ASTHMA

Neutrophil ghosts worsen asthma

Marsha Wills-Karp

Neutrophil cytoplasts are linked to amplification of T helper 17–mediated inflammation and severe asthma.

Asthma is a heterogeneous inflammatory disease that plagues modern societies. The disease often differs in severity of airway obstruction and symptoms, frequency of exacerbations and hospitalizations, treatment responses, and inflammatory profiles. Whereas most asthmatics present with eosinophilic inflammation, a subset of patients with a neutrophilic inflammatory profile may present with a more severe disease that is resistant to corticosteroid therapy.

Despite the clinical importance of severe asthma, there is a limited understanding of the drivers of neutrophilic inflammation and the mechanisms by which polymorphonuclear cells (PMNs, or neutrophils) may worsen asthma symptoms. In this issue of *Science Immunology*, Krishnamoorthy *et al.* (*1*) provide new and unexpected insight into the causal link between neutrophil cytoplasts and severe asthma that could fuel development of new therapies.

Asthma has traditionally been associated with aberrant T helper 2 (T_H2) immune responses to innocuous environmental antigens, resulting in eosinophilic inflammatory responses in the lung, impaired lung function [reductions in forced expiratory volume (FEV1)], and excessive mucus production (*2*). However, recent unbiased cluster analyses of cellular and clinical data have revealed the existence of both T_H2-high [interleukin-13 (IL-13), IL-5, and IL-4] and T_H2-low endotypes (*3*). The T_H2-low endotypes have been associated with either mixed neutrophilic/eosinophilic or predominantly neutrophilic pulmonary inflammation. Neutrophilic counts and the levels of the neutrophil chemoattractant IL-8 have been reported to be the only biomarkers that distinguish severe or moderate asthma from mild asthma (*4*). The T_H17-derived cytokine IL-17A, which is a potent regulator of neutrophil recruitment, has been implicated in severe disease pathology and steroid-resistant airway inflammation (*5*). Together, these findings underscore the need to better understand the mechanisms driving these aberrant neutrophilic inflammatory responses, with the ultimate goal of the development of alternative therapies to improve clinical outcomes.

Neutrophils are the most abundant type of leukocyte in peripheral human blood and have long been known to wield a double-edged sword as both pathogen killers and maestros of resolution and tissue repair. They serve as a critical frontline defense against invading pathogens by directly phagocytosing their targets or by releasing toxic components via degranulation. PMNs are traditionally thought of as short-lived, terminally differentiated cells, but they can persist in inflamed tissues through the actions of multiple survival factors and cause tissue damage. As such, the recruitment and activation of neutrophils are tightly regulated to balance their powerful effector functions with their potential to destroy tissue.

Our understanding of neutrophil biology has been advanced by the discovery of neutrophil extracellular trap (NET) formation, a process by which neutrophils externalize weblike chromatin strands decorated with antimicrobial peptides, proteases, and cytotoxic enzymes (*6*). Like PMNs themselves, NETs can entrap and kill microbes and initiate inflammatory responses.

The formation of NETs (a process referred to as NETosis) is stimulated by a variety of microbes [fungi, viruses, bacteria, and bacterial components such as lipopolysaccharide (LPS)] and host factors [granulocyte-macrophage colony-stimulating factor (GM-CSF), IL-8, and C5a], which causes the neutrophil nuclear envelope to disintegrate to allow mixing of chromatin with granular proteins. NETosis requires the presence of enzymes from neutrophil granules [myeloperoxidase (MPO), neutrophil elastase (NE), and peptidyl arginine deiminase (PAD4)]. NE and MPO degrade histones and promote chromatin decondensation, and PAD4 mediates chromatin decondensation by hypercitrullinating positively charged arginines on specific histones. Unlike cells undergoing apoptosis, PMNs do not necessarily die after extrusion of their DNA during NETosis. Instead, PMN cytoplasts (ghosts) devoid of DNA and cytoplasm are generated and have been shown to maintain many, but not all, functions of intact PMNs (*7*). Emerging evidence suggest that the persistence of NETs in tissues contributes to the pathogenesis of a number of inflammatory disorders, including those of the lung (*1*, *8*).

