Selective eosinophil necroptosis contributes to airway inflammation and remodeling in asthma

To the Editor,

Asthma is a widespread inflammatory disease characterized by airway inflammation, remodeling, and hyperresponsiveness (AHR),\(^{1,2}\) that eosinophils are assumed to play essential roles in the development of asthma and are associated with asthma severity.\(^{3,4}\) Recently, accumulating evidence demonstrates that programmed cell death plays critical roles in eosinophil degranulation and pathological processes. Necroptosis is characterized by membrane rupture and DAMPs release for inflammation,\(^{5,6}\) but its association with eosinophils remains poorly understood. In this study, we aimed to investigate the effects of eosinophil necroptosis in asthma, and further explore its potential application in asthma treatment.

First of all, higher levels of lactate dehydrogenase (LDH) were detected in the serum of asthma patients than healthy controls (Figure 1A; Table S1). Pearson correlation coefficients analysis showed that serum LDH levels were positively correlated with the percentage of blood eosinophils (Figure 1B), but negatively correlated with blood neutrophils in asthmatic patients (Figure 1C). Immunofluorescence staining showed that p-MLKL was mainly detected in BALF\(^{+}\) cells in asthmatic patients (Figure 1E,F) and OVA/CFA-induced model mice (Figure 1D and S1F). Meanwhile, same phenomenon was also detected in OVA/Alum-induced asthmatic model mice (Data not shown). All of these results indicate that necroptosis is involved in the process of asthma.

Immunofluorescence staining showed that p-MLKL was mainly detected in EMBP\(^{+}\) cells in asthmatic patients (Figure 1E,F) and OVA/CFA-induced mice (Figure S1F). Same phenomenon was confirmed in OVA/Alum-induced asthmatic model mice (Data not shown). ImageStream FCM analysis showed most of p-MLKL signaling in SiglecF\(^{+}\) but not Ly6G\(^{+}\) cells (Figure 1G), with higher MFI of p-MLKL in eosinophils than neutrophils (Figure 1H). All of these results proved that eosinophils but not neutrophils are the predominant targets of necroptosis in asthma.

Based on the Human Protein Atlas (HPA) database, lower level of CASP 8 and higher level of RIPK1 (Figure 1I) were detected in eosinophils than neutrophils, which could be one of the compelling arguments supporting eosinophils’ greater susceptibility to necroptosis.

To investigate the role of eosinophil necroptosis in asthma, we adoptively transferred eosinophils to the airways of OVA/CFA-induced mice (Figure S2A). WT eosinophil transference induced higher AHR than model mice, while no significant difference was detected between models and Mlkl\(^{-/-}\) eosinophil transference (Figure 2A), demonstrating that eosinophil necroptosis plays a positive role in AHR.

It was further confirmed that after WT but not Mlkl\(^{-/-}\) eosinophil transference, the inflammatory response was obviously upregulated, including pathological changes (Figure 2B), as well as more cell counts, higher levels of LDH and inflammatory cytokines in BALF (Figure 2C and S2C).

Considering the potential clinical application, we tested Nec-1 (inhibitor of necroptosis) in vivo (Figure S3A). Nec-1 treatment significantly attenuated AHR (Figure 2D) and pathology (Figure 2E and S3C–F). Furthermore, Nec-1 medication primarily suppressed type 2 immune response (IL-4 and IL-5) (Figure 2F), as well as pro-inflammatory cytokine TNF-\(\alpha\) and eosinophil-specific chemokine Eotaxin-1 (Figure 2G), but less influence on the type 17 immune response or other inflammation (Figure S3G).

We further investigated the interaction between eosinophils and epithelial cells in vitro. BALF from asthma patients could directly promote necroptosis of EoL-1 (Because of the medical ethics, PBS treatment was used as negative control. Figure 2H and S4A). In the EoL-1 and 16HBE co-culture system, 16HBE cells were induced to death (PI positive staining, Figure 2I and S4B,C) and mucin (MUC5AC) expression (Figure 2J and S4D) after stimulation with asthma patients’ BALF. Data suggested eosinophils to be induced to necroptosis in the airway, and further communicated with epithelium for epithelial injury and mucin secretion.

