Homeostatic and early-recruited CD101^{-} eosinophils suppress endotoxin-induced acute lung injury

Chen Zhu[1,6], Qing-Yu Weng[1,6], Ling-Ren Zhou[1,6], Chao Cao[2], Fei Li[1], Yin-Fang Wu[1], Yan-Ping Wu[1], Miao Li[1], Yue Hu[1], Jia-Xin Shen[1], Xue-Fang Xiong[1,3], Fen Lan[1], Li-Xia Xia[1], Bin Zhang[1], Hao Zhang[1], Man Huang[4], Song-Min Ying[1], Hua-Hao Shen[1,5,7], Zhi-Hua Chen[1,7] and Wen Li[1,7]

Affiliations: [1]Key Laboratory of Respiratory Disease of Zhejiang Province, Dept of Respiratory and Critical Care Medicine, Second Affiliated Hospital of Zhejiang University School of Medicine, Hangzhou, China. [2]Dept of Respiratory Medicine, Ningbo First Hospital, Ningbo, China. [3]Dept of Respiratory Medicine, The Central Hospital of Lishui City, Lishui, China. [4]Dept of Central Intensive Care Unit, Second Affiliated Hospital of Zhejiang University School of Medicine, Hangzhou, China. [5]State Key Lab for Respiratory Diseases, Guangzhou, China. [6]These authors contributed equally to this article. [7]These authors contributed equally to this article as lead authors and supervised the work.

Correspondence: Wen Li, Key Laboratory of Respiratory Disease of Zhejiang Province, Dept of Respiratory and Critical Care Medicine, Second Affiliated Hospital of Zhejiang University School of Medicine, Hangzhou, Zhejiang, 310009, China. E-mail: liwen@zju.edu.cn

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Eosinophils, a type of immune cell easily overlooked in nonallergic inflammatory diseases, could reside and be rapidly recruited into lungs to suppress endotoxin-induced acute lung injury
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ABSTRACT

Introduction: Acute lung injury (ALI) is a fatal but undertreated condition with severe neutrophilic inflammation, although little is known about the functions of eosinophils in the pathogenesis of ALI. Our objectives were to investigate the roles and molecular mechanisms of eosinophils in ALI.

Methods: Pulmonary eosinophils were identified by flow cytometry. Mice with abundant or deficient eosinophils were used. Cellularity of eosinophils and neutrophils in bronchoalveolar lavage fluid, inflammatory assessment, and survival rate were determined. Human samples were also used for validating experimental results.

Results: Blood eosinophils were increased in surviving patients with acute respiratory distress syndrome (ARDS) independent of corticosteroid usage. There existed homeostatic eosinophils in lung parenchyma in mice and these homeostatic eosinophils, originating from the bone marrow, were predominantly CD101^{-}. More CD101^{-} eosinophils could be recruited earlier than lipopolysaccharide (LPS)-initiated neutrophilic inflammation. Loss of eosinophils augmented LPS-induced pulmonary injury. Homeostatic CD101^{-} eosinophils ameliorated, while allergic CD101^{+} eosinophils exacerbated, the neutrophilic inflammation induced by LPS. Likewise, CD101 expression in eosinophils from ARDS patients did not differ from healthy subjects. Mechanistically, CD101^{-} eosinophils exhibited higher levels of Alox15 and Protectin D1. Administration of Protectin D1 isomer attenuated the neutrophilic inflammation.

Conclusions: Collectively, our findings identify an uncovered function of native CD101^{-} eosinophils in suppressing neutrophilic lung inflammation and suggest a potential therapeutic target for ALI.

