with DC/neutrophil ratio of 1:0.5, 1:2, and even an excess ratio of 1:10 (Fig. 6B and Fig. S6). DCs from both HDM/LPS and HDM/Veh had similar responses, supporting a pivotal role for the cytoplasts in T_H17 differentiation. Because cytoplasts were present in BALF and MLN (Fig. 1), these results suggest that cytoplasts but not neutrophils in proximity to DCs can directly educate DCs to induce antigen-specific T_H17 differentiation. The cytoplasts also triggered the expression of IL-13 from CD4⁺ T cells, although to a lesser extent than IL-17 (Fig. 6B). The DC–cytoplasts interaction was contact-dependent to trigger IL-17 and IL-13 production because the antigen-specific cytokine generation in vitro was abrogated when the cytoplasts were separated from DCs and T cells with a transwell culture system

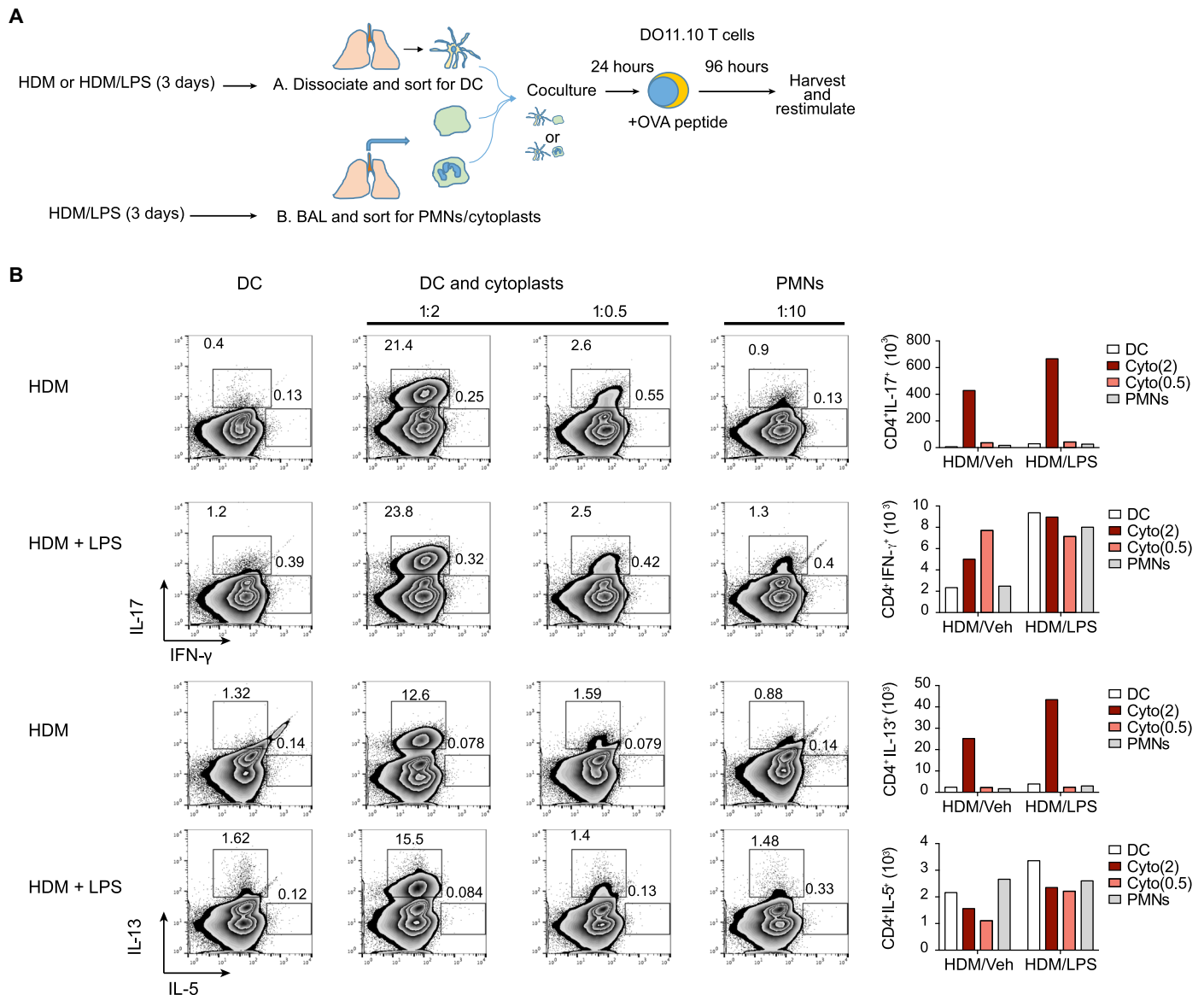


Fig. 6. Lung cytoplasts interact with DCs to induce antigen-specific T lymphocytes. (A) Schematic diagram showing the antigen-specific T cell activation protocol. Briefly, DCs were harvested from the lungs of HDM/Veh- or HDM/LPS-sensitized mice and were incubated overnight with cytoplasts or neutrophils (PMNs) at the indicated cell ratios (top of plots) of DC/cytoplasts (1:2 and 1:0.5) and DC/PMNs (1:10). The HDM/Veh and HDM/LPS DCs were then cocultured with naïve CD4⁺ T cells from DO11.10 mice in the presence of ovalbumin (OVA) peptide, and T cells were restimulated and stained for intracellular cytokines (see Materials and Methods). **(B)** Representative flow cytometry plots showing T cells expressing the indicated cytokines. Numbers outside the box represent percentage of CD4⁺ T cells. Bar graphs (right) show the absolute cell count for the number of cells expressing the indicated cytokine. Data are representative of two experiments.

(fig. S6). Conditioned media from cytoplast culture (24 hours) also failed to promote IL-17 and IL-13 production by the T cells (fig. S6). The data from these experiments highlight a bridging function for cytoplasts in programming an adaptive immune response in T cells that is contact-dependent and distinct from neutrophils.

Select cytoplast surface proteins are distinct from intact neutrophils

To begin to dissect the distinct DC responses to cytoplasts and neutrophils, we analyzed several surface proteins on cytoplasts and neutrophils by flow cytometry. Both cytoplasts and neutrophils displayed

very low levels of annexin V expression (Fig. 7), and expression of CD32 (Fc γ RII) was below the limits of detection on both cell populations. The expression of Toll-like receptor 4 (TLR4) on neutrophils and cytoplasts was comparable (Fig. 7). Notably, cytoplasts did not significantly express DC-SIGN (specific intercellular adhesion molecule-3-grabbing nonintegrin, also known as CD209), but cytoplasts did display substantial expression of major histocompatibility complex class II (MHCII), which was in contrast with neutrophils (Fig. 7). Even with this limited array of surface proteins, it is evident that there are select differences in cytoplasts and neutrophils that may account for their distinct and overlapping functions.

