Locally instructed CXCR4\textsuperscript{hi} neutrophils trigger environment-driven allergic asthma through the release of neutrophil extracellular traps

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Low exposure to microbial products, respiratory viral infections and air pollution are major risk factors for allergic asthma, yet the mechanistic links between such conditions and host susceptibility to type 2 allergic disorders remain unclear. Through the use of single-cell RNA sequencing, we characterized lung neutrophils in mice exposed to a pro-allergic low dose of lipopolysaccharide (LPS) or a protective high dose of LPS before exposure to house dust mites. Unlike exposure to a high dose of LPS, exposure to a low dose of LPS instructed recruited neutrophils to upregulate their expression of the chemokine receptor CXCR4 and to release neutrophil extracellular traps. Low-dose LPS-induced neutrophils and neutrophil extracellular traps potentiated the uptake of house dust mites by CD11b\textsuperscript{+}Ly-6C\textsuperscript{+} dendritic cells and type 2 allergic airway inflammation in response to house dust mites. Neutrophil extracellular traps derived from CXCR4\textsuperscript{hi} neutrophils were also needed to mediate allergic asthma triggered by infection with influenza virus or exposure to ozone. Our study indicates that apparently unrelated environmental risk factors can shape recruited lung neutrophils to promote the initiation of allergic asthma.
initiation of allergic asthma in mice, we used a model of exposure to HDM in mice pre-exposed to a pro-allergic environmental factor, namely a low dose of LPS\(^1\). Different doses of LPS, ranging from 0.1 ng to 10\(\mu\)g, were administered intranasally (i.n.) to groups of BALB/c mice, which were exposed 1 and 8 d later to 40 and 10\(\mu\)g of HDM i.n., respectively. At day 11, airway eosinophilia, a feature of type 2 allergic asthma\(^1\), was virtually absent in vehicle pre-exposed HDM-treated mice, but it reached a peak in mice pre-exposed to 10\(\mu\)g of LPS and returned to baseline with 10\(\mu\)g of LPS (Supplementary Fig. 1a,b). Single exposures to either 100 ng or 10\(\mu\)g of LPS were chosen to model a pro-allergic, ‘hygienic’ (LPS\(^0\)) or a protective\(^2\), ‘nonhygienic’ (LPS\(^\ast\)) environment, respectively. LPS\(^\ast\) mice treated with HDM (LPS\(^\ast\)-HDM mice) developed features of allergic asthma 3 d after the second HDM instillation, including increased bronchial hyperreactivity to methacholine (Fig. 1a), airway eosinophilia (Fig. 1b), a higher secretion of IL-4, IL-5 and IL-13 by total LN cells restimulated with HDM (Fig. 1c), perivascular and peribronchial leukocyte infiltration (Fig. 1d,e), and increased bronchial mucus production (Fig. 1f) when compared to vehicle-HDM or LPS\(^\ast\)-HDM mice. Of note, CD45\(^+\)CD11b\(^+\)Ly-6G\(^+\) neutrophils, quantified by flow cytometry, were massively recruited into the lungs of LPS\(^0\) and LPS\(^\ast\) mice between 6 and 24 h after LPS exposure (Supplementary Fig. 1c,d). Thus, low-dose exposure to LPS induced a lung environment that promoted type 2 immunity to HDM and the development of allergic asthma.

scRNA-seq identifies particular neutrophils in LPS\(^\ast\) mice. To investigate the transcriptional diversity of lung neutrophils, we performed scRNA-seq. Twenty-four hours after vehicle, LPS\(^0\) and LPS\(^\ast\) i.n., neutrophils were first enriched from lung single-cell suspensions pooled from 3 BALB/c mice per condition by negative selection with MACS and were then FACS sorted as CD45\(^+\)CD11b\(^+\)Ly-6G\(^+\) neutrophils from LPS\(^0\) and LPS\(^\ast\) mice, respectively. Nonlinear dimensional reduction and graph-based clustering of single cells pooled from vehicle, LPS\(^0\) and LPS\(^\ast\) mice identified 6 transcriptionally distinct clusters of neutrophils, all characterized by high expression of S100a8, S100a9 and Csf3r transcripts (cluster 0 through cluster 5; Fig. 2a and Supplementary Fig. 2a–c). With the 10x Genomics platform\(^3\), cells were subjected to single-cell droplet encapsulation, scRNA-seq and quality-control filtering (Supplementary Fig. 2d,e). A total of 1,406, 2,146 and 2,746 cells were analyzed in vehicle, LPS\(^0\) and LPS\(^\ast\) mice, respectively. Nonlinear dimensional reduction and graph-based clustering of single cells pooled from vehicle, LPS\(^0\) and LPS\(^\ast\) mice identified 6 transcriptionally distinct clusters of neutrophils, all characterized by high expression of S100a8, S100a9 and Csf3r transcripts (cluster 0 through cluster 5; Fig. 2a and Supplementary Fig. 2f,g). Neutrophils from vehicle lungs were grouped in one cluster (cluster 0; Fig. 2b–d and Supplementary Fig. 3a–d), while neutrophils from LPS\(^0\) and LPS\(^\ast\) mice were segregated into five additional clusters (clusters 1–5, Fig. 2b–d). Neutrophils in cluster 1 were almost uniquely found in lungs from LPS\(^0\) mice, while neutrophils in clusters 2, 3 and 5 were nearly exclusively present in lungs from LPS\(^\ast\) mice, and neutrophils in cluster 4 were equally distributed between LPS\(^0\) and LPS\(^\ast\) mice (Fig. 2b–d). We performed a differential expression analysis, and we defined a common,
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neutrophils from LPSlo mice as compared to neutrophils from vehicle or LPSm mice did not (Fig. 3e and Supplementary Fig. 4a–d). Thus, lung neutrophils induced by a pro-allergic, low dose of LPS are transcriptionally distinct from those induced by a high LPS dose.

LPSm locally triggers NET-releasing CXCR4hi neutrophils. To validate the scRNA-seq findings and identify the neutrophils induced by LPSm treatment in vivo, we exposed BALB/c mice to vehicle, LPSlo or LPSm and assessed the expression of CXCR4 and Lamp-1 on lung CD45CD11bLy-6G neutrophils 24 h later. We found a significant upregulation of CXCR4 and Lamp-1 on lung neutrophils from LPSm mice as compared to neutrophils from vehicle or LPSm mice (Fig. 3a,b). The expression of CD49d, an integrin antigen reported to be highly expressed on CXCR4hi blood neutrophils25, was also upregulated on lung neutrophils from LPSm mice (Fig. 3a,b). Next, we performed time-course analyses of CXCR4 expression on bone marrow (BM), blood and lung CD45CD11bLy-6G neutrophils isolated 6, 12, 18, 24 and 48 h after treatment in vehicle, LPSm and LPSm mice. In the lung, CXCR4 expression on CD45CD11bLy-6G neutrophils started to increase 18 h after LPSm, reached a peak at 24 h and returned to baseline after 48 h (Fig. 3c). Expression of CXCR4 on lung CD45CD11bLy-6G neutrophils from LPSm mice was similar to that of vehicle mice (Fig. 3c). Of note, while we detected a rhythmic oscillation in the expression of CXCR4 on blood neutrophils, the oscillation was not affected by treatment with LPS, regardless of the dose (Fig. 3c). Expression of CXCR4 on BM CD45CD11bLy-6G neutrophils was similar in LPSm and vehicle mice (Fig. 3c), indicating that low-dose LPS administered i.n. instructs the neutrophils locally and not at distant sites. Morphologically, CXCR4hiCD49dhi neutrophils sorted from the lungs of LPSm mice were smaller and displayed a higher nucleus/cytoplasm ratio and a hypersegmented nucleus compared to LPSm mice (Fig. 3c). Of note, the expression of CD49d was also upregulated on lung neutrophils from LPSm mice (Fig. 3a,b). The expression of CD49d, an integrin antigen reported to be highly expressed on CXCR4hi blood neutrophils25, was also upregulated on lung neutrophils from LPSm mice (Fig. 3a,b). Next, we performed time-course analyses of CXCR4 expression on bone marrow (BM), blood and lung CD45CD11bLy-6G neutrophils isolated 6, 12, 18, 24 and 48 h after treatment in vehicle, LPSm and LPSm mice. In the lung, CXCR4 expression on CD45CD11bLy-6G neutrophils started to increase 18 h after LPSm, reached a peak at 24 h and returned to baseline after 48 h (Fig. 3c). Expression of CXCR4 on lung CD45CD11bLy-6G neutrophils from LPSm mice was similar to that of vehicle mice (Fig. 3c).