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Introduction

Acute lung injury (ALI)/acute respiratory distress syndrome (ARDS) is a clinical disorder characterised by increased pulmonary vascular permeability, massive inflammation, and subsequent pulmonary oedema and refractory hypoxia [1, 2], leading to high mortality and morbidity. During the process of ALI/ARDS, accumulated inflammatory cells and cytokines induce the disruption of the capillary endothelial and alveolar epithelial barrier, and promote subsequent pulmonary oedema and hypoxia [3]. Despite a wide variety of risk factors for ARDS [4], inhibition of pulmonary inflammation represents a common therapeutic strategy for this critical syndrome.

Eosinophils are known as the terminal effector cells during parasite infection or allergic inflammation, but growing evidence has demonstrated their regulatory effects in various immune responses [5]. It has been reported that eosinophils interact with dendritic cells to synergistically promote the T-helper cell type 2 immune response in the allergic airway [6, 7]. Interestingly, a recent clinical investigation showed that ALI survivors exhibited an increased number of eosinophils in the lung compared with nonsurvivors [8], suggesting a protective role of eosinophils in ALI. Conversely, patients with eosinophil-dominant diseases, such as asthma, appear to be at high risk for infection [9]. Hence, the eventual functions of eosinophils and their molecular mechanisms in the pathogenesis of ALI require further investigations.

Tissue-resident cells frequently demonstrate unique functions in maintaining the milieu of certain organs or systems. Lung-resident alveolar macrophages are known to display a distinctive origin, surface markers and functions [10, 11]. While the resident eosinophil subgroup was mostly identified in tissues like the gastrointestinal tract, adipose tissue, uterus, spleen, thymus or mammary glands [12], Mesnil *et al.* [13] have recently demonstrated that there are also homeostatic eosinophils in the lung. This study also implies that SiglecF^{+}CD125^{+} eosinophils display distinctive subgroups distinguished by CD101. Nonetheless, little is known about the origination and functions of lung homeostatic eosinophils, especially in noneosinophilic diseases.

The present study aims to explore the origination and function of lung eosinophils in an endotoxin-induced ALI model using eosinophil-deficient PHIL mice. We also provide evidence that homeostatic CD101^{-} and allergy-induced CD101^{+} eosinophils exert distinct functions in ALI through different molecular mechanisms.

Methods

Details of methods are available in the supplementary material.

Statistics analysis

The statistical graphs of each experiment were prepared using Prism version 7.0 (GraphPad, La Jolla, CA, USA) and data are presented as mean with standard deviation. For statistics analysis, the two-tailed t-test and one-way ANOVA were used, unless otherwise specified. For survival curve analysis, the Mantel–Cox test was used. Categorical variables in patient information were analysed by Pearson's Chi-squared tests. Significance was accepted at $p<0.05$.

Results

Increased blood eosinophil cellularity in surviving ARDS patients is independent of corticosteroid usage

We first examined the clinical relevance of eosinophils in ALI. It is ethically not correct to undertake lung biopsy of ALI/ARDS patients due to the potential risks. Thus, we performed a retrospective analysis referring to all the diagnosed ARDS inpatients of the Second Affiliated Hospital of Zhejiang University School of Medicine (Hangzhou, China) from 2012 to 2018. Brief demographic profiles are summarised in supplementary table S1 and the inclusion criteria are presented in figure 1a. We did not observe any differences in the amount of blood eosinophils among healthy controls, surviving ARDS patients and nonsurviving ARDS patients at basal levels. Interestingly, the surviving individuals displayed an elevated eosinophil cellularity following clinical treatment, whereas the nonsurvivors did not (figure 1b). We further analysed the doses of corticosteroid used in these patients and found that there were insignificant differences in the levels of corticosteroid usage, either intravenous (*i.v.*) (systemically) or inhaled, between surviving and nonsurviving ARDS patients (figure 1c and d). These data suggest that increased levels of blood eosinophils are associated with an increased survival in ARDS patients, independent of the usage of corticosteroid.