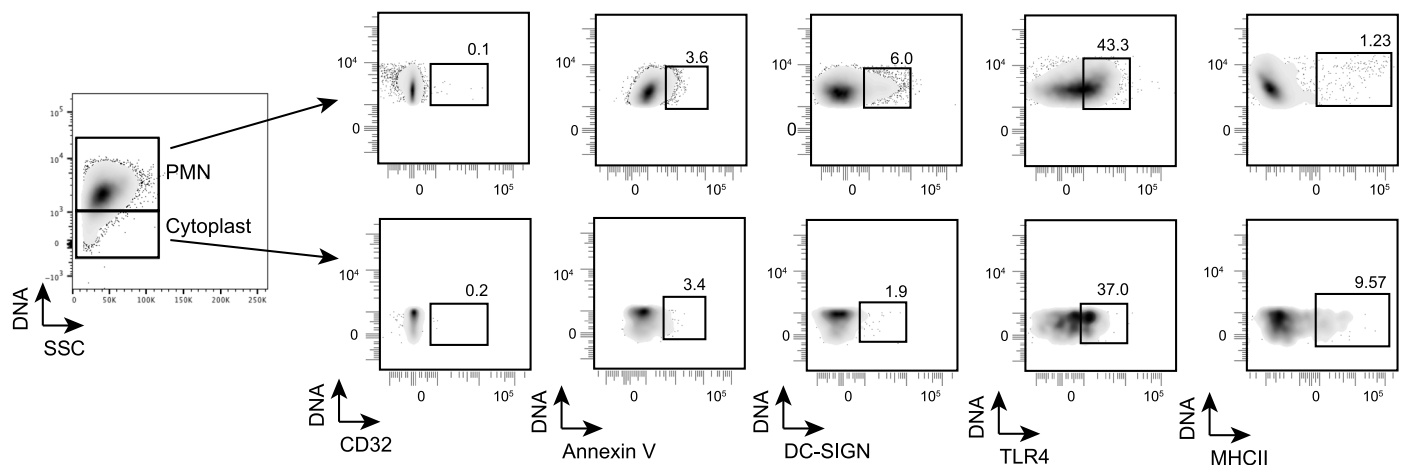


Fig. 7. Select cytoplasmic surface proteins are distinct from intact neutrophils. BALF from HDM/LPS-sensitized mice were stained with different antibodies to detect the expression of these markers on neutrophils and cytoplasts. The expression of the markers is shown in the flow cytometry plots along with the percentage of positivity for each cell population. This experiment was performed twice.

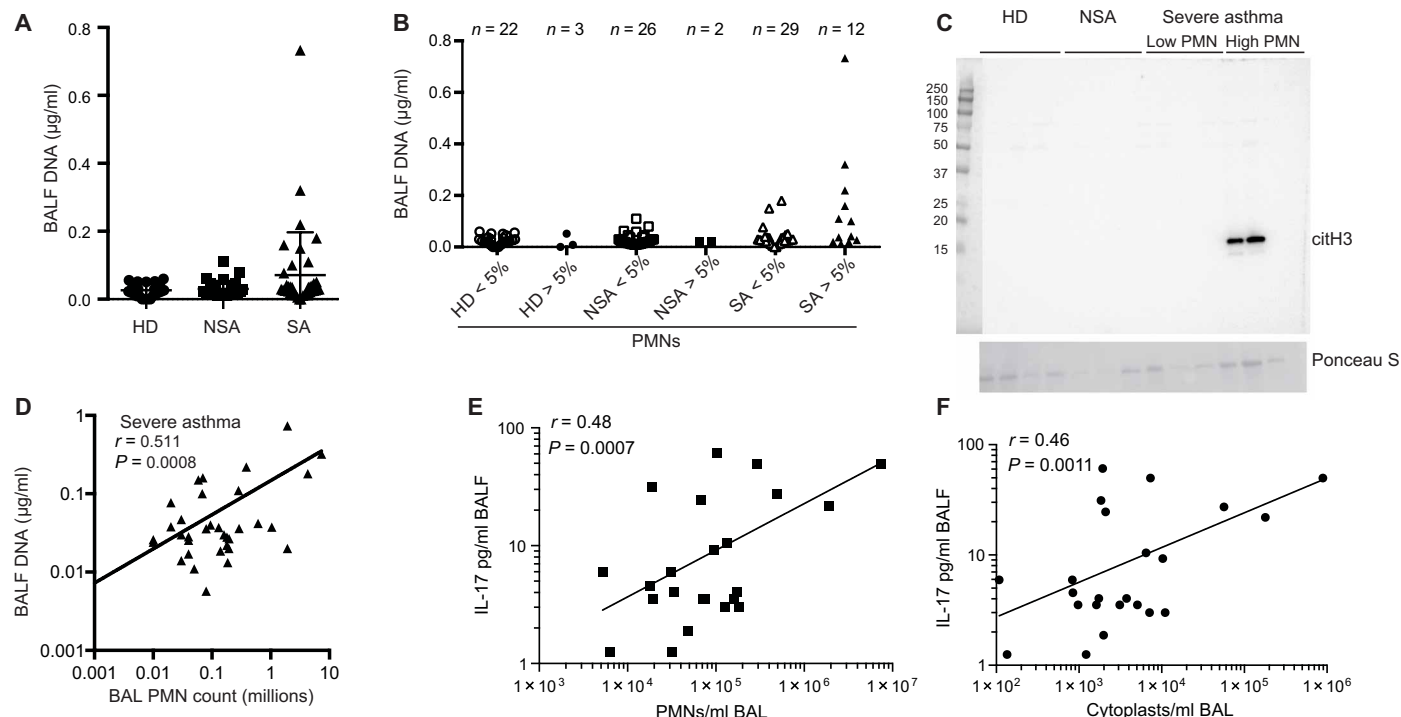


Fig. 8. Lung NETosis in SA correlates with BALF IL-17. (A) PicoGreen assays of BALF DNA in biospecimens from HDs and patients with NSA and SA. (B) BALF DNA levels were further stratified into low neutrophil (PMN) (<5%) and high PMN (>5%). (C) Representative Western blot showing citH3 in BALFs from HD, NSA, and SA with low or high PMN count (top) and Ponceau S stain as a loading control (bottom). (D) Correlation between BALF DNA and PMN count in SA. (E) Correlation between BALF IL-17 levels and number of PMNs. (F) Correlation between BALF IL-17 levels and number of BALF cytoplasts. Pearson correlation r values and significance are noted for each correlation, and regression lines are shown.