Lung CXCR4hiCD49dhi neutrophils sorted from LPSm mice released NETs ex vivo, while lung CXCR4hiCD49dhi neutrophils from vehicle or LPSm mice did not (Fig. 3e and Supplementary Fig. 4a–d). In addition, the amount of free double-stranded DNA (dsDNA) and of NE–DNA complexes, which are characteristic of NETs24, were higher in the bronchoalveolar lavage fluid (BALF) of LPSm mice 24 h after LPS than in the vehicle and LPSm mice (Fig. 3f,g), while the amounts of Cit-H3, a modified form of histone H3 implicated in chromatin decondensation and NET formation31, were significantly increased in the lungs of LPSm mice (Fig. 3h). High-resolution confocal microscopy indicated the presence of extracellular MPO-Cit-H3+ NETs in the lungs of LPSm mice, unlike in vehicle or LPSm mice (Fig. 3j,k). Notably, neutrophil depletion with an antibody against Ly-6G was associated with an absence of NETs 24 h after LPS in the lungs of anti-Ly-6G-treated LPSm mice (Supplementary Fig. 5), indicating that NETs were exclusively derived from neutrophils. Concordant with the upregulation of CXCR4, NETs were detected at 18 h after low-dose LPS, but not detected after 48 h (Supplementary Fig. 6a,b). These observations indicate that neutrophils recruited to the lungs of LPSm mice had a CXCR4hiCD49dLamp-1hi phenotype that was only detected in the lungs, and were prone to release NETs, while lung neutrophils recruited in LPSm mice were phenotypically similar to steady-state neutrophils and did not release NETs.

CXCR4hi neutrophils and NETs trigger allergic airway inflammation in LPSm mice. To address whether the effect of low-dose LPS on HDN-induced allergic airway inflammation was mediated by neutrophils, LPSm-HDM mice were administered Sch527123, an antagonist of the chemokine receptor CXCR2 (anti-CXCR2) (ref. 27), orally 2 h before and 4 and 8 h after LPSm, and 2 h before and 4 h after i.n. administration of 40 µg of HDN, to inhibit the LPS-induced neutrophil recruitment to the lung2. Treatment with anti-CXCR2 significantly reduced numbers of lung CD45CD11bLy-6G neutrophils 24 h after LPS treatment in LPSm mice (Fig. 4a,b). LPSm-HDM mice treated with anti-CXCR2 had significantly reduced airway eosinophilia (Fig. 4c), HDN-specific type 2 immune responses (Fig. 4d), perivascular and peribronchial inflammation (Fig. 4e,f) and mucus cell production (Fig. 4g,h) compared to vehicle-LPSm–HDM mice. In a different approach, we isolated lung neutrophils from vehicle, LPSm or LPSm mice by MACS negative selection and FACS CD45 sorting 24 h after LPS (vehicle, LPSm and LPSm neutrophils, respectively) and adoptively transferred 5 × 105 neutrophils in the trachea of naive recipients together with 40 µg of HDN, which were exposed to 10 µg of HDN i.n. 7 d later and analyzed 11 d after transfer. Transfer of LPSm neutrophils was sufficient to trigger airway eosinophilia (Fig. 4i), HDN-specific type 2 immunity (Fig. 4j), peribronchial inflammation (Fig. 4k,l) and increased mucus production (Fig. 4m,n) in HDN-treated recipient mice, while all these features were significantly lower in mice that received vehicle or LPSm neutrophils.

Fig. 2 | scRNA-seq analysis of the lung neutrophil compartment 24 h after pro-allergic low or protective high LPS exposure. a, tSNE plots depicting the transcriptional identity of lung neutrophils merged from vehicle, LPSm and LPSm mice 24 h after treatment, analyzed by scRNA-seq (n = 3 pooled mice per group). b, tSNE plots depicting the transcriptional identity of lung neutrophils from the three separate experimental conditions, as in a. c, Pie charts depicting the relative contribution of each neutrophil cluster to the pool of neutrophils in lungs of mice, as in a. Insets indicate average percentage of neutrophils among total lung cells. d, Absolute numbers of lung neutrophils per cluster in mice, as in a. e, volcano plot depicting the differentially expressed genes between LPSm and LPSm lung neutrophils of mice, as in a. Transcripts characteristic of the common LPS, the LPSm– and LPSm-specific signatures are colored in black, red and blue, respectively. f, PANTHER GO enrichment tests on the genes of the LPSm (left) and LPSm (right) signatures. g, h, Dot plots showing average expression of genes of the LPSm (g) and LPSm (h) signatures within neutrophil clusters. d, Data show mean ± s.e.m. (n = 3 mice per group). P values were calculated with a two-way ANOVA with Tukey’s post hoc test (d), a likelihood ratio test based on zero-inflated data to identify positive and negative markers of a single cluster compared to some or all other clusters (e) or a two-tailed Mann–Whitney U-test with Benjamin–Hochberg false discovery rate (FDR) correction (f). The symbol * within a given cluster in d indicates that neutrophil numbers in that cluster are significantly different from the ones of the same cluster in the two other experimental conditions. **p < 0.01.
To test the contribution of NETs to the initiation of allergic airway inflammation in LPS\textsuperscript{lo}-HDM mice, we targeted the NETs with 4 daily intraperitoneal (i.p.) injections of DNase, which degrades NETs\textsuperscript{18,29}, starting 1 d before LPS\textsuperscript{lo} treatment, or with 8 i.p. injections of either the NE inhibitor GW-311616 (NEi)\textsuperscript{15}, or an inhibitor of the arginine deiminase PAD4 (Cl-amidine)\textsuperscript{30}, every 12 h starting 1 d before LPS\textsuperscript{lo} treatment. Treatments with DNase, NEi and Cl-amidine resulted in a significant decrease of...
NET volume in LPS<sup>b</sup> mice 24 h after LPS (Fig. 5a,b). Most features of type 2 allergic airway inflammation, such as airway eosinophilia (Fig. 5c), HDM-specific type 2 immunity (Fig. 5d), peribronchial inflammation (Fig. 5e,f) and increased mucus production (Fig. 5g,h) were significantly reduced in DNase-, NEi- or Cl-amidine-treated LPS<sup>b</sup>-HDM mice compared to vehicle LPS<sup>b</sup>-HDM mice (Fig. 5i), HDM-specific type 2 immunity (Fig. 5d), and mucus production in NEi-treated LPS<sup>b</sup>-HDM mice compared to vehicle LPS<sup>b</sup>-HDM mice (Fig. 5g,h). These observations indicate that lung neutrophils and NETs from LPS<sup>b</sup> mice were mediating the pro-allergic effects of low-dose LPS on HDM-induced allergic asthma.

**Fig. 3 Pro-allergic low-dose LPS instructs lung neutrophils to upregulate CXCR4, CD49d and Lamp-1 and release NETs.**

**a.** Representative histograms of CXCR4, CD49d and Lamp-1 expression by lung CD45<sup>+</sup>CD11b<sup>+</sup>Ly-6G<sup>+</sup> neutrophils from vehicle, LPS<sup>b</sup> and LPS<sup>c</sup> mice 24 h after treatment.

**b.** MFI showing quantification of CXCR4, CD49d and Lamp-1 expression by lung CD45<sup>+</sup>CD11b<sup>+</sup>Ly-6G<sup>+</sup> neutrophils, as in a. Kinetic analysis of CXCR4 expression by bone marrow, blood and lung CD45<sup>+</sup>CD11b<sup>+</sup>Ly-6G<sup>+</sup> neutrophils from mice, as in a. Representative photographs of FACS-sorted CXCR4<sup>+</sup>/CD49d<sup>+</sup>/Lamp-1<sup>+</sup> lung neutrophils from mice, as in a.

**c.** Confocal microscopy stainings of Cit-H3<sup>+</sup> lung neutrophils from mice, as in a. Kinetic analysis of CXCR4 expression by bone marrow, blood and lung CD45<sup>+</sup>CD11b<sup>+</sup>Ly-6G<sup>+</sup> neutrophils from mice, as in a. Representative photographs of FACS-sorted CXCR4<sup>+</sup>/CD49d<sup>+</sup>/Lamp-1<sup>+</sup> lung neutrophils from mice, as in a.

**d.** Representative blots of Cit-H3 in lung protein extracts of mice, as in a.

**e.** ELISA measurement of NE–DNA complexes in the BALF of mice, as in a.

**f.** Levels of extracellular dsDNA in the BALF of vehicle, LPS<sup>b</sup> and LPS<sup>c</sup> mice 24 h after treatment. ELISA measurement of NE–DNA complexes in the BALF of mice, as in a. Representative blots of Cit-H3 in lung protein extracts of mice, as in a.