Krishnamoorthy *et al.* (*1*) explored the role of NETs in neutrophilic inflammation in mice cosensitized with the aeroallergen house dust mite (HDM) and the bacterial component LPS (Fig. 1). LPS/HDM cosensitization drove neutrophilic lung inflammation, whereas allergen sensitization alone resulted in a dominant eosinophil influx. LPS-driven neutrophilia was associated with elevations in lung-draining lymph node IL-17A levels. Of note, eosinophil numbers were suppressed in HDM/LPS-treated mice despite sustained levels of type 2 cytokines. HDM/LPS treatment of mice greatly elevated NET-associated DNA (hypercitrullinated histone H3) in bronchoalveolar lavage fluids (BALFs) but not in mice exposed only to HDM/Veh. Interestingly, enucleated neutrophil cytoplasts ($CD45^+CD11b^+Ly6g^+DNA^-$) were evident in the lungs of HDM/LPS-sensitized and HDM/LPS-challenged mice and virtually absent in the BALF of HDM/Veh-exposed mice.

These studies demonstrate that NETs and neutrophil cytoplasts are formed in the allergic lung after exposure to an aeroallergen in conjunction with a bacterial trigger, thus resembling what is observed in bacterially driven exacerbations of asthma.

Evidence that NETosis was responsible for both the marked neutrophilia and NET formation observed after LPS/HDM exposure was provided in PAD4-deficient mice (*1*). PAD4 deficiency led to a marked reduction in LPS/HDM-induced neutrophilic inflammation, NETosis (H3-DNA), and the numbers of cytoplasts present in the lung. Most impressively, the loss of NET formation resulted in protection against the development

Department of Environmental Health and Engineering, Johns Hopkins Bloomberg School of Public Health, Baltimore, MD 21205, USA. Email: mwkarp@jhu.edu