Cytoplasts correlated with IL-17 levels in the BALF of severe asthmatics

To determine whether NETosis was operative in human asthma, we first measured DNA levels in BALFs from patients with SA ($n = 41$) and non-SA (NSA; $n = 28$) and healthy donors (HDs; $n = 25$) in the National Heart, Lung, and Blood Institute's Severe Asthma Research Program-3 (SARP-3; table S1). BALF DNA was measurable in a

subset of asthmatic individuals (Fig. 8A), particularly in samples from patients meeting the SARP-3 criteria for SA (see Materials and Methods). Increased levels of DNA in SA were principally identified in BALFs with neutrophils $\geq 5\%$ (Fig. 8B). To more confidently identify DNA from NETosis, we detected citH3 by Western blot in SA BALF with high neutrophil counts (Fig. 8C). BALF levels of DNA in SA were strongly correlated with neutrophil count (Fig. 8D). In addition to BALF DNA,

BAL cells from SA ($n = 28$) and NSA ($n = 19$) patients were analyzed for cytoplasts by flow cytometry. Cytoplasts were identified as CD45⁺ CD66b⁺ CD16⁺ DNA⁻ with low side scatter (gating strategy in fig. S7). There was a strong positive correlation between BAL neutrophils and cytoplasts, and both cell types were associated with BALF IL-17 levels (Fig. 8, E and F). Of the asthmatic patients with high BAL neutrophils and DNA, there was an increased association with frequent asthma exacerbations (>4/year) and sinusitis (table S2). Together, these translational findings indicate that a subset of severe asthmatic individuals with neutrophil-predominant inflammation have active lung NETosis with an association between cytoplasts and IL-17 levels, suggesting that, similar to the preclinical model with HDM/LPS (Figs. 1 to 3), these enucleated cytoplasts can skew an adaptive immune response toward T_H17 to propagate lung neutrophilia.

DISCUSSION

Asthma is a heterogeneous disorder (2, 24, 25). Although eosinophilia and type 2 inflammation are prominent in at least 50% of asthma, some severe asthmatic patients have substantial neutrophilia that is likely to indicate distinct pathogenic mechanisms (26–28). To understand how LPS exposure during sensitization could influence later responses to allergen challenge, we characterized the acute responses to sensitization and identified a marked increase in lung neutrophils and NETosis in the animals exposed to allergen with LPS. In addition to NETs, the resulting neutrophil cytoplasts were also detectable in the lung and MLNs. PAD4 KO mice had reduced NETosis during sensitization and decreased neutrophils and T_H17 responses to later allergen challenge. DNase treatment decreased BALF DNA levels and lung macrophages; however, lung neutrophils were not decreased. There was a trend for reduced allergen-initiated BAL eosinophilia that did not reach significance. Together, these findings implicate the neutrophil cytoplasts as an underlying mechanism for the allergen-mediated T_H17 responses. Administration of neutralizing antibody for either IL-17A or IL-17F given during allergen sensitization can attenuate murine allergic lung inflammation and airway hyperreactivity (29), and here, administration of anti-IL-17 antibody during the sensitization phase significantly decreased BAL total leukocytes and neutrophilia. IL-17 neutralizing antibody did not affect lung cytoplast numbers, supporting a role for IL-17A downstream from cytoplasts for allergen-initiated lung neutrophil recruitment. With neutrophils and neutrophil cytoplasts present in respiratory tissues, immune functions for both cell types were examined after isolation by fluorescence-activated cell sorting (FACS). The enucleated cytoplasts were smaller in size compared with neutrophils and retained membrane integrity (i.e., excluded trypan blue). Albeit smaller than neutrophils, the cytoplasts (3 to 4.5 μ m) were significantly larger than microvesicles or exosomes. The isolated cytoplasts were chemokinetic but differed from neutrophils by not exhibiting chemotaxis to LT β . The cytoplasts were mobile in the microfluidic chamber and exhibited random motion via pseudopods, consistent with the published properties of neutrophil cytoplasts from NETosis during bacterial infection (9). The phagocytic index of the cytoplasts for *E. coli* particles and for killing *S. pneumoniae* was comparable to neutrophils and similar to properties of neutrophil cytoplasts generated by ex vivo enucleation, which remain capable of phagocytosis of staphylococci (30–32). The phagocytic capacity of neutrophil cytoplasts after NETosis suggests sufficiently intact cytoskeleton and membrane integrity for functional cell responses. The comparable

TLR4 staining in cytoplasts and neutrophils after LPS exposure also suggests a similar mechanism for sensing Gram-negative bacteria. Notably, NETosis and neutrophil cytoplasts have also been detected with Gram-positive bacteria, suggesting the presence of other TLR molecules on cytoplasts.

DCs are critical antigen-presenting cells that instruct CD4⁺ T cells to differentiate with a specific phenotype (33). The chemokinesis of cytoplasts and their presence in the MLNs suggested cytoplast trafficking via lung lymphatics and their potential for spatiotemporal regulation of T cell priming. When cytoplasts were incubated with DCs, they promoted differentiation of “naïve CD4⁺ T cells to produce IL-17 in an antigen-specific and dose-dependent manner. The levels of IL-17 induced by the cytoplasts were about twofold higher than IL-13. Notably, neutrophils did not trigger this T_H17 immune response, suggesting that DCs were responding to the activated cytoplasts in a distinct manner than to the intact neutrophils. The antigen-specific nature of this response supports a directed role for this cell-cell interaction between the DCs and cytoplasts that is not simply a generalized alarm signal. Increased expression of MHCII on cytoplasts indicates the potential capacity to process and present antigen. The loss of augmented IL-17 and IL-13 production when DCs and cytoplasts were separated in culture by transwell suggests that antigen presentation via MHCII during cytoplast contact with DCs is a potential mechanism for cytoplast-mediated DC programming of T cell cytokine production. One caveat is that adherent NET fragments on cytoplasts cannot be entirely excluded. If present, cytoplast-associated surface DNA would be another potential mechanism for cell activation. DC-SIGN expression on cytoplasts was low, so the mechanisms for T cell skewing to IL-17 production were likely independent of DC-SIGN. The ability of neutrophil cytoplasts to trigger a regulated T_H17-driven immune response is reminiscent of previous reports that infected apoptotic cells can also promote T_H17 differentiation (34).

To translate the murine findings to human asthma, we studied BAL samples from comprehensively phenotyped healthy and asthmatic individuals. DNA, including hypercitrullinated histones consistent with NETs, was present in a subset of severe asthmatic patients with high BAL neutrophil counts. In SA, BALF levels of DNA correlated to BAL neutrophils. Neutrophil cytoplasts were also detected in SA BAL with high neutrophil counts. BAL neutrophils and cytoplasts correlated with BALF levels of IL-17. Compared with asthmatics with lower BAL neutrophil counts, the asthma patients with increased BAL neutrophils and NETosis were associated with frequent exacerbations and sinusitis—asthma comorbidities linked to infections. Together, these findings support the notion that a subset of severe asthmatic patients has neutrophil-enriched inflammatory responses that are driven by cytoplast skewing to T_H17 adaptive inflammation. Several studies have identified NETs in biospecimens from asthmatic patients (35), and some have linked the DNA to type 2 responses (36). In sharp contrast, intact neutrophil cytoplasts after NETosis have not been previously detected in human asthma or after murine responses to allergen. Neutrophil cytoplasts have been detected after NETosis in vivo with murine Gram-positive bacterial infection and in human Gram-positive abscesses (9), suggesting that the findings here have uncovered a mechanistic link between innate and adaptive immune responses that is relevant more broadly to neutrophilic diseases. Many severe asthmatic patients do not benefit from anti-inflammatory therapy with corticosteroids, which can prolong neutrophil survival and delay their clearance

from asthmatic lungs (37, 38). A phase 2a anti-IL-17 receptor A antibody clinical trial did not demonstrate improvement in asthma symptoms in moderate to severe asthmatics (39); however, stratification of the asthmatic patients by neutrophils and IL-17 levels was not performed. Markers of NETosis, such as DNA-citrullinated histones or neutrophil cytoplasts in sputum, may provide an opportunity for increased precision or alternate therapeutic strategies to disrupt IL-17 pathways in subsets of asthma patients in future clinical research.