**g.** Confocal microscopy stainings of Cit-H3 in lung protein extracts of mice, as in a.

**h.** Western blot of lung protein extracts from mice, as in a.

**i.** Quantification of normalized Cit-H3 levels of lung neutrophils from mice, as in a. Representative blots of Cit-H3 in lung protein extracts of mice, as in a.

**j.** Confocal microscopy stainings of Cit-H3 in lung protein extracts of mice, as in a.

**k.** Quantification of NET volume in lung sections of mice, as in a. Representative blots of Cit-H3 in lung protein extracts of mice, as in a.

Values were calculated with a one-way ANOVA with Tukey’s post hoc test. *P < 0.05; **P < 0.01; ***P < 0.001. OD, optical density; NS, not significant. Scale bar, 10 μm.
Fig. 4 | LPS<sup>lo</sup> neutrophils mediate susceptibility to HDM-induced type 2 allergic airway inflammation. 

**a**, Representative dot plots of lung CD45<sup>+</sup>CD11b<sup>hi</sup>Ly-6G<sup>hi</sup> neutrophils from vehicle or LPS<sup>lo</sup> mice treated or not with Sch527123, a CXCR2 antagonist (anti-CXCR2), 2 h before and 4, 8 and 20 h after LPS, and analyzed 24 h after LPS. Insets indicate percentage of cells within the gate. 

**b**, Absolute numbers of lung CD45<sup>+</sup>CD11b<sup>hi</sup>Ly-6G<sup>hi</sup> neutrophils, as in **a**. Eosinophil cell counts in the BALF, assessed 3 d after the second HDM administration in LPS<sup>lo</sup>-HDM mice treated or not with anti-CXCR2. 2 h before and 4 and 8 h after LPS<sup>lo</sup>, and 2 h before and 4 h after i.n. sensitization with HDM. 

**c**, ELISA measurement of cytokine production by HDM-restimulated BLN cells of mice, as in **a**. Inflammatory score estimating perivascular and peribronchial inflammation, quantified from H&E-stained lung sections of mice, as in **a**. 

**d**, Representative H&E staining of lung sections of mice as in **i**. 

**e**, Representative PAS staining of lung sections of mice, as in **i**. 

**f**, Representative PAS-stained epithelial cells per bronchi showing airway mucus production in mice, as in **i**. 

**g**, Quantification of PAS-stained epithelial cells per bronchi showing airway mucus production in mice, as in **i**. 

**h**, Eosinophil cell counts in BALF, assessed 3 d after the second HDM administration in LPS<sup>lo</sup>-HDM mice treated or not with anti-CXCR2, 2 h before and 4 and 8 h after LPS<sup>lo</sup>, and 2 h before and 4 h after i.n. sensitization with HDM. 

**i**, ELISA measurement of cytokine production by HDM-restimulated BLN cells of mice, as in **a**. Inflammatory score estimating perivascular and peribronchial inflammation, quantified from H&E-stained lung sections of mice, as in **i**. 

**j**, Quantification of PAS-stained epithelial cells per bronchi showing airway mucus production in mice, as in **i**. 

**k**, Inflammatory score estimating perivascular and peribronchial inflammation, quantified from H&E-stained lung sections of mice, as in **i**. 

**l**, Representative PAS-staining of lung sections of mice, as in **i**. 

**m**, Quantification of PAS-stained epithelial cells per bronchi showing airway mucus production in mice, as in **i**.
NETs promote HDM uptake by CD11b\(^{+}\)Ly-6C\(^{-}\) DCs. Given the predominant role played by lung DCs in the induction of Th2 responses to HDM, we looked at their ability to take up HDM on exposure to vehicle, LPS\(^{\text{iv}}\) or LPS\(^{\text{hi}}\). Twenty-four hours after LPS, we administered 40\(\mu\)g of fluorescent-labeled AF647-HDM i.n., and the numbers of lung CD45\(^{+}\)CD11c\(^{+}\)MHC-I\(^{+}\)HDM\(^{+}\) DCs were quantified 24 h later by flow cytometry. Lungs of LPS\(^{\text{iv}}\) mice exposed to AF647-HDM had greater numbers of CD11b\(^{+}\)Ly-6C\(^{-}\) DCs, and to a lesser extent CD11b\(^{+}\)Ly-6C\(^{+}\) DCs, that were AF647-HDM\(^{+}\) than the lungs of vehicle or LPS\(^{\text{iv}}\) mice (Fig. 6a–c). Of note, the numbers of CD11b\(^{+}\)Ly-6C\(^{+}\)AF647-HDM\(^{+}\) DCs were significantly reduced in DNase-, NEi- or Cl-amidine-treated LPS\(^{\text{iv}}\)-AF647-HDM mice as compared to vehicle LPS\(^{\text{iv}}\)-AF647-HDM mice (Fig. 6d), suggesting that NETs promoted HDM uptake by CD11b\(^{+}\)Ly-6C\(^{-}\) DCs directly or indirectly.

To assess whether LPS\(^{\text{hi}}\) neutrophils modulated AF647-HDM uptake by CD11b\(^{+}\)Ly-6C\(^{-}\) DCs directly, we co-cultured vehicle, LPS\(^{\text{iv}}\) or LPS\(^{\text{hi}}\) neutrophils with bone marrow–derived DCs (BMDCs), which contained both CD11b\(^{+}\)Ly-6C\(^{-}\) and CD11b\(^{+}\)Ly-6C\(^{+}\) BMDCs (Fig. 6e) and are known to induce type 2 sensitization to HDM when pulsed with HDM and reinjected into recipient animals. Co-culture of AF647-HDM-treated BMDCs with LPS\(^{\text{iv}}\) neutrophils increased the uptake of AF647-HDM by CD11b\(^{+}\)Ly-6C\(^{-}\) BMDCs, and to a lesser extent by CD11b\(^{+}\)Ly-6C\(^{+}\) BMDCs, as compared to AF647-HDM-treated BMDCs alone (Fig. 6f). Co-culture of AF647-HDM-treated BMDCs with vehicle or LPS\(^{\text{hi}}\) neutrophils promoted a marginal increase in AF647-HDM uptake by CD11b\(^{+}\)Ly-6C\(^{-}\) BMDCs compared to AF647-HDM-treated CD11b\(^{+}\)Ly-6C\(^{-}\) BMDCs alone, which was significantly lower than that elicited by the co-culture
of AF647-HDM-treated BMDCs with LPS\textsuperscript{s} neutrophils (Fig. 6f). AF647-HDM uptake, especially by CD11b\textsuperscript{+}Ly-6C\textsuperscript{−} BMDCs, was significantly diminished when DNase was added to the co-culture of BMDCs with LPS\textsuperscript{s} neutrophils (Fig. 6f), indicating that the process was dependent on NETs. Co-culture of BMDCs with LPS\textsuperscript{s} neutrophils, but not with vehicle or LPS\textsuperscript{h} neutrophils, promoted the expression of the pro-T\textsubscript{17} co-stimulatory molecule CD86 (refs\textsuperscript{34,35}) on CD11b\textsuperscript{+}Ly-6C\textsuperscript{−} BMDCs, and this effect was not affected by DNase treatment (Fig. 6f).

Thus, LPS\textsuperscript{s} neutrophils acted on CD11b\textsuperscript{+}Ly-6C\textsuperscript{−} DCs directly to promote AF647-HDM uptake through NET-dependent mechanisms.
Fig. 7 | Influenza virus infection and ozone exposure instruct recruited lung CXCR4⁺ neutrophils to release NETs. a,c, Representative histograms of CXCR4, Lamp-1 and CD49d expression by lung CD45⁺CD11b⁺Ly-6G⁺ neutrophils 7 d after influenza virus (PR8) infection (a) or 24 h after 3 daily ozone exposures (c). b,d, MFI showing quantification of CXCR4, CD49d and Lamp-1 expression by lung CD45⁺CD11b⁺Ly-6G⁺ neutrophils as in a and c, respectively. e,f, Kinetic analysis of CXCR4 expression by bone marrow, blood and lung CD45⁺CD11b⁺Ly-6G⁺ neutrophils after PR8 infection (e) or ozone exposure (f), as in a. g,k, Levels of extracellular dsDNA in the BALF of PBS-injected and PR8-infected mice (g), 7 d after PR8, or air- and ozone-exposed mice, 24 h after 3 daily ozone exposures (k). h,l, ELISA measurement of NE-DNA complexes in the BALF of mice, as in g and k, respectively. i,m, Confocal microscopy stainings of Cit-H3+MPO+ NETs on lung sections of mice, as in g and k, respectively. Pictures are representative of one of >5 lungs analyzed. j,n, Quantification of NET volume in lung sections of mice, as in g and k, respectively. (b–k,m) Data show mean ± s.e.m. and are pooled from 2–3 independent experiments (b,d,g–i,k–m) (b,d,g,h,i,k,l,m; n = 6,6,5,5,6,8,5,6) mice per group, respectively) or 2 independent experiments per time point analyzed (e,f) (n = 4 mice per time point). P values were calculated using an unpaired two-tailed Student's t-test (b,d,g–i,k–m) or a one-way ANOVA that compares PR8-infected or ozone-exposed versus control counterparts (e,f). **P < 0.01; ***P < 0.001. NS, not significant; OD, optical density. Scale bars, 50 μm.