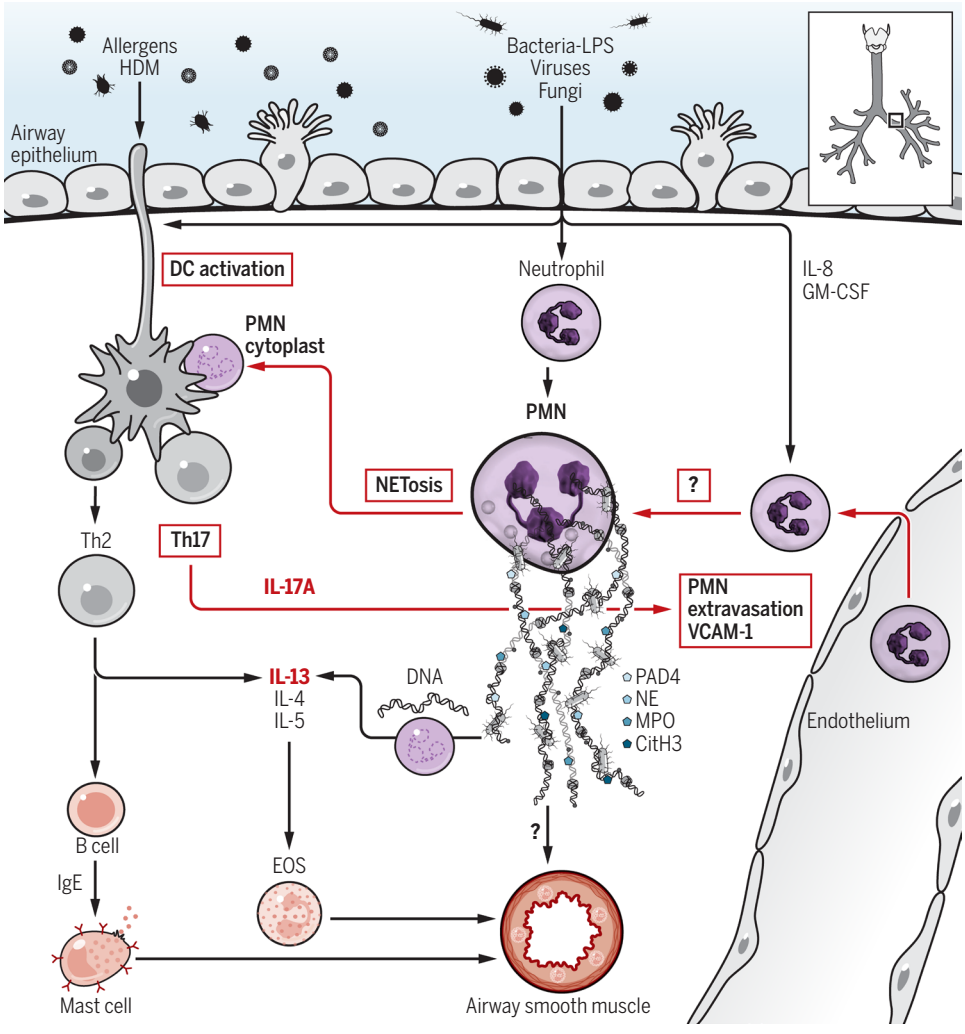

Fig. 1. NETosis exacerbates allergic inflammation. Exposure to allergens in susceptible individuals drives T_H2 cytokine production, resulting in immunoglobulin E (IgE) antibody production, eosinophilic inflammation, mucus hypersecretion, and AHR. Coincident microbial infections lead to the recruitment of PMNs and the initiation of NETosis, which releases DNA into the extracellular space and generates neutrophil cytoplasts. These cytoplasts migrate to the lymph node and, through their interactions with DCs, drive T_H17 cell differentiation. The newly generated T_H17 cells release IL-17A, which amplifies the neutrophilic inflammatory response through its ability to up-regulate GM-CSF, IL-8, and vascular cell adhesion molecule–1 (VCAM-1) expression, leading to increased PMN differentiation and extravasation from the bloodstream into airway tissues. Together, these processes drive exacerbation of allergic asthma.

CREDIT: A. KITTERMAN/AAAS

of allergen-driven airway hyperresponsiveness (AHR), mucus cell metaplasia, and the expansion of IL-17–producing cells in the lungs. Interestingly, this protection occurred without reductions in type 2–mediated immune responses (eosinophilia; IL-13$^+$ cells). Together, these results suggest that products of NETosis (DNA, NE granules, and cytoplasts) are able to enhance asthma symptoms independently of type 2 immune responses.

Previous studies have suggested that elevated extracellular DNA levels resulting from NETosis were the likely culprit in increasing asthma symptoms (*8*). Deoxyribonuclease (DNase) treatment of HDM/LPS-sensitized mice degraded NETs but reduced the levels of DNA and NETs in the lungs; it did not have an impact on neutrophilic inflammation in this model, suggesting that these two events are independently regulated (*1*). A study by Toussaint *et al.* (*9*) demonstrated that DNase treatment prevented rhinovirus (RV)–induced exacerbation of type 2–mediated allergic airway inflammation, but they observed that the DNA and NETs were of neutrophil, not eosinophil, origin. PMN depletion and NE inhibition protected mice from type 2 immunopathology and RV-driven accentuation of AHR, and injection of mouse genomic DNA in the lungs of HDM-sensitized mice was sufficient to recapitulate many features of RV-induced type 2 immune responses and asthma pathology. Likewise, they reported a substantial correlation between the release of double-stranded DNA after RV infection and type 2 inflammation in humans. The differences in the contribution of DNA to lung inflammation in these two studies are unknown but may be due to the nature of the stimulus-live RV infection versus LPS. Nonetheless, these studies suggest that extracellular DNA likely accentuates type 2 immunity, not neutrophilic inflammation, even though DNA may accumulate as a result of a neutrophil-dependent process.