In summary, during allergen-mediated responses, the presence of endotoxin can trigger lung NETosis with functional roles for the enucleated cytoplasts to convey specific signals of neutrophil activation that can serve as pivotal effectors for initiation of T_H17 differentiation in a transition from innate to adaptive immune responses. Cell-cell interactions between neutrophil cytoplasts and DCs are spatiotemporally regulated and can elicit antigen-specific T cell responses with direct relevance to disease pathogenesis for some patients with SA and non-type 2 inflammation. Our translational results also suggest a broader role for NETosis-derived cytoplasts in initiating T_H17 -driven adaptive immune responses to pathogens that are crucial to host defense.

MATERIALS AND METHODS

Mice

Balb/c mice used for the asthma model were purchased from Charles River Laboratories. C57BL/6 mice, C.Cg-Tg(DO11.10)10Dlo/J mice, and control BALB/c mice were purchased from the Jackson Laboratory. PAD4 KO mice (C57BL/6 background) (22) were obtained as a gift from Y. Wang's laboratory and were bred at Boston Children's Hospital. The mice were backcrossed for more than 10 generations on the C57BL/6 background. Unless otherwise stated, age-matched male animals were used at 8 to 12 weeks of age. The animal protocols were approved by the Animal Review Committee at Harvard Medical School and Boston Children's Hospital.

Fifteen-day model of allergic lung inflammation

For the intranasal instillations, animals were sedated using isoflurane carried by oxygen. For allergen sensitization, 25 μ g of HDM (B70 source material, Greer Laboratories) with or without 1 μ g of LPS (*E. coli* O55:B5, Sigma-Aldrich) was instilled (intranasally) in 30 μ l of normal saline once daily for 3 days (days 0 to 2). In some experiments, mice were sacrificed on day 3, and BAL was performed using PBS containing 0.6 mM EDTA. MLNs were also harvested on day 3 from some mice for analyses.

In other experiments, DNase (500 μ g per mouse in 50 μ l of PBS) or PBS control was instilled intranasally 6 hours after each sensitization (days 0 to 2). For allergen challenge, mice were rested for 4 days after sensitization (days 3 to 6). In some experiments, MLNs were harvested on day 7 to study T lymphocyte differentiation. Beginning on day 7, 25 μ g of HDM in 25 μ l of saline was instilled (intranasally) once daily for 8 days (days 7 to 14). Mice were then sacrificed 24 hours later on day 15, BAL was performed, and MLNs were harvested for analyses. BALFs were centrifuged (400g, 10 min). Cell pellets were resuspended for analysis by flow cytometry or cytospin and staining with Diff-Quik stain to determine leukocyte differential counts.

In the anti-IL-17 experiments, two doses of anti-IL-17A (clone eBioMM17F3, Thermo Fisher Scientific) or control rat immunoglobulin G were administered to the mice (80 μ g, 100 μ l) via the intraperitoneal

route 24 hours before the first sensitization with HDM/LPS (protocol day -1) and then during HDM/LPS sensitization (protocol day 1; fig. S3A). The sensitization phase was completed with HDM/LPS, followed by challenge with HDM. Twenty-four hours after the last HDM challenge, the cellular profile in the BALF was analyzed.

For AHR, the protocol was performed as described previously (40). Briefly, anesthetized mice were mechanically ventilated using a flexiVent (SCIREQ), and aerosolized methacholine (0, 3, 10, and 30 mg/ml) was delivered in-line via the inhalation port for 10 s. Lung resistance was calculated to the baseline dose of methacholine (dose 0, PBS control).

Cell-free supernatants were subjected to analysis by Quant-iT PicoGreen double-stranded DNA quantitation assay (Thermo Fisher Scientific) or immunoblot analysis for citH3 to detect NETs. For Western blot, rabbit polyclonal antibody to histone H3 (citrulline 2 + 8 + 17, Abcam) was used. For MLN T lymphocyte analysis, MLNs were dissociated to achieve single-cell suspensions using a 5-ml polypropylene round-bottom tube with a 0.3- μ m filter cap. Harvested MLNs were crushed using the plunger of 1-ml syringe and ice-cold PBS with 2% heat-inactivated fetal bovine serum (FBS). Cells from day 3 mice were then counted and analyzed for neutrophil and cytoplast infiltration. MLN cells harvested from day 7 mice were counted, and 2×10^6 cells in 2 ml of RPMI with 10% FBS, 1 mM Na pyruvate, $1 \times$ penicillin/streptomycin mix, and gentamicin (5 μ g/ml). MLN cells were then restimulated with phorbol 12-myristate 13-acetate (PMA; 50 ng/ml), ionomycin (500 ng/ml), and GolgiStop for 96 hours at 37°C in a humidified incubator with 5% CO₂. Cells were then harvested, dissociated, and stained for intracellular cytokine staining using the FoxP3 Staining Kit (eBioscience), as per the manufacturer's protocol.

Flow cytometry

Cell suspensions were stained in ice-cold PBS with or without 2% FBS on ice. The following antibodies were used for staining mouse cells: CD45 (clone 30-F11, BioLegend), Ly6G (clone 1A8, BD Biosciences), CD11c (clone HL3, BD Biosciences), CD11b (clone M1/70, BioLegend), Siglec F (clone E50-2440, BD Biosciences), CD3e (clone 145-2C11, eBioscience), CD4 (clone GK1.5, BioLegend), IFN (clone XMG1.2, BioLegend), IL-17 (clone eBio17B7, eBioscience), IL-5 (clone TRFK5, BioLegend), IL-13 (clone eBio13A, eBioscience), CD25 (clone PC61.5, eBioscience), CD44, CD62L (clone MEL-14, BD Biosciences), MHCII I-A/I-E (clone M5/114.15.2, eBioscience), DC-SIGN/CD209 (R&D Systems), CD32 (clone D34-485, eBioscience), TLR4/CD284 (clone SA15-21, BioLegend), and annexin V (BioLegend). Neutrophils were defined as CD45⁺CD11b⁺Ly6G⁺DNA⁺, and cytoplasts were defined as CD45⁺CD11b⁺Ly6G⁺DNA⁻. Naïve T lymphocytes were defined as CD4⁺CD25⁻CD44^{low}CD62L^{hi}. Lung DCs were defined as CD45⁺CD11c⁺MHCII⁺autofluorescence^{low}. The following antibodies were used to stain human cells: anti-CD45 PE-Cy7 (HI30), anti-CD66b (G10F5), and anti-CD16 APC-Cy7 (3G8) (all from BioLegend). Vybrant DyeCycle Ruby (Life Technologies) was used to stain intracellular DNA. Data were acquired on BD FACSCanto II or BD LSRFortessa and analyzed using FlowJo v10. For cell sorting, FACSARIA was used.