Other pro-allergic factors promote allergic asthma via NETs. Next, we tested whether NET-prone CXCR4⁺ neutrophils were associated with additional pro-allergic conditions. Besides low exposure to microbial products, respiratory viral infections and air pollution also represent risk factors for allergic asthma in humans.

In mice, acute respiratory infection with influenza virus or exposure to ozone promote allergic airway inflammation to HDM or ovalbumin, respectively. BALB/c mice infected i.n. with 5 plaque-forming units (PFU) of influenza A virus H1N1 strain PR8/34 (PR8) had decreased body weight at day 7 (Supplementary Fig. 7a,b) and
increased lung viral RNA expression (Supplementary Fig. 7c) and numbers of CD45⁺CD11b⁺Ly-6G⁺ neutrophils (Supplementary Fig. 7d) between 3 and 7 d after infection. Similarly, BALB/c mice exposed for 3 to 2 ppm ozone during 3 h also had increased lung neutrophil numbers 24 h after the last exposure (Supplementary Fig. 7e,f). Seven days after PR8 or 24 h after ozone exposure, these neutrophils had increased expression of CXCR4, Lamp-1 and CD49d as compared to neutrophils from uninfected or air-exposed control mice (Fig. 7a–d), suggesting shared phenotypical similarities with LPS⁹ neutrophils. In addition, CXCR4⁴ neutrophils were only found in the lung and could not be detected in the blood or BM (Fig. 7e,f), suggesting local imprinting. NETs were also found in the lungs of mice infected with PR8 or exposed to ozone (Fig. 7g–n and Supplementary Fig. 7g–k), suggesting that exposure to other pro-inflammatory factors triggered the accumulation of NET-prone CXCR4⁴ neutrophils in the lungs.

Next, we instilled PR8-infected mice i.n. with 40 µg of HDM 7 d after PR8 and with 10 µg of HDM 7 d later (PR8-HDM). To test the contribution of NETs to PR8-triggered allergic asthma, PR8-HDM mice were treated daily for 12 d with DNase i.p., starting 5 d after PR8. Alternatively, PR8-HDM mice were treated every 12 h with NEi or Cl-amidine i.p. for the same duration as for DNase. Three days after the HDM challenge, we observed increased susceptibility of PR8-HDM mice to develop airway eosinophilia and HDM-specific IgG1 (Fig. 8a), HDM-specific type 2 immunity (Fig. 8b) and goblet cell hyperplasia (Fig. 8c,d), while administration of HDM without pre-exposure to PR8 did not induce features of allergic asthma and treatments of PR8-HDM mice with DNase, NEi and Cl-amidine resulted in a significant decrease of nearly all features of allergic asthma compared to vehicle PR8-HDM mice, except for LN production of IL-13, which was similar in DNase-treated and vehicle PR8-HDM mice (Fig. 8b).

In addition, we observed higher numbers of lung CD11b⁺Ly-6C⁺-AF647⁻HDM⁺ DCs in PR8-AF647-HDM mice than in infected AF647-HDM-treated controls 24 h after AF647-HDM, these numbers were significantly decreased in DNase-, NEi- and Cl-amidine-treated PR8-AF647-HDM mice compared to vehicle PR8-AF647-HDM controls (Fig. 8e).

We also instilled ozone-exposed mice i.n. with 40 µg of HDM 1 d after the last ozone treatment and with 10 µg of HDM 7 d later (ozone-HDM). Some ozone-HDM mice were also treated daily for 4 d with DNase i.p., starting the first day of ozone exposure. Alternatively, ozone-HDM mice were treated every 12 h with NEi or Cl-amidine i.p. for the same duration as for DNase. Three days after the HDM challenge, we observed increased susceptibility of ozone-HDM mice to develop airway eosinophilia and HDM-specific IgG1 (Fig. 8f), HDM-specific type 2 immunity (Fig. 8g), goblet cell hyperplasia (Fig. 8h,i) and peribronchial inflammation (Supplementary Fig. 8), while administration of HDM without pre-exposure to ozone did not induce features of allergic asthma and treatments of ozone-HDM mice with DNase, NEi and Cl-amidine resulted in a significant decrease of all the features of allergic asthma tested (Fig. 8f–i and Supplementary Fig. 8). In addition, while we observed higher numbers of lung CD11b⁺Ly-6C⁺-AF647⁻HDM⁺ DCs in ozone-AF647-HDM mice than in air-exposed AF647-HDM-treated controls 24 h after AF647-HDM, these numbers were significantly decreased in DNase-, NEi- and Cl-amidine-treated ozone-AF647-HDM mice compared to vehicle ozone-AF647-HDM controls (Fig. 8i).

These observations suggested that NETs promoted AF647-HDM uptake by lung CD11b⁺Ly-6C⁺ DCs and mediated allergic airway inflammation triggered by flu infection or ozone exposure.

Discussion

Here we showed that neutrophils have an important role in type 2 allergic immunity and link pro-allergic environmental conditions and host allergic susceptibility. We showed that airway exposure to low-dose LPS, influenza virus infection and ozone exposure in mice induced the accumulation of NET-releasing CXCR4⁴ neutrophils in the lung. These neutrophils promoted the uptake of HDM by lung CD11b⁺Ly-6C⁺ DCs and increased susceptibility to allergic asthma.

LPS measurements have been used in epidemiological studies related to the 'hygiene hypothesis', suggesting that an LPS-rich environment is associated with reduced risk of atopy and asthma, while LPS-poor environments represent a risk factor for the development of asthma⁸,⁹. The 'hygienic' model used here is an adaptation of a previous model¹¹, in which priming with a low or high dose of LPS biased the immune response to ovalbumin towards a Th2 or Th1 profile, respectively. Here, we found that exposure to a low dose of LPS in BALB/c mice potentiated HDM-induced type 2 allergic asthma, while priming with a higher dose of LPS¹² had no substantial effect.

Airway exposure to low-dose LPS in mice triggered NET-releasing CXCR4⁴ neutrophils in the lung. These neutrophils shared similarities with 'aged' neutrophils, a subset of NET-prone CXCR4⁴ neutrophils found in the blood⁹,¹⁰. Kinetic analyses of CXCR4 expression on lung, blood and BM neutrophils indicated that neutrophils were recruited to the lung and instructed locally. This indicates that CXCR4⁴ neutrophils, rather than representing a subset, are in a transient state of activation that depends on a local stimulus (that is, low-dose LPS). Our data argue in favor of the notion that neutrophils are heterogeneous, plastic and adaptable to context-specific cues to exert particular functions⁴. We found that these locally instructed CXCR4⁴ neutrophils represented early and critical players in the initiation of type 2 allergic asthma against HDM allergens. Given the central role of epithelial-derived alarmins, including IL-33, IL-25 and TSLP in the initiation of type 2 allergic immunity, it would be interesting to test their respective contributions to the imprinting of CXCR4⁴ neutrophils.

We used DNase, NEi and Cl-amidine to assess the functional importance of NETs in the initiation of allergic asthma. None of these approaches are fully specific for NETs⁹. Cl-amidine inhibits the activity of PAD4, which is implicated in chromatin reorganization and is expressed in many cell types. Treatment with Cl-amidine may lead to decreased activity of citrullinated histone proteins rather than NET impairment¹³. In addition, the biological activity of the inhibitors may not be optimal in the tissue and could be influenced by factors that are inherent to the experimental models used. However, treatment with DNase, NEi and Cl-amidine impaired NETs and had the same global outcome on the development of HDM-induced asthma, suggesting that NETs could be mediating the onset of allergic airway inflammation to HDM in the models tested here.

Low-dose LPS, influenza virus infection and ozone exposure were associated with an increased uptake of fluorescent-labeled HDM by CD11b⁺Ly-6C⁺ DCs, an important step in the initiation of HDM-specific type 2 responses¹⁴,¹⁵. Allergen uptake was significantly reduced in NET-targeted mice, supporting the idea that NETs can directly or indirectly modulate this process. Ex vivo co-culture experiments with lung neutrophils and BMDCs further indicated that NETs derived from LPS⁹ neutrophils could directly promote HDM uptake by CD11b⁺Ly-6C⁺ DCs. Our study is in accordance with reports that extracellular host DNA, the main NET component, is a potent signal that promotes the activation of DCs and type 2 immune responses¹⁶,¹⁷.