Because NETosis appeared to be important in the regulation of IL-17A, the authors tested the hypothesis that NETosis and cytoplast generation were IL-17–dependent (*1*). Interestingly, antibody blockade of IL-17A before sensitization of mice with HDM/LPS reduced the influx of PMNs into the lungs but did not affect lung cytoplast generation. These findings suggest that IL-17 production is downstream of cytoplast generation and that other as-yet-unknown LPS-dependent facts drive cytoplast generation, which in turn leads to IL-17A production and PMN recruitment and activation.

Because PMN cytoplasts were found in the lung-draining lymph nodes of HDM/LPS-sensitized mice, Krishnamoorthy *et al.* (*1*) postulated that cytoplasts may influence dendritic cell (DC)–driven CD4$^+$ T cell differentiation. In vitro coculture of lung DCs with cytoplasts, but not intact PMNs, triggered antigen-specific IL-17 production from naïve CD4$^+$ T cells. These findings suggest that cytoplasts, but not intact PMNs, have developed properties through NETosis that drive T cell differentiation. Although the exact signals emanating from cytoplasts that instruct T cell programming were not identified in this study, the ability of cytoplasts to drive T_H17 differentiation was shown to require contact between DCs and cytoplasts and to be associated with elevated levels of major histocompatibility complex class II expression on cytoplasts. This observation supported the possibility that cytoplasts may present antigen to T cells, which has been previously proposed but not proven. Further studies are needed to elucidate the exact signals that cytoplasts convey to DCs

and/or T cells and whether they do present antigen.

To validate their findings in human asthmatics, Krishnamoorthy *et al.* (*1*) examined the association of features of NETosis with asthma severity in a well-characterized cohort of asthmatics enrolled in the National Heart, Lung, and Blood Institute's Severe Asthma Research Program. Similar to their findings in mice, hypercitrullinated histone H3 DNA was only present in the BALF of severe asthmatics with high BALF neutrophil counts and not in patients with severe asthma with low BALF PMN numbers. These results suggested that neutrophils and NETosis may be parameters that identify a subset of severe asthma patients. The presence of PMNs and cytoplasts correlated with BALF IL-17 levels, thus providing a link between neutrophilia, NETosis, and T_H17. Interestingly, when patients were stratified into groups in which their BALF contained DNA with either low or high levels of PMNs, no link was observed between lung function or asthma symptom scores and markers of NETosis. However, associations between high PMN/DNA content in BALF and more frequent asthma exacerbations and sinusitis were observed. The lack of association between PMN/DNA levels and lung function may have been due to the small number of individuals in this subgroup because the studies in $PAD4^{-/-}$ mice suggested that NETosis was required for LPS-driven AHR. These results are consistent with a previous study showing no association between NETs and AHR in human asthmatics (*10*). Unfortunately, an association between cytoplast generation and lung function was not examined in either of these studies. Inclusion of biomarkers of NETosis into subsequent studies may provide an opportunity to direct anti–IL-17A treatment to the subset of patients in which disruption of this pathway may be useful.