Neutrophil/cytoplast, DC, and T cell coculture assay

Two cohorts of mice were sensitized with HDM/LPS and HDM/Veh. Mice were sacrificed on day 3. BAL was performed in one cohort, and CD45⁺CD11b⁺Ly6G⁺DNA⁺ neutrophils and CD45⁺CD11b⁺Ly6G⁺DNA⁻

cytoplasts were flow-sorted from BALFs. From second cohort, harvested lungs were dissociated, and CD45⁺CD11c⁺MHCII⁺ DCs were flow-sorted. These DCs were then cocultured with either cytoplasts (DC/cytoplast cell ratios of 1:0.5 or 1:2) or neutrophils (DC/neutrophil cell ratio of 1:0.5, 1:2, or 1:10) overnight. The next day, the spleens of DO11.10 were harvested and crushed using a 70- μ m cell strainer and a plunger from a 5-ml syringe with ice-cold PBS with 2% FBS to obtain single-cell suspension. Cells were then centrifuged (800g, 6 min, 4°C). Red blood cells (RBCs) were then lysed using RBC lysis buffer (eBioscience), as per the manufacturer's instructions. Cells were then washed and resuspended in PBS with 2% FBS, stained, and sorted for CD4⁺CD25⁻CD44^{lo}CD62L^{hi} naïve T lymphocytes. Naïve T lymphocytes were then added to the DCs (T cells/DC, 10:1), together with ovalbumin peptide (5 μ g/ml). In select experiments, the cytoplasts were separated from the DC/T cells by transwell. The cytoplasts were added to the transwell, allowing the flow of the media but preventing the cytoplasts from directly interacting with DCs and T cells. In addition, conditioned supernatant from cytoplast culture for 24 hours was added to the DC/T cell coculture to determine the potential role of soluble factors from cytoplasts in T cell differentiation. The cells were then cultured for 96 hours, and T cell differentiation was assessed by restimulating with PMA (50 ng/ml), ionomycin (500 ng/ml), and GolgiStop. Cells were then harvested, dissociated, and stained for intracellular cytokine staining using the FoxP3 Staining Kit (eBioscience), as per the manufacturer's protocol.

Design and fabrication of the microfluidic assay

The in vitro microfluidic assay enables investigation of chemotaxis of neutrophils and cytoplasts with high temporal and spatial resolution. It has three key components: (i) cell loading channel, (ii) chemoattractant chambers, and (iii) an array of migration channels, which bridge the first two components. The cell loading channel and the chemoattractant chambers have a dimension of 200 μ m $\times$ 100 μ m ($h \times w$). The migration channels have a dimension of 10 μ m $\times$ 10 μ m $\times$ 500 μ m ($h \times w \times l$). Each device has 10 identical units with a total number of 90 migration channels, which allows observation of hundreds of cells in one experiment.

Device priming

First, the microfluidic device was placed in a desiccator under vacuum for 20 min to generate a negative pressure in the device. Immediately after the degassing process, 5 μ l of complete RPMI containing 100 nM LTB₄ (Cayman Chemical, Ann Arbor, MI) and 100 nM fibronectin (R&D Systems, Minneapolis, MN) was introduced into the cell loading channel through the inlet port using a pipette. The temporary negative pressure pulled the media into the migration channels and chemoattractant chambers filling the device within 10 min. The cell loading channel was then washed with 10 μ l of fresh media to establish a chemogradient from the chemoattractant chambers (highest chemokine concentration) to the cell loading channel (lowest chemokine concentration). One microliter of mice neutrophils or cytoplasts at a concentration of 2×10^7 /ml was introduced into a device. Afterward, the device was immersed by pipetting 4 ml of media into the well and was ready for the following time-lapse imaging.

Time-lapse imaging and analysis

The six-well plate was mounted on a Nikon Eclipse Ti microscope equipped with a biochamber to maintain the temperature and gas conditions at 37°C and 5% CO₂. For each condition, 20 images were taken at different locations in two identical devices using a 10 $\times$ lens

in bright field. The experiment was imaged for 6 hours with a 3-min interval between two imaging cycles. The sizes of neutrophils and cytoplasts were measured using Fiji ImageJ. The recruitment of neutrophils or cytoplasts was analyzed by counting the number of cells that entered the migration channels. The velocities and trajectories of neutrophil chemotaxis and cytoplast chemokinesis were measured using TrackMate in Fiji ImageJ.

Phagocytosis assay

Neutrophils and cytoplasts were cell-sorted (see cell sorter methods) and plated on serum-coated slides (10⁵ cells per slide). The cells were allowed to adhere to the slides at 37°C for 1 hour. *E. coli* particles conjugated to pHrodo Red dye (100 μ g/ml; Life Technologies) were added to neutrophils and cytoplasts. Slides were captured with Zeiss epifluorescence microscopy with filter sets for indicator dye of pHrodo Red (green excitation) or DAPI (blue excitation) 60 min after the addition of *E. coli* particles. Total cell count was obtained by enumerating cells on light microscopy. Phagocytosis index was determined by counting the number of cells stained positive for pHrodo Red relative to total cells.

Bacterial killing

Frozen stocks of *S. pneumoniae* were plated and grown for 12 hours at 37°C and 5% CO₂. Fresh colonies were grown in 10 ml of Todd Hewitt Broth (0.5% of yeast extract) until log phase [OD₆₀₀ (optical density at 600 nm) = 0.4 arbitrary units; $\sim 1.5 \times 10^9$ colony-forming units (CFU)]. After centrifugation (2000 rpm, 20 min), cells were washed with sterile PBS and resuspended in 1 ml of RPMI with 5% FBS. Sorted neutrophils or cytoplasts were incubated with 1 ml of solution of log-phase *S. pneumoniae* serotype 1 with a multiplicity of infection of 2 for 30, 60, and 120 min at 37°C and 5% CO₂. At each time point, 50 μ l of the culture supernatant was serially diluted and plated on blood agar plates. The plates were incubated overnight at 37°C and 5% CO₂, and after counting the colonies, results were expressed as CFU of *S. pneumoniae* per milliliter.

Human patients

Adult patients 18 years of age and older with asthma and healthy controls were recruited to the SARP-3 (NCT01606826) between November 2012 and October 2014 by seven research centers in the United States. Patients were defined as having SA or NSA, as defined by the European Respiratory Society/American Thoracic Society guidelines (41). Healthy individuals were nonsmokers with no history of lung disease, atopic disease, or allergic rhinitis. Written informed consent was obtained after institutional review board approval at each site. BAL was performed by instilling warm saline (three 50 ml of aliquots) into the right middle lobe. BALF was recovered, and BAL cells and cell-free BAL supernatant were frozen and stored at -80°C or liquid nitrogen and later shipped to Brigham and Women's Hospital for analysis. Patient demographic information is provided in table S1.