Whether CXCR4⁴ neutrophils and NETs can trigger type 2 sensitization against other type 2-inducing stimuli, such as fungal proteases, food allergens, helminths or additional pro-allergic factors (such as other respiratory viruses, exhaust particles, cigarette smoke) in BALB/c or other genetic backgrounds remains unclear¹⁸. In humans, neutrophils are important immune-modulating cells in the effector phase of severe asthma, which is characterized by neutrophilic inflammation, high expression of IL-17 and resistance to corticosteroids¹⁹–²⁰. Whether particular NET-releasing neutrophils contribute to the initiation of type 2 allergic asthma in humans remains unknown. A prospective Danish study found that the
number of respiratory infections in early life, but not the particular viral trigger, was associated with asthma development. The authors postulated that a host-derived factor might underlie the increased allergic susceptibility. Our results suggest that the accumulation of NET-prone neutrophils could be such a host-associated factor. In addition, blood neutrophils from Hutterite children, who are raised in an environment poor in LPS and are prone to develop allergies, have higher expression of CXCR4 than blood neutrophils from Amish children, who live in LPS-rich homes and are protected from allergies. While lung neutrophils have not been assessed in these
children, it is tempting to speculate that environmental risk factors may promote allergic airway sensitization in humans by inducing NET-prone CXCR4\(^+\) neutrophils, which increase host susceptibility for mount type 2 allergic responses towards aeroallergens.

### Online content

Any methods, additional references, Nature Research reporting summaries, source data, statements of code and data availability and associated accession codes are available at https://doi.org/10.1038/s41590-019-0496-9.

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### Author contributions

C. Radermecker, T.M. and F.B. conceived the project. T.M., C.Ruscitti and S.L.J. were involved in experiments aiming at detecting and inhibiting NETs. C.V., M.T. and F.B. conceived the project. T.M., F.B., M.T., C.Radermecker and C.S. contributed to experiments involving ozone exposure and invasive measurements of airway function. T.M. analyzed scRNA-seq data with the help of the F.R.S.-FNRS, S.L.J. and F.B. and contributed equally to this work. P.M., C.Ruscitti and J.S. were involved in (bone marrow-derived) dendritic cell-related experiments. M.T. and S.L.J. were involved in experiments aiming at detecting and inhibiting NETs. C.V., F.P., N.R. and D.C. contributed to experiments involving ozone exposure and invasive measurements of airway function. T.M. analyzed scRNA-seq data with the help of the GIGA Genomics Platform. C. Radermecker and T.M. prepared the figures, and T.M. wrote the manuscript. All authors provided feedback on the manuscript.

### Competing interests

The authors declare no competing interests.

### Additional information

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Methods

Mice. Female BALB/c mice were purchased from Janvier Laboratories. Age-
matched, 6- to 10-week-old mice were used for experiments. Mice were housed
under specific pathogen-free conditions and maintained in a 12-h light-dark
cycle with food and water ad libitum. All animal experiments described in this
study were reviewed and approved by the Institutional Animal Care and Use
Committee of the University of Liège. The ‘Guide for the Care and Use of
Laboratory Animals,’ prepared by the Institute of Laboratory Animal Resources,
National Research Council, and published by the National Academy Press, as
well as European and local legislations, were followed carefully.

Reagents and antibodies. 2.4G2 Fc receptor-blocking antibodies were produced
in house. Anti-mouse FITC- and V500-conjugated anti-CD45.2 (clone 104), PE-
conjugated anti-CD3 (clone 142-2C11), PE-conjugated anti-CD19 (clone 1D3),
PE-conjugated anti-NK-1.1 (clone pK136), PE-conjugated anti-Siglec-F (clone
E50-244C11), PE-conjugated anti-Ly-6C (clone RE606), and PE-conjugated anti-
CD11b (clone M170), PE-Cy7-conjugated anti-Ly-6G (clone 1A8), APC-Cy7-conjugated anti-CD11c (clone HL3), PE-CF594-conjugated anti-Ly-
6C (clone 49-44), FITC-conjugated anti-CD103 (clone M290), PE-conjugated
anti-Lamp-1 (CD107a) (clone ID107a) (clone 1D4B) and PE-conjugated streptavidin antibodies
were purchased from BD Biosciences. Anti-mouse PE-conjugated anti-CCR4
(clone 2B11), anti-mouse PE-conjugated anti-CD49d (clone R1-2) and anti-mouse
anti-CD86 biotin (clone GL-1) antibodies were purchased from eBioscience.
Anti-
mouse PerCp-Cy5.5-conjugated anti-MHC-II (Ia-e) (clone M5/M4.15.2) and
BV421-conjugated anti-CD46 (clone S4.5-7.1) antibodies were purchased from
Biolegend.

Unconjugated goat anti-mouse MPO (catalog no. AF3667) antibodies were
purchased from R&D systems; unconjugated rabbit anti-mouse-citullinated
Histone 3 (catalog no. Ab5103) antibodies were purchased from Abcam. Donkey
anti-rabbit and anti-goat IgG antibodies conjugated with Alexa Fluor 568 and
Alexa Fluor 488, respectively, were purchased from Thermo Fisher.

Anti-mouse IgG1kgoat anti-rabbit immunoglobulins/HRP and rabbit anti-
mouse immunoglobulins/HRP were purchased from Agilent.

Additional reagents can be found in the sections below.

Model of low-dose-LPS triggered allergic asthma to HDM. Isoflurane-
anesthetized BALB/c wild-type mice were instilled i.n. with different doses of
LPS ranging from 0.1 ng to 10 μg, two of which were chosen as low (100 ng) or
high (10 μg) doses (LPS from Escherichia coli O55:B5, Sigma-Aldrich). Vehicle
mice were instilled i.n. with 50 μl of PBS. One day later (day 1), mice were
administered i.n. with 40 μg of HDM (HDM pteronyssinus, Greer laboratories) in
50 μl of PBS. Seven days later (day 8), all mice were challenged by i.n. instillation
of 10 μg HDM in 50 μl of PBS. Three days after the HDM challenge (day 11),
we estimated bronchial hyperresponsiveness to methacholine by assessing
dynamic airway resistance in anesthetized animals subjected to increased doses
of methacholine with a FlexiVent small animal ventilator42. Additional reagents can be found in the sections below.

Cell isolation, staining and flow cytometry. To obtain single-lung-cell
suspensions, lungs were perfused with 10 ml of PBS through the right ventricle,
and red blood cells were lysed with red blood cell lysis buffer (eBioscience). BM cells were examined under the microscope for counting and viability testing.

For scRNA-seq, adoptive transfer and co-culture experiments, single-lung-cell
suspensions were obtained from lungs of vehicle, LPS- and LPS mice 24 h after
treatment. Neutrophils were first enriched by negative selection with a MACS
Negative Selection Isolation kit (Miltenyi Biotec) and cultured ex vivo with LPS as
a stimulus. Cells were examined under the microscope for counting and viability
testing. Viability was above 95% for all three samples. Cell preparations were centrifuged at 300g for 5 min. For scRNA-seq, neutrophils were isolated from lung single-cell
suspensions as pooled from 3 BALB/c female wild-type mice per condition and were
resuspended in calcium- and magnesium-free PBS containing 400 μg/ml bovine
serum albumin (BSA, Sigma-Aldrich).

For cytologic examination and immunofluorescence ex vivo, CD45+CD11b+Ly-
6C+CD49d+ neutrophils from vehicle and LPS mice and CD45+CD11b+Ly-
6C+CD49d+ neutrophils from LPS mice were FACs sorted 24 h after LPS.

Analysis scRNA-seq samples. Cell Ranger (v.2.1.0) (10x Genomics) was used
to demultiplex Illumina BCL files to FASTQ files (cellranger mkfastq), to perform
alignment (to mouse GRCm38/mm10 genome), filtering, UMI counting and to
produce gene–barcode matrices (cellranger count).

Subsequent analysis used R bioconductor (v.3.4.2.) and the R package Seurat
(v.2.1.0). We first performed a quality-control analysis and selected cells for further
analysis (see Supplementary Fig. 2d,e). Gene counts were normalized, and highly
variable genes were calculated. Cell–cell variation in the number of detected UMI
was regressed out with the ScaleData function. Linear dimensional reduction was performed on the scaled data with the ‘RunPCA’ function. To identify the number of statistically significant principal components (PCs) to include for subsequent analyses, we used the ‘JackStraw’ function, which implements a resampling test inspired by the jackstraw procedure44. PCs 1:11 were used in the subsequent analyses. We have also performed analyses including lower and higher numbers of
PCs (1:8 to 1:13) and did not find any substantial change in the results obtained.