Findings from Krishnamoorthy *et al.* (*1*) provide a plausible explanation for the role that neutrophils may play in the pathogenesis of severe asthma. NETosis and cytoplast generation likely occur as a result of exposure to microbial products or infections (bacteria, viruses, and fungi) in allergen-sensitized individuals, but not as a result of allergen sensitization per se. Although extruded DNA likely up-regulates type 2 immune responses, neutrophil cytoplasts activate the T_H17 arm of the immune system, further enhancing PMN recruitment and activation and presumably leading to tissue damage that triggers asthma exacerbations through mechanisms yet to be determined. The coincident use of steroids, which prolongs the survival of PMNs, further propagates this cycle. Many questions remain regarding the role of NETs and PMN cytoplasts in asthma pathogenesis: What are the precise signals directing NETosis? How do IL-17A and/or PMNs drive exacerbations of asthma? And why do only certain asthmatics mount these neutrophilic inflammatory responses, despite all individuals experiencing microbial infections? Nonetheless, blockade of this process may provide some benefit in the subset of asthma patients with this phenotype. Broad suppression of neutrophil function is not a realistic approach, but targeting specific actions of PMNs may be warranted to disrupt this destructive cycle. The availability of multiple ways to interfere with NETosis and cytoplast generation (IL-1, IL-8, MPO and NE inhibitors, PAD4 inhibitors, and anti-histone antibodies) may provide a means to control or speed up clearance of NETs and cytoplasts and could represent a promising new therapeutic direction for the treatment of severe, neutrophilic asthma.

REFERENCES

1. N. Krishnamoorthy, D. N. Douda, 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. Bleecker, M. Castro, S. 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).
2. M. Wills-Karp, Immunologic basis of antigen-induced airway hyperresponsiveness. *Annu. Rev. Immunol.* **17**, 255–281 (1999).
3. N. R. Bhakta, P. G. Woodruff, Human asthma phenotypes: From the clinic, to cytokines, and back again. *Immunol. Rev.* **242**, 220–232 (2011).
4. K. Hosoki, S. Ying, C. Corrigan, H. Qi, A. Kurosky, K. Jennings, Q. Sun, I. Boldogh, S. A. Sur, Analysis of a panel of 48 cytokines in BAL fluids specifically identifies IL-8 levels as the only cytokine that distinguishes controlled asthma from uncontrolled asthma, and correlates inversely with FEV_1. *PLOS ONE* **10**, e0126035 (2015).
5. L. McKinley, J. F. Alcorn, A. Peterson, R. B. Dupont, S. Kapadia, A. Logar, A. Henry, C. G. Irvin, J. D. Piganelli, A. Ray, J. K. Kolls, T_H17 cells mediate steroid-resistant airway inflammation and airway hyperresponsiveness in mice. *J. Immunol.* **181**, 4089–4097 (2008).
6. V. Brinkmann, U. Reichard, C. Goosmann, B. Fauler, Y. Uhlemann, D. S. Weiss, Y. Weinrauch, A. Zychlinsky, Neutrophil extracellular traps kill bacteria. *Science* **303**, 1532–1535 (2004).
7. O. Z. Cheng, N. Palaniyar, NET balancing: A problem in inflammatory lung diseases. *Front. Immunol.* **4**, 1 (2013).
8. M. Toussaint, D. J. Jackson, D. Swieboda, A. Guedán, T.-D. Tsourouktsoglou, Y. M. Ching, C. Radermecker, H. Makrinioti, J. Aniscenko, 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).
9. A. K. Gupta, M. B. Joshi, M. Philippova, P. Erne, P. Hasler, S. Hahn, T. J. Resink, Activated endothelial cells induce neutrophil extracellular traps and are susceptible to NETosis-mediated cell death. *FEBS Lett.* **584**, 3193–3197 (2010).
10. R. Dworski, H.-U. Simon, A. Hoskins, S. Yousefi, Eosinophil and neutrophil extracellular DNA traps in human allergic asthmatic airways. *J. Allergy Clin. Immunol.* **127**, 1260–1266 (2011).

10.1126/sciimmunol.aau0112

Citation: M. Wills-Karp, Neutrophil ghosts worsen asthma. *Sci. Immunol.* **3**, eaau0112 (2018).

Science Immunology

Neutrophil ghosts worsen asthma

Marsha Wills-Karp

Sci. Immunol. **3**, eaau0112.
DOI: 10.1126/sciimmunol.aau0112