BALF IL-17 measurement

IL-17 was measured in cell-free BALF from asthma patients using a flow cytometry bead-based immunoassay (LEGENDplex, BioLegend).

Statistics

Results are expressed as mean $\pm$ SEM or mean $\pm$ SD, as stated in the figure legends. Statistical differences were calculated by two-tailed

unpaired Student's *t* test between two groups and by parametric one-way analysis of variance (ANOVA) between three or more groups (human cohorts). For non-normal data distribution, a Mann-Whitney *U* test or nonparametric one-way ANOVA was used. Correlations were evaluated by Pearson's correlation coefficient (*r*). For table S2, comparisons between categorical variables were assessed by χ^2 test. *P* < 0.05 was defined as statistically significant.

SUPPLEMENTARY MATERIALS

immunology.sciencemag.org/cgi/content/full/3/26/eaao4747/DC1

Fig. S1. A mouse model of neutrophilic airway inflammation is associated with the neutrophil recruitment and T_H17 activation during sensitization.

Fig. S2. Gating strategy to identify neutrophils and cytoplasts.

Fig. S3. Anti-IL-17 administration during the sensitization phase blunts neutrophilia without affecting cytoplast generation.

Fig. S4. Protease-free DNase does not affect neutrophilia.

Fig. S5. Citrullinated histone identified on neutrophils from WT mice.

Fig. S6. Cytoplast promotes T cell responses via contact-dependent mechanism.

Fig. S7. Gating strategies for detection of cytoplasts in human BALF from severe asthmatics.

Table S1. Patient characteristics.

Table S2. Neutrophil and DNA high asthma patient symptoms, lung function, and comorbidities.

Movie S1. Murine lung neutrophils were flow-sorted after HDM/LPS treatment (3-day exposure), and chemotaxis to LTB₄ was monitored in a microfluidic chamber.

Movie S2. Murine lung cytoplasts were flow-sorted after HDM/LPS treatment (3-day exposure), and chemotaxis to LTB₄ was monitored in a microfluidic chamber.