Cells were clustered via the ‘FindClusters’ function. Several cluster resolutions were tested, and the resolution of 0.25 was chosen, because higher resolutions created additional subdivisions or clusters containing singlets, which were considered not relevant. To visualize the data, nonlinear dimensional reduction was used, and
SNSe plots were created by use of the ‘RanTSNE’ function, with the number of
dimensions to use set to 11 (PCs 1:11). The aforementioned analyses were performed on the individual Seurat objects (encompassing data from vehicle, LPS- and LPS mice) but also on a merged Seurat object that encompassed merged data from vehicle, LPS- and LPS mice. Differential expression analysis between clusters was performed with the ‘FindMarkers’ function. A value of 0.25 was attributed to the min.pct argument, which requires a gene to be detected at least in 25% in either of the two groups of cells. Only DE genes with an adjusted
P value < 10−5 were retained. To define the transcriptional signature of steady-
state neutrophils (cluster 0) (see Supplementary Fig. 3e,f), lists of differentially expressed genes common to clusters 0, 1 and 4 were generated, and genes commonly found in each of the 3 lists were retained. To define the common ‘LPS signature’ (see Supplementary
Fig. 3e,f), lists of the significantly upregulated genes in cluster 1 compared to
cluster 0 (that is, LPS−-induced), cluster 0 compared to clusters 2, 3 and 5 (that is, LPS−-induced), and cluster 0 compared to cluster 4 were generated, and genes commonly found in each of the 3 lists were retained. To define cluster-specific gene signatures, only genes that did not belong to the
common LPS signature and that were significantly upregulated in that specific
cluster as compared to each of the others were retained. Statistical enrichment tests for Gene Ontology (GO) biological processes were performed by using the GO
cluster as compared to each of the others were retained. Statistical enrichment tests
for Gene Ontology (GO) biological processes were performed by using the GO
functions in the R package clusterProfiler. GO terms were considered as
significant if they were enriched (P < 0.05) and with a false discovery rate
(FDR) < 0.05.

For scRNA-seq, adoptive transfer and co-culture experiments, single-lung-cell
suspensions were obtained from lungs of vehicle, LPS- and LPS mice 24 h after
treatment. Neutrophils were first enriched by negative selection with a MACS
Negative Selection Isolation kit (Miltenyi Biotec) and then sorted as CD45+
Ly-6ChighCD11bloLy-6G- cells, CD45+Ly-6ChiCD11bloLy-6G- cells, and CD45+
Ly-6ChiCD11bhiLy-6Ghi neutrophils from vehicle and LPS mice and CD45+CD11bhiLy-
6C+CD49d+ neutrophils from LPS mice were FACs sorted 24 h after LPS.

Neutrophil cytologic examination. Cytologic examination of FACS-sorted
neutrophils was performed on cytopsin preparations stained with Hemacolor
(Merck KgaA). Sections were examined with a FSX100 microscope (Olympus).

Quantifications of neutrophil size and cytoplasm/nucleus ratio were performed with the ImageJ software.

Immunofluorescence. To assess the ability of neutrophils to form NETs ex vivo, 2.5 x 104 FACS-sorted neutrophils were seeded and cultured ex vivo on slides
(Nunc Lab-Tek II Chamber Slide system, Sigma) precoated with poly-L-lysine
hydrobromide (Sigma) for 24 h in supplemented D-MEM medium (Gibco).

Supernatants were removed and chambers were rinsed with PBS. Slides were
then fixed with paraformaldehyde 10%, rinsed twice with PBS and permeabilized in PBS 0.5% triton X-100. Slides were then blocked and stained as described below.

To identify NETs from lung tissues, lungs were collected without performing BALF and fixed with 4% paraformaldehyde in PBS. Lung tissues were paraffin-embedded, and lung sections were cut (2-mm-thick sections) for immunofluorescence staining. After deparaffinization and rehydration, tissue sections were boiled for 20 min in 10 mM sodium carbonate buffer for antigen retrieval. Lung sections were permeabilized in PBS 0.5% triton X-100. Sections were incubated with a blocking buffer (PBS with 2% BSA and 2% of donkey serum (Sigma-Aldrich)) for 1 h at room temperature (RT) and stained in blocking buffer with rabbit anti-mouse antibodies directed against citrullinated histone H3 (1:100 in blocking buffer) and with goat anti-mouse antibodies directed against MPO (1:40 in blocking buffer) during 1 h at RT. After washing samples with PBS, secondary donkey anti-rabbit IgG antibodies conjugated with AlexaFluor 568 (1:200 in blocking buffer) and donkey anti-goat IgG antibodies conjugated with AlexaFluor 488 (1:200 in blocking buffer) were added in blocking buffer containing DAPI (1:1,000) and incubated for 2 h in the dark at RT. Finally, samples were mounted with 10 µl of ProLong Antifade reagent (ThermoFisher) on glass slides and stored at RT in the dark overnight.

All samples were analyzed by fluorescence microscopy with standard filter sets. Controls were stained with secondary antibody only, and nonspecific fluorescent staining was not detected when secondary antibodies were tested alone. Images were acquired on a Zeiss LSM 880 Airyscan Elyra S.I. confocal microscope (Zeiss) and processed with the ImageJ software. To quantify the volume of NETs released by neutrophils ex vivo (see Supplementary Fig. 4g) or present in the lung tissue (see Figs. 3g, 5a, h and 7, 1). Z stack pictures were acquired and Imaris software was used. Briefly, we performed a three-dimensional reconstruction of structures staining double positive for H3-Cit (red) and MPO (green), and Imaris provided quantification of the volume of these structures, expressed as mm³ per 10⁴ neutrophils or ex vivo neutrophils (counted in the bright-field channel and staining positive for intracellular MPO associated with the granules) or per 10 mm² of lung tissue.

DsdNA measurement in BALF. DsdNA was measured in the acellular fraction of the BALF, which was obtained after a double centrifugation and supernatant collection. Levels of dsDNA were determined with Quant-iT PicoGreen dsDNA reagent (Invitrogen) according to the manufacturer’s protocol.

Western blotting. Lung tissues were homogenized in RIPA buffer supplemented with a cocktail of protease inhibitors (Complete, Roche). An equivalent amount of protein per sample was resolved on a 4–20% gel (Mini-PROTEAN TGX Precast Gels, biorad) and electroblotted on Invitronol PVDF membranes (Life Technologies). Membranes were blocked 1 h at RT in TBS-Tween 0.1% containing 5% of dry milk and stained overnight at 4°C with primary antibody anti-citrullinated H3 (1:1,000 in 0.1% TBS-Tween and 5% BSA). The membranes were then incubated 2 h at RT with appropriate HRP-associated secondary antibodies in TBS-Tween 0.1% containing 5% BSA. Equal loading was confirmed by probing for HSP90a.

Measurement of NE–DNA complexes in BALF. Nunc plates (ThermoFisher) were coated overnight with anti-neutrophil elastase antibodies (1:2,000, Abcam, ab21595) and washed 3 times with PBS-Tween 0.05%. Plates were blocked 1 h at RT with PBS-RNase, washed 3 times, and PBS was then loaded for 2 h at RT and plates were then washed 5 times. Mouse anti-dsDNA antibodies were added for 1 h at RT followed, after 5 washes, by incubation with biotinylated rat anti-mouse IgG polyclonal antibodies for 90 min at RT. After extensive washing, secondary streptavidin–HRP (BD pharmingen, catalog no. 554066) was added for 30 min at RT, followed by 5 washes. The relative amount of NE–DNA complexes was revealed with TMB substrate solution (Sigma-Aldrich).

Antibody-mediated neutrophil in vivo depletion. Neutrophils were depleted by i.p. injection of 500 µg of anti-Ly-6G antibody (InVivo Plus anti-mouse Ly-6G, clone 1A8, Bioxell) intraperitoneally (i.p.) 1 day before (day –1), day 0 and 1 day after LPS exposure (day 1) (see Supplementary Fig. 5a). Mice were killed 3 h later.

Inhibition of LPS-induced lung neutrophil recruitment. To inhibit LPS-induced neutrophil recruitment, an antagonist of the chemokine receptor CXCR2 (that is, Sch527123, MedChem Express) was given orally, by gavage, at the dose of 3 mg kg⁻¹ 2 h before, 4 h after and 8 h after LPS treatment, as well as 2 h before and 4 h after i.n. administration of 40 µg of HDM. Seven days later, mice were challenged with 10 µg of HDM, and features of allergic airway inflammation were assessed 3 d later.