In a summary, we highlighted the phenomenon that necroptosis selectively targets eosinophils but not neutrophils, which is associated with the pathological process of airway inflammation, epithelial damage, and remodeling (Figure S4E). Our findings expand current understanding of eosinophils in the pathogenesis of asthma and provide novel strategies for clinical application.

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Abbreviation: BALF, Bronchoalveolar Lavage Fluid; CFA, Complete Freund’s adjuvant; DAMPs, damage-associated molecular patterns; EMBP, Eosinophil Major Basic Protein; MFI, mean fluorescence intensity; MLKL, Mixed Lineage Kinase Domain Like Pseudokinase; OVA, Ovalbumin; RIPK1 Receptor-interacting protein kinase 1

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FIGURE 1  Necroptosis selectively concentrates in eosinophils but not neutrophils in the process of asthma. (A) LDH test in serum from asthma patients \( (n = 16) \) and healthy controls \( (n = 12) \). (B and C) Linear correlation between serum LDH and the percentage of blood eosinophils (B) or neutrophils (C) through Pearson correlation coefficient test. (D) Immunoblot in lungs of OVA/CFA-induce mice, and grayscale of p-MLKL through ImageJ \( (n = 4) \). (E and F) Immunofluorescence staining in Pulmonary specimens from asthma patients and healthy controls (E); the number of p-MLKL+ cells and the ratio of necroptotic eosinophils (F) through ImageJ. (G and H) Images of necroptotic eosinophils and neutrophils in pulmonary cell suspension of OVA/CFA-induced mice through IF staining and imageStream flow cytometry (G); mean fluorescence intensity (MFI) of p-MLKL in eosinophils and neutrophils \( (n = 7/\text{group}) \) (H). (I) Relative mRNA levels in neutrophils and eosinophils through the HPA database. Data are presented as mean \( \pm \) SD. \(* p < .05, ** p < .01, *** p < .001\), as analyzed by two-tailed unpaired student’s t test.
Figure 2: Role of eosinophil necroptosis in asthma. (A–C) Bone marrow induced eosinophils from wild-type or Mikl₁⁻/⁻ mice were adoptively transferred into OVA/CFA-induced mice. AHR assessment (n = 4/group) (A); HE and PAS staining in lung sections (B); total cell counts in BALF, LDH levels in BALF and cytokine levels in pulmonary homogenates through ELISA (C). (D–G) OVA/CFA-induced mice intratracheally treated with Nec-1 (2 mg/kg) or PBS as control. AHR assessment (n = 3–4/group) (D); total cell counts in BALF (n = 4/group), inflammatory scores and PAS⁺ ratio through ImageJ (n = 6/group) (E); cytokine levels in lung homogenates by ELISA (n = 4–5/group) (F and G). (H) Immunoblot in EoL-1 treated with asthmatic patients’ BALF or PBS as control. (I and J) 16HBE co-cultured with EoL-1, followed with BALF or PBS treatment for 12 h, and IF stained with PI/Hoechst or anti-MUC5AC/DAPI. The PI⁺ ratio (I) and MFI of MUC5AC (J) in total cells through ImageJ. Data are presented as mean ± SD. *p < .05, **p < .01, ***p < .001, as analyzed by One-way ANOVA test (A and D) or two-tailed unpaired Student’s t-test.
AUTHOR CONTRIBUTION
A. H performed the experiments and analyzed data; J. C, J. G, Y. H, and H. X performed Western blotting and analysis; H. C and Y. W performed animal experiments; Q. C and S. X performed LDH test; H. L and C. O performed histological H&E staining and IHC analysis; Q. Z and A. T provided consults; J.Y designed and supervised the project; A. H and J. Y wrote the manuscript.

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CONFLICTS OF INTERESTS
All of authors declared no competing financial interests.

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