REFERENCES AND NOTES

- C. H. Fanta, Asthma. *N. Engl. J. Med.* **360**, 1002–1014 (2009).
- S. T. Holgate, Innate and adaptive immune responses in asthma. *Nat. Med.* **18**, 673–683 (2012).
- B. D. Levy, P. J. Noel, M. M. Freemer, M. M. Cloutier, S. N. Georas, N. N. Jarjour, C. Ober, P. G. Woodruff, K. C. Barnes, B. G. Bender, C. A. Camargo Jr., G. L. Chupp, L. C. Denlinger, J. V. Fahy, A. M. Fitzpatrick, A. Fuhlbrigge, B. M. Gaston, T. V. Hartert, J. K. Kolls, S. V. Lynch, W. C. Moore, W. J. Morgan, K. C. Nadeau, D. R. Ownby, J. Solway, S. J. Szefer, S. E. Wenzel, R. J. Wright, R. A. Smith, S. C. Erzurum, Future research directions in asthma. An NHLBI working group report. *Am. J. Respir. Crit. Care Med.* **192**, 1366–1372 (2015).
- S. E. Wenzel, Asthma phenotypes: The evolution from clinical to molecular approaches. *Nat. Med.* **18**, 716–725 (2012).
- J. F. Alcorn, C. R. Crowe, J. K. Kolls, T_H17 cells in asthma and COPD. *Annu. Rev. Physiol.* **72**, 495–516 (2010).
- W. Al-Ramli, D. Préfontaine, F. Chouiali, J. G. Martin, R. Olivenstein, C. Lemiere, Q. Hamid, T_H17-associated cytokines (IL-17A and IL-17F) in severe asthma. *J. Allergy Clin. Immunol.* **123**, 1185–1187 (2009).
- D. C. Newcomb, J. Y. Cephus, M. G. Boswell, J. M. Fahrenholz, E. W. Langley, A. S. Feldman, W. Zhou, D. E. Dulek, K. Goleniewska, K. B. Woodward, C. M. Sevin, R. G. Hamilton, J. K. Kolls, R. S. Peebles Jr., Estrogen and progesterone decrease *let-7f* microRNA expression and increase IL-23/IL-23 receptor signaling and IL-17A production in patients with severe asthma. *J. Allergy Clin. Immunol.* **136**, 1025–1034 e11 (2015).
- Y.-H. Wang, M. Wills-Karp, The potential role of interleukin-17 in severe asthma. *Curr. Allergy Asthma Rep.* **11**, 388–394 (2011).
- B. G. Yipp, B. Petri, D. Salina, C. N. Jenne, B. N. V. Scott, L. D. Zbytniuk, K. Pittman, M. Asaduzzaman, K. Wu, H. C. Meijndert, S. E. Malawista, A. de Boisleury Chevance, K. Zhang, J. Conly, P. Kubes, Infection-induced NETosis is a dynamic process involving neutrophil multitasking in vivo. *Nat. Med.* **18**, 1386–1393 (2012).
- S. E. Malawista, G. Van Blaricom, Phagocytic capacity of cytokine-plasts from human blood polymorphonuclear leukocytes. *Blood Cells* **12**, 167–177 (1986).
- A. M. Rich, K. N. Giedd, P. Cristello, G. Weissmann, Granules are necessary for death of neutrophils after phagocytosis of crystalline monosodium urate. *Inflammation* **9**, 221–232 (1985).
- D. Roos, A. A. Voetman, L. J. Meerhof, Functional activity of enucleated human polymorphonuclear leukocytes. *J. Cell Biol.* **97**, 368–377 (1983).
- V. Brinkmann, A. Zychlinsky, Neutrophil extracellular traps: Is immunity the second function of chromatin? *J. Cell Biol.* **198**, 773–783 (2012).
- A. Caudrillier, K. Kessenbrock, B. M. Gilliss, J. X. Nguyen, M. B. Marques, M. Monestier, P. Toy, Z. Werb, M. R. Looney, Platelets induce neutrophil extracellular traps in transfusion-related acute lung injury. *J. Clin. Invest.* **122**, 2661–2671 (2012).
- G. M. Thomas, C. Carbo, B. R. Curtis, K. Martinod, I. B. Mazo, D. Schatzberg, S. M. Cifuni, T. A. Fuchs, U. H. von Andrian, J. H. Hartwig, R. H. Aster, D. D. Wagner, Extracellular DNA traps are associated with the pathogenesis of TRALI in humans and mice. *Blood* **119**, 6335–6343 (2012).
- M. M. Stein, C. L. Hrusch, J. Gozdz, C. Igartua, V. Pivniouk, S. E. Murray, J. G. Ledford, M. Marques dos Santos, R. L. Anderson, N. Metwali, J. W. Neilson, R. M. Maier, J. A. Gilbert, M. Holbreich, P. S. Thorne, F. D. Martinez, E. von Mutius, D. Vercelli, C. Ober, A. I. Sperling, Innate immunity and asthma risk in Amish and Hutterite farm children. *N. Engl. J. Med.* **375**, 411–421 (2016).
- M. Arora, S. L. Poe, T. B. Oriss, N. Krishnamoorthy, M. Yarlagadda, S. E. Wenzel, T. R. Billiar, A. Ray, P. Ray, TLR4/MyD88-induced CD11b⁺Gr-1^{int} F4/80⁺ non-migratory myeloid cells suppress Th2 effector function in the lung. *Mucosal Immunol.* **3**, 578–593 (2010).
- H.ammad, M. Chieppa, F. Perros, M. A. Willart, R. N. Germain, B. N. Lambrecht, House dust mite allergen induces asthma via Toll-like receptor 4 triggering of airway structural cells. *Nat. Med.* **15**, 410–416 (2009).
- S. R. Clark, A. C. Ma, S. A. Tavener, B. McDonald, Z. Goodarzi, M. M. Kelly, K. D. Patel, S. Chakrabarti, E. McAvoy, G. D. Sinclair, E. M. Keys, E. Allen-Vercor, R. Devinney, C. J. Doig, F. H. Y. Green, P. Kubes, Platelet TLR4 activates neutrophil extracellular traps to ensnare bacteria in septic blood. *Nat. Med.* **13**, 463–469 (2007).
- D. N. Doua, R. Jackson, H. Grasmann, N. Palaniyar, Innate immune collectin surfactant protein D simultaneously binds both neutrophil extracellular traps and carbohydrate ligands and promotes bacterial trapping. *J. Immunol.* **187**, 1856–1865 (2011).
- L. E. Noges, J. White, J. C. Cambier, J. W. Kappler, P. Marrack, Contamination of DNase preparations confounds analysis of the role of DNA in alum-adjuvanted vaccines. *J. Immunol.* **197**, 1221–1230 (2016).
- P. Li, M. Li, M. R. Lindberg, M. J. Kennett, N. Xiong, Y. Wang, PAD4 is essential for antibacterial innate immunity mediated by neutrophil extracellular traps. *J. Exp. Med.* **207**, 1853–1862 (2010).
- Y. Wang, M. Li, S. Stadler, S. Correll, P. Li, D. Wang, R. Hayama, L. Leonelli, H. Han, S. A. Grigoryev, C. D. Allis, S. A. Coonrod, Histone hypercitrullination mediates chromatin decondensation and neutrophil extracellular trap formation. *J. Cell Biol.* **184**, 205–213 (2009).
- A. Ray, M. Raundhal, T. B. Oriss, P. Ray, S. E. Wenzel, Current concepts of severe asthma. *J. Clin. Invest.* **126**, 2394–2403 (2016).
- J. L. Simpson, T. V. Grissell, J. Douwes, R. J. Scott, M. J. Boyle, P. G. Gibson, Innate immune activation in neutrophilic asthma and bronchiectasis. *Thorax* **62**, 211–218 (2007).
- J. V. Fahy, Type 2 inflammation in asthma — Present in most, absent in many. *Nat. Rev. Immunol.* **15**, 57–65 (2015).
- P. Gao, P. G. Gibson, K. J. Baines, I. A. Yang, J. W. Upham, P. N. Reynolds, S. Hodge, A. L. James, C. Jenkins, M. J. Peters, J. Zhang, J. L. Simpson, Anti-inflammatory deficiencies in neutrophilic asthma: Reduced galectin-3 and IL-1RA/IL-1β. *Respir. Res.* **16**, 5 (2015).
- H. Parfrey, N. Farahi, L. Porter, E. R. Chilvers, Live and let die: Is neutrophil apoptosis defective in severe asthma? *Thorax* **65**, 665–667 (2010).
- P. Chenuet, L. Fauconnier, F. Madouri, T. Marchiol, N. Rouxel, A. Ledru, P. Mauny, R. Lory, C. Uttenhove, J. van Snick, Y. Iwakura, F. di Padova, V. Quesniaux, D. Togbe, B. Ryffel, Neutralization of either IL-17A or IL-17F is sufficient to inhibit house dust mite induced allergic asthma in mice. *Clin. Sci.* **131**, 2533–2548 (2017).
- S. E. Malawista, R. R. Montgomery, G. van Blaricom, Evidence for reactive nitrogen intermediates in killing of staphylococci by human neutrophil cytoplasts. A new microbicidal pathway for polymorphonuclear leukocytes. *J. Clin. Invest.* **90**, 631–636 (1992).
- S. E. Malawista, R. R. Montgomery, G. Van Blaricom, Microbial killing by human neutrophil cytokine-plasts: Similar suppressive effects of reversible and irreversible inhibitors of nitric oxide synthase. *J. Leukoc. Biol.* **60**, 753–757 (1996).
- S. E. Malawista, G. Van Blaricom, M. G. Breitenstein, Cryopreservable neutrophil surrogates. Stored cytoplasts from human polymorphonuclear leukocytes retain chemotactic, phagocytic, and microbicidal function. *J. Clin. Invest.* **83**, 728–732 (1989).
- M. Merad, P. Sathe, J. Helft, J. Miller, A. Mortha, The dendritic cell lineage: Ontogeny and function of dendritic cells and their subsets in the steady state and the inflamed setting. *Annu. Rev. Immunol.* **31**, 563–604 (2013).
- M. B. Torchinsky, J. Garaude, A. P. Martin, J. M. Blander, Innate immune recognition of infected apoptotic cells directs T_H17 cell differentiation. *Nature* **458**, 78–82 (2009).
- T. K. Wright, P. G. Gibson, J. L. Simpson, V. M. McDonald, L. G. Wood, K. J. Baines, Neutrophil extracellular traps are associated with inflammation in chronic airway disease. *Respirology* **21**, 467–475 (2016).
- M. Toussaint, D. J. Jackson, D. Swieboda, A. Guedán, T.-D. Tsourouktsoglou, Y. M. Ching, C. Radermecker, H. Makrinioti, J. Aniskenko, N. W. Bartlett, M. R. Edwards, R. Solari, F. Farnir, V. Papayannopoulos, F. Bureau, T. Marichal, S. L. Johnston, Host DNA released by NETosis promotes rhinovirus-induced type-2 allergic asthma exacerbation. *Nat. Med.* **23**, 681–691 (2017).
- M. G. Duval, C. Barnig, M. Cernadas, I. Ricklefs, N. Krishnamoorthy, N. L. Grossman, N. R. Bhakta, J. V. Fahy, E. R. Bleeker, M. Castro, S. C. Erzurum, B. M. Gaston, N. N. Jarjour, D. T. Mauger, S. E. Wenzel, S. A. Comhair, A. M. Coverstone, M. L. Fajt, A. T. Hastie,