Neutrophil adoptive transfers. After MACS enrichment and FACS sorting, neutrophils were resuspended in calcium- and magnesium-free PBS at the concentration of 2×10⁶ cells/ml. Five hundred thousand (5×10⁴) cells, together with 40 µg of HDM, were injected intratracheally to naive mice in 50 µl of PBS. Seven days later, mice were challenged with 10 µg of HDM, and features of allergic airway inflammation were assessed 3 d later.

Degradation of NETs and inhibition of NET formation in vivo. The formation of NETs was prevented in vivo by i.p. injection of Nei (GW-31161 hydrochloride, Axon Medchem) (2.5 µg g⁻¹ 200 µl of PBS) or of Cl-amidine (10x stock solution in DMSO, Sanbio) (10µg kg⁻¹ in 200 µl of 1% DMSO v/v in PBS), twice daily from day –1 to day +2 (LPS model) or from day 0 to day 3 (ozone model). Alternatively, NETs were degraded by injecting 1,000 IU DNase I (Sigma-Aldrich) i.p. in 200 µl of HBSS once daily from day –1 to day +2 (model of LPS exposure) or from day 0 to day 7 (PR8 infection model) or from day 0 to day 3 (ozone model). In pilot experiments, we compared the responses of LPS⁵–HDM, PR8-HDM or ozone-HDM mice with their vehicle-treated counterparts (either HBSS, PBS or 1% DMSO v/v, used separately) and confirmed that vehicle did not affect the response. In subsequent experiments, vehicle LPS⁵–HDM, PR8-HDM or ozone-HDM mice were treated randomly with either HBSS, PBS or DMSO 1% v/v.

AF647–HDM uptake by lung DC subsets. To assess HDM uptake by lung DCs, LPS⁵ or ozone-exposed mice were administered i.n. with 40 µg of HDM labeled with Alexafluor 647 (AF647–HDM) (Alexa Fluor 647 Protein Labeling Kit, ThermoFisher) 24 h after treatment. Alternatively, PR8-infected mice were injected i.n. with AF647–HDM at day 7 after infection. Twenty-four hours later, lung-single-cell suspensions were obtained, and lung DCs were selected as CD45⁺CD11c⁺CD11b⁺ and used separately) and confirmed that vehicle did not affect the response. In subsequent experiments, vehicle LPS⁵–HDM, PR8-HDM or ozone-HDM mice were treated randomly with either HBSS, PBS or DMSO 1% v/v.

Neutrophils/BMDCs co-culture experiments. To generate BMDCs, BM cells were isolated from naive BALB/c mice and were grown for 7 d in GM-CSF-containing medium, as described ¹. Vehicle, LPS⁵ and LPS⁵ neutrophils were isolated 24 h after treatment. In total, 2.5×10⁶ neutrophils were seeded into 48-well plates previously coated with poly-(I:C)-biotin (Sigma) in supplemented D-MEM medium (Gibco) and allowed to adhere for 2 h. BMDCs were then added to neutrophils cultures at a ratio of 1:1. Finally, AF647–HDM (500 nm-1) and DNsase I (400 µl ml⁻¹, Sigma-Aldrich) were added to the culture. Twelve hours later, cells were collected, washed, resuspended in FACS medium and stained for flow cytometry analysis.

Influenza A virus infection protocol. Influenza A virus strain A/PR8/34 (H1N1) was kindly provided by F. Trottée (Institut Pasteur). The viral stock suspension (10⁵ PFU ml⁻¹) was diluted, and 5 PFU were administered i.n. to isoflurane-anesthetized mice in 50 µl of PBS.

Assessment of viral mRNA expression. Lung apical lobes were excised, and total mRNA was isolated from homogenized tissue according to the Imgen protocol (www.imgen.org). cDNA was obtained with RevertAid First Strand cDNA Synthesis Kit (ThermoFisher Scientific), and qPCR was performed in duplicate with Taqq Universal SYBR green supermix (Biorad) and ABI 7900HT Fast Real-Time PCR System (Applied Biosystems). Primer sequences were as follows: 5′-TTCACCACTGCTTCTCTC-3′ and 5′-CCCATCTCATTCTGC-3′ for viral NS1, 5′-AGCCAGTTATCAACCAAG-3′ and 5′-ACCAAAGAAACGCAAGAG-3′ for housekeeping gene Ubc, 5′-TGCGGAGGATGTGGTGCC-3′ and 5′-AAATGTTGAGGCACTCCG-3′ for housekeeping gene GAPDH, 5′-CATGCTGCGTACGG-5′ and 5′-AATGCTG5′ GGAACTG-3′ for housekeeping gene B2m. Expression levels of NS1 were normalized relative to the three control genes (Ubc, B2m and Gapdh).

Model of PR8-triggered allergic asthma to HDM. Seven days after infection, mice were administered i.n. with 40 µg of HDM. Seven days later (day 7), all mice were challenged by i.n. instillation of 10µg of HDM. Three days after the challenge, animals were killed and features of allergic airway inflammation were assessed, as described above. In addition, levels of HDM-specific IgG1 were quantified, as described ¹.

Model of ozone-triggered allergic asthma to HDM. Naive mice were exposed for 3 consecutive days to 2 ppm ozone during 3 h. Twenty-four hours after the last ozone exposure, mice were administered i.n. with 40 µg of HDM. Seven days later, mice were challenged with 10 µg of HDM, and features of allergic airway inflammation were assessed 3 d later as described above.

Statistical analysis. Respect of the assumptions of normal distribution of residuals and homoscedasticity was verified, and data were presented as mean±s.e.m., as well as individual values, unless otherwise indicated. Data from independent experiments were pooled for analysis in each data panel, unless otherwise indicated. Statistical analyses were performed with Prism 6 (GraphPad Software). We considered a P value lower than 0.05 as significant.

Reporting Summary. Further information on research design is available in the Nature Research Reporting Summary linked to this article.
Data availability
The scRNA-seq data provided in this manuscript have been deposited in the ArrayExpress database at EMBL-EBI (https://www.ebi.ac.uk/arrayexpress/experiments/E-MTAB-6902) under accession number E-MTAB-6902. The figures that have associated scRNA-seq data are the following: Fig. 2, Supplementary Figs. 2 and 3. The source data underlying Fig. 1a–d,e,f, 2d, 3b,c,f,g,i,k, 4b–e,g,i–k,m, 5b–e,g, 6b,d,f, 7b–i,k–m and 8a–c,e,f–h,j and Supplementary Figs. 1b,d, 4c,d, 5b,c,e,g, 6b, 7b–d,f,g,i,j and 8a are provided as a Source Data file. Any data that support the findings of this study are available from the corresponding authors upon request.

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Reporting Summary

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Statistics

For all statistical analyses, confirm that the following items are present in the figure legend, table legend, main text, or Methods section.

- The exact sample size (n) for each experimental group/condition, given as a discrete number and unit of measurement
- A statement on whether measurements were taken from distinct samples or whether the same sample was measured repeatedly
- The statistical test(s) used AND whether they are one- or two-sided
- A description of all covariates tested
- A description of any assumptions or corrections, such as tests of normality and adjustment for multiple comparisons
- A full description of the statistical parameters including central tendency (e.g. means) or other basic estimates (e.g. regression coefficient) AND variation (e.g. standard deviation) or associated estimates of uncertainty (e.g. confidence intervals)
- For null hypothesis testing, the test statistic (e.g. F, t, r) with confidence intervals, effect sizes, degrees of freedom and P value noted
  - Give P values as exact values whenever suitable.
- For Bayesian analysis, information on the choice of priors and Markov chain Monte Carlo settings
- For hierarchical and complex designs, identification of the appropriate level for tests and full reporting of outcomes
- Estimates of effect sizes (e.g. Cohen’s d, Pearson’s r), indicating how they were calculated

Our web collection on statistics for biologists contains articles on many of the points above.

Software and code

Policy information about availability of computer code

Data collection

Cell Ranger software (v1.2.0) (10x Genomics) was used to demultiplex Illumina BCL files to FASTQ files (cellranger mkfastq), to perform alignment (to mouse GRCm38/mm10 genome), filtering, UMI counting and to produce gene – barcode matrices (cellranger count).

Data analysis

Single cell analysis used R bioconductor (version 3.4.2.), and the R package Seurat (version 2.1.0). Statistical enrichment tests for GO biological processes were performed with PANTHER 13.1. Flow cytometry analysis were performed with FlowJo (version 10.1, Tree Star, Ashland, USA). Immunofluorescence images were acquired on a Zeiss LSM 880 Airyscan Elyra S.1. confocal microscope (Zeiss) and analyzed using the ImageJ (version 15.1n) and Imaris (version 7.5.2) softwares. Statistics: GraphPad Prism 6 for PC, R Bioconductor.