- M. W. Johansson, M. C. Peters, B. R. Phillips, E. Israel, B. D. Levy, Natural killer cell-mediated inflammation resolution is disabled in severe asthma. *Sci. Immunol.* **2**, eaam5446 (2017).
38. M. Wang, P. Gao, X. Wu, Y. Chen, Y. Feng, Q. Yang, Y. Xu, J. Zhao, J. Xie, Impaired anti-inflammatory action of glucocorticoid in neutrophil from patients with steroid-resistant asthma. *Respir. Res.* **17**, 153 (2016).
 39. W. W. Busse, S. Holgate, E. Kerwin, Y. Chon, J. Feng, J. Lin, S.-L. Lin, Randomized, double-blind, placebo-controlled study of brodalumab, a human anti-IL-17 receptor monoclonal antibody, in moderate to severe asthma. *Am. J. Respir. Crit. Care Med.* **188**, 1294–1302 (2013).
 40. O. Haworth, M. Cernadas, R. Yang, C. N. Serhan, B. D. Levy, Resolvin E1 regulates interleukin 23, interferon- γ and lipoxin A₄ to promote the resolution of allergic airway inflammation. *Nat. Immunol.* **9**, 873–879 (2008).
 41. K. F. Chung, S. E. Wenzel, J. L. Brozek, A. Bush, M. Castro, P. J. Sterk, I. M. Adcock, E. D. Bateman, E. H. Bel, E. R. Bleeker, L.-P. Boulet, C. Brightling, P. Chanez, S.-E. Dahlen, R. Djukanovic, U. Frey, M. Gaga, P. Gibson, Q. Hamid, N. N. Jajour, T. Mauad, R. L. Sorkness, W. G. Teague, International ERS/ATS guidelines on definition, evaluation and treatment of severe asthma. *Eur. Respir. J.* **43**, 343–373 (2014).

Acknowledgments: We thank G. Zhu for the technical support. We thank Y. Qui and Y.-D. Lin for the technical help with flow cytometry sorting of cells. **Funding:** The work was supported, in part, by the National Heart, Lung, and Blood Institute (E.I. and B.D.L., U10 HL109172; E.R.B., U10 HL109164; M. Castro, U10 HL109257; S.C.E., U10 HL109250; J.V.F., U10 HL109146; B.M.G., U10 HL109250; N.N.J., U10 HL109168; S.W., U10 HL109152; D.T.M., U10 HL109086) and by R01GM092804 (to D.I.), R35HL135765 (to D.D.W.) and R01HL122531 (to B.D.L.). In addition, this program is supported through NIH National Center for Advancing Translational Sciences awards (UL1 TR001420 to Wake Forest University, UL1 TR000427 to the University of

Wisconsin, UL1 TR001102 to Harvard University, and UL1 TR000454 to Emory University); a Canadian Institutes of Health Research postdoctoral fellowship (to D.N.D.), K12 HD047349 (to M.G.D.), and K08 HL130540 (to R.-E.E.A.); and a fellowship from the German Society for Pediatric Pneumology (to I.R.). **Author contributions:** N.K., D.N.D., T.R.B., I.R., M.G.D., L.T., R.-E.E.A., X.W., and D.I. designed and performed the experiments, analyzed the data, and wrote the manuscript. N.K., D.N.D., T.R.B., I.R., M.G.D., L.T., R.-E.E.A., and X.W. performed the statistical analysis. K.M. and D.D.W. provided the PAD4 KO mice, analyzed the data, and wrote the manuscript. M. Castro, E.I., D.T.M., E.R.B., M. Cernadas, S.C.E., B.M.G., N.N.J., S.W., E.D., and J.V.F. collected the specimens, analyzed the data, and wrote the manuscript. B.D.L. conceived the study, designed the experiments, analyzed the data, and wrote the manuscript. **Competing interests:** The authors declare that they have no competing interests related to the publication of this manuscript. **Data and materials availability:** PAD4 KO mice are available from Yanming Wang under a material agreement with Penn State University. All data supporting the findings of this study are available within the article.

Submitted 25 July 2017

Resubmitted 9 March 2018

Accepted 5 July 2018

Published 3 August 2018

10.1126/sciimmunol.aao4747

Citation: N. Krishnamoorthy, D. N. Doua, T. R. Brüggemann, I. Ricklefs, M. G. Duvall, R.-E. E. Abdulnour, K. Martinod, L. Tavares, X. Wang, M. Cernadas, E. Israel, D. T. Mauger, E. R. Bleeker, M. Castro, S. C. Erzurum, B. M. Gaston, N. N. Jarjour, S. Wenzel, E. Dunican, J. V. Fahy, D. Irimia, D. D. Wagner, B. D. Levy, National Heart, Lung, and Blood Institute Severe Asthma Research Program-3 Investigators, Neutrophil cytoplasts induce T_H17 differentiation and skew inflammation toward neutrophilia in severe asthma. *Sci. Immunol.* **3**, eaao4747 (2018).

Neutrophil cytoplasts induce T_H17 differentiation and skew inflammation toward neutrophilia in severe asthma

Nandini Krishnamoorthy, David N. Douma, Thayse R. Brüggemann, Isabell Ricklefs, Melody G. Duvall, Raja-Elie E. Abdounour, Kimberly Martinod, Luciana Tavares, Xiao Wang, Manuela Cernadas, Elliot Israel, David T. Mauger, Eugene R. Bleecker, Mario Castro, Serpil C. Erzurum, Benjamin M. Gaston, Nizar N. Jarjour, Sally Wenzel, Eleanor Dunican, John V. Fahy, Daniel Irimia, Denisa D. Wagner, Bruce D. Levy and National Heart, Lung, and Blood Institute Severe Asthma Research Program-3 Investigators

Sci. Immunol. **3**, eaao4747.
DOI: 10.1126/sciimmunol.aao4747

Nefarious neutrophil cytoplasts

In addition to DNA release, neutrophil extracellular trap (NET) formation can result in enucleated cells called cytoplasts. Krishnamoorthy *et al.* examined how neutrophil cytoplasts contribute to asthmatic inflammation in mouse models of allergic lung inflammation and in asthmatic patients. Airway exposure of mice to LPS with house dust mite allergen induced NET formation in the lung that was associated with IL-17 production upon subsequent exposure to allergen. Cytoplasts and not neutrophil DNA released in NETosis triggered neutrophilia upon allergen exposure, and cytoplasts alone were sufficient to induce IL-17 production by antigen-specific T cells. Cytoplasts also correlated with IL-17 levels in bronchoalveolar lavage fluid from severe asthmatics. These findings provide insight into how neutrophil cytoplasts can contribute to asthma severity.

ARTICLE TOOLS

<http://immunology.sciencemag.org/content/3/26/eaao4747>

SUPPLEMENTARY MATERIALS

<http://immunology.sciencemag.org/content/suppl/2018/08/02/3.26.eaao4747.DC1>

REFERENCES

This article cites 41 articles, 11 of which you can access for free
<http://immunology.sciencemag.org/content/3/26/eaao4747#BIBL>

Use of this article is subject to the [Terms of Service](#)