For manuscripts utilizing custom algorithms or software that are central to the research but not yet described in published literature, software must be made available to editors/reviewers. We strongly encourage code deposition in a community repository (e.g. GitHub). See the Nature Research guidelines for submitting code & software for further information.

Data

Policy information about availability of data

All manuscripts must include a data availability statement. This statement should provide the following information, where applicable:

- Accession codes, unique identifiers, or web links for publicly available datasets
- A list of figures that have associated raw data
- A description of any restrictions on data availability

The scRNA-seq data provided in this manuscript have been deposited in the ArrayExpress database at EMBL-EBI (https://www.ebi.ac.uk/ arrayexpress/ experiments/ E-MTAB-6902) under accession number E-MTAB-6902. The figures that have associated raw data are the following: Fig. 2, Supplementary Figs. 2,3.
Life sciences study design

All studies must disclose on these points even when the disclosure is negative.

| Sample size | We assume that variance will be equal between experimental groups. We estimate that a difference will be biologically significant between two groups if a variation of at least 50% is observed between the mean of the two groups. If we want to compare two means of 1500 and 3000 with a standard deviation of 750 (example from the number of eosinophils in the bronchoalveolar lavage fluid of asthmatics and controls mice), the results of power analysis for expecting effects show that for a power of 0.9 (and $\alpha<0.05$) in a t student test, the required sample size would be 3-5 animals. In most of the experiments, 3 to 10 mice/group was sufficient to identify differences between groups with at least 90% power and a 5% significance level. |
|---|---|
| Data exclusions | No data was excluded from the analyses. |
| Replication | For each experiment, each experimental group was composed of 3-5 mice constituting biological replicates. Each experiments have been repeated two to four times. All attempts at replication were successful and gave similar readout. |
| Randomization | Female age-matched BALB/c mice were randomly allocated into experimental groups. |
| Blinding | Blinding was only relevant for histological analysis of H&E and PAS stained-lung sections. All other conclusions were made based on quantitative parameters and statistical significance. |

Reporting for specific materials, systems and methods

We require information from authors about some types of materials, experimental systems and methods used in many studies. Here, indicate whether each material, system or method listed is relevant to your study. If you are not sure if a list item applies to your research, read the appropriate section before selecting a response.

### Materials & experimental systems

| n/a | Involved in the study |
|---|---|
| ☑ | Antibodies |
| ☑ | Eukaryotic cell lines |
| ☑ | Palaeontology |
| ☑ | Animals and other organisms |
| ☑ | Human research participants |
| ☑ | Clinical data |

### Methods

| n/a | Involved in the study |
|---|---|
| ☑ | ChIP-seq |
| ☑ | Flow cytometry |
| ☑ | MRI-based neuroimaging |

### Antibodies

- **Antibodies used**
  - Anti-mouse FITC-, and V500-conjugated anti-CD45.2 (clone 104, dilution 1/100, #561874, #562130), PE-conjugated anti-CD3 (clone 142-2C11, dilution 1/100, #553064), PE-conjugated anti-CD19 (clone 1D3, dilution 1/300, #553786), PE-conjugated anti-NK1.1 (clone 1H6, dilution 1/300, # 553165), PE-conjugated anti-Siglec-F (clone E50-2440, dilution 1/500, #552126), PE-conjugated anti-Ly6C (clone 1A8, dilution 1/500, #551461), eFluor450- and PE-Cy7-conjugated anti-CD11b (clone M1/70, dilution 1/300, #560455, #561098), PE-Cy7-conjugated anti-Ly6G (clone 1A8, dilution 1/200, #560501), APC-Cy7-conjugated anti-CD11c (clone HL3, dilution 1/150, #561241), PE-Cy7-conjugated anti-Ly6G (clone 1A8, dilution 1/200, #560501), APC-Cy7-conjugated anti-CD11c (clone HL3, dilution 1/150, #561241), PE-CF594-conjugated anti-Ly6C (clone 49-44, dilution 1/200, #562728), FITC-conjugated anti-CD103 (clone M290, dilution 1/100, #557494) and PE-conjugated anti-Lamp-1 (CD107a) (clone 1D4B, dilution 1/100, #558661), Anti-mouse PE-conjugated anti-CXCR4 (clone 2B11, dilution 1/100, #561734) antibodies were purchased from BD biosciences. Anti-mouse PE-conjugated anti-CD49d (clone R1-2, dilution 1/100, #12-0982-81) antibodies were purchased from Biolegend. Anti-mouse PerCP-Cy5.5-conjugated anti-MHC-II (Ia-Lc) (clone M5/M4, dilution 1/100, #107626) and BV421-conjugated anti-CD64 (clone X54-5/7.1, dilution 1/100, #139309) antibodies were purchased from Biolegend. Unconjugated Goat anti-myeloperoxidase (dilution 1/40, #AF3667) was purchased from R&D systems and unconjugated rabbit anti-histone H3 citrullinated (dilution 1/100, #ab5103) was purchased from abcam.

- **Validation**
  - All the antibodies are from commercial sources and purchased from BD pharmingen, Ebioscience, Biolegend, R&D systems and abcam. The data sheets were provided by the manufacturer, in which the validation of antibodies for the specificity to the mouse antigen and for the usage for flow cytometry/western blot/confocal microscopy was confirmed. Statements about validation are found in manufacturer's website.
Animals and other organisms

Policy information about studies involving animals; ARRIVE guidelines recommended for reporting animal research

| Laboratory animals | Mus musculus, BALB/c, female, 6 to 10 weeks |
|--------------------|---------------------------------------------|
| Wild animals       | The study did not involve wild animals.     |
| Field-collected samples | The study did not involve samples collected from the field. |
| Ethics oversight   | All animal experiments described in this study were reviewed and approved by the Institutional Animal Care and Use Committee of the University of Liège. The “Guide for the Care and Use of Laboratory Animals”, prepared by the Institute of Laboratory Animal Resources, National Research Council, and published by the National Academy Press, as well as European and local legislations, were followed carefully. |

Note that full information on the approval of the study protocol must also be provided in the manuscript.

Flow Cytometry

Plots

Confirm that:
- The axis labels state the marker and fluorochrome used (e.g. CD4-FITC).
- The axis scales are clearly visible. Include numbers along axes only for bottom left plot of group (a ‘group’ is an analysis of identical markers).
- All plots are contour plots with outliers or pseudocolor plots.
- A numerical value for number of cells or percentage (with statistics) is provided.

Methodology

Sample preparation

To obtain single-lung-cell suspensions, lungs were perfused with 10 ml of PBS through the right ventricle, cut into small pieces, and digested for 1 h at 37°C in HBSS containing 1 mg/ml collagenase A (Roche) and 0.05 mg/ml DNase I (Roche). Blood was collected in an EDTA-containing tube (100 mM), and red blood cells were lysed with RBC lysis buffer (eBioscience). BM cells were isolated by flushing femurs with PBS. Staining reactions were performed at 4°C after incubation with 2.4G2 Fc receptor blocking antibodies to avoid non-specific binding. Anti-mouse Lamp-1 intracellular staining was performed using the Foxp3/Transcription factor Staining Buffer Set from eBiosciences according to the manufacturer’s instructions. Briefly, after extracellular staining, cells were resuspended in 200 μl of Fix/Perm buffer for 45 minutes at 4°C in the dark and then washed with 200 μl of permeabilization buffer. Cells were then stained for intracellular protein in permeabilization buffer for 35-40 minutes.

Instrument

Cell phenotyping and sorting were performed on a FACSCANTO II and a FACSARIA III (BD Biosciences), respectively.

Software

FlowJo (version 10.1, Tree Star, Ashland, USA)

Cell population abundance

Purity was between 90 and 95% and was determined by flow cytometry after sorting.

Gating strategy

Lung dendritic cells (DCs) were selected as CD45+CD3e-CD19-Siglec-F-Ly6G-NK1.1-MHCII+CD11c+ cells and were then subdivided into CD11b-CD103+, CD11b+Ly-6C- and CD11b+Ly-6C+ subsets; lung neutrophils were selected as SSchighCD45.2 +CD11bhighly-G6high cells. Bone marrow dendritic cells (BMDCs) were selected as MHC-II+CD11c+ cells and further divided between CD11b+Ly-6C+ and CD11b+Ly-6C- subsets.

Tick this box to confirm that a figure exemplifying the gating strategy is provided in the Supplementary Information.