Homeostatic and early-induced eosinophils during ALI are localised in lung parenchyma

To assess the possible function of eosinophils in the pathogenesis of ALI, we examined the homeostasis and flux of lung eosinophils in lipopolysaccharide (LPS)-induced ALI. In our study, eosinophils were

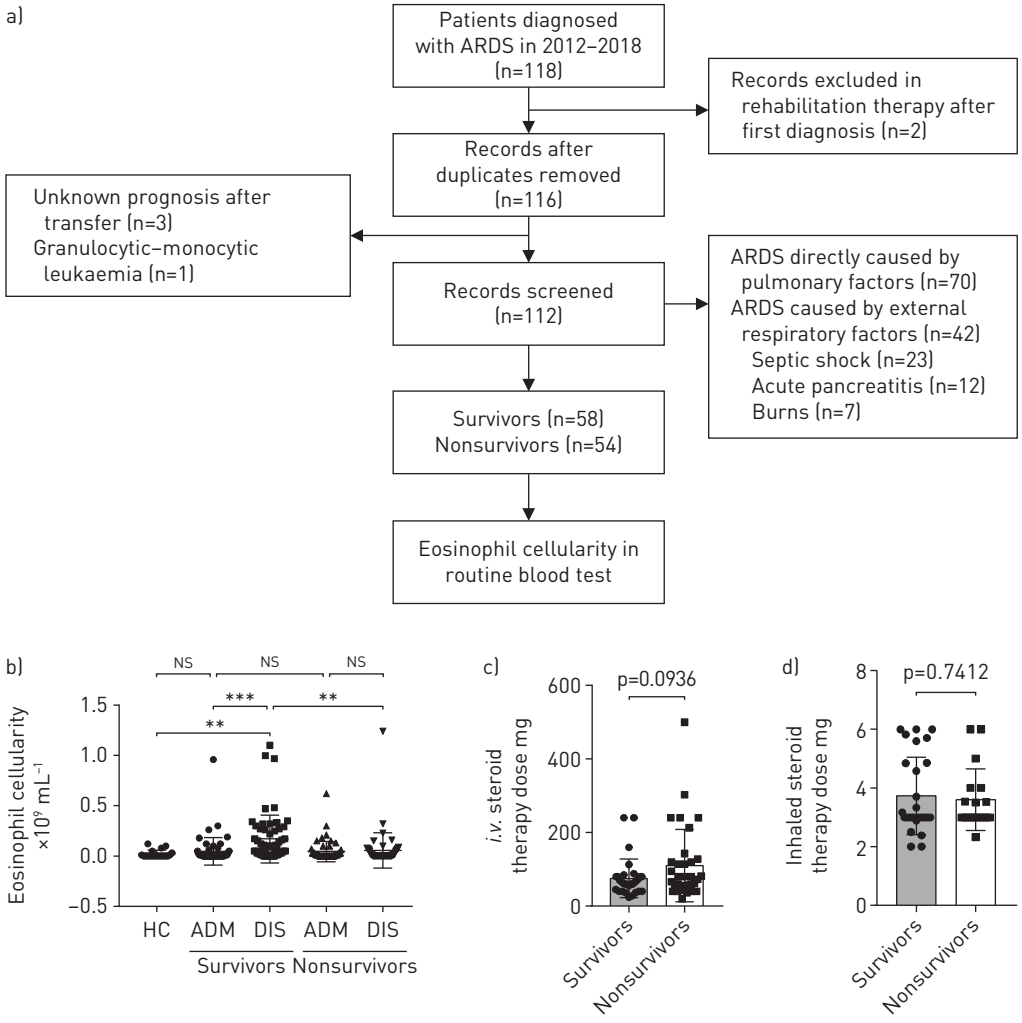
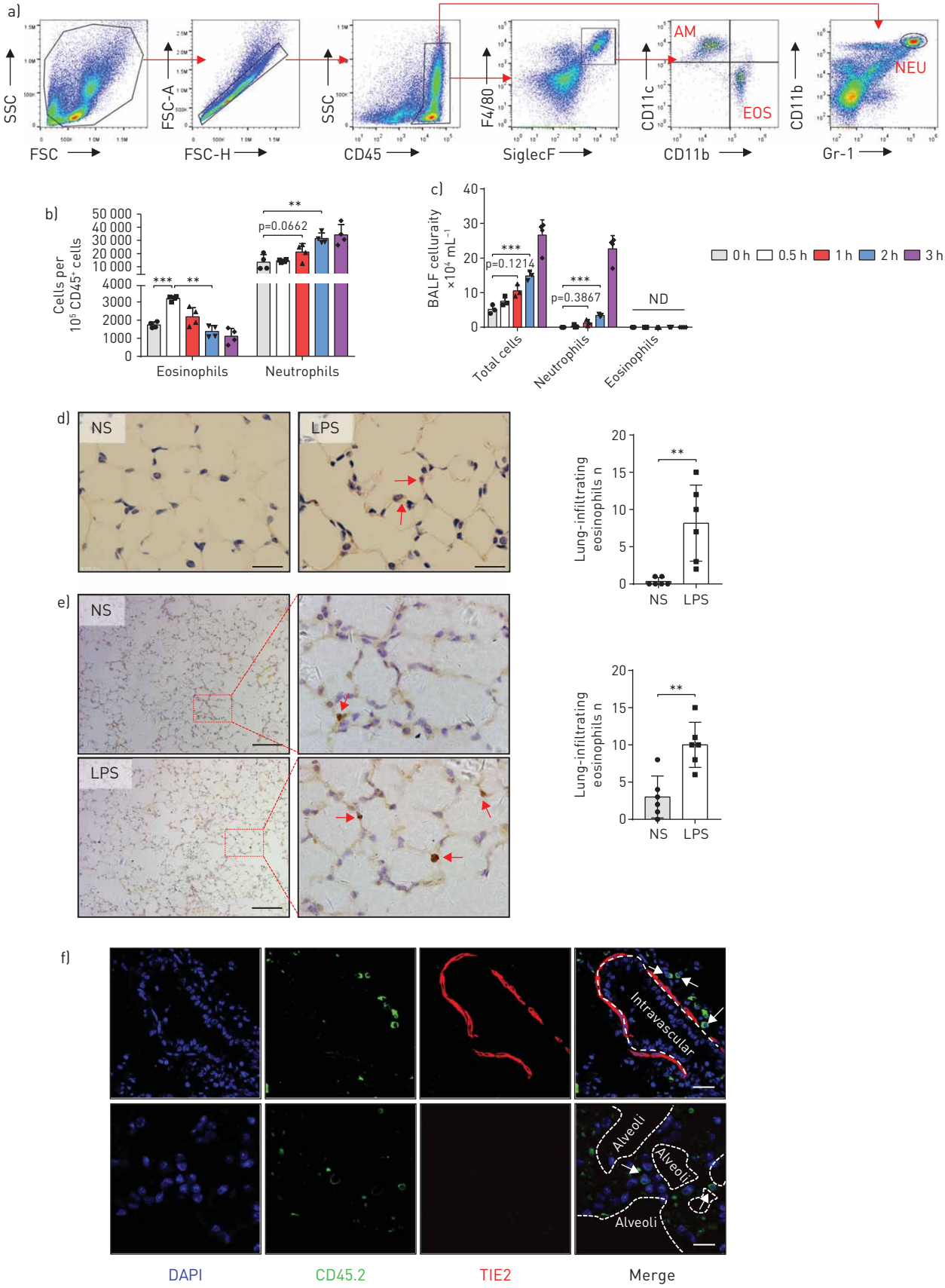


FIGURE 1 Retrospective analysis of eosinophil cellularity and prognosis in acute respiratory distress syndrome (ARDS) inpatients. HC: healthy control; ADM: admission (within 24 h of hospitalisation); DIS: discharge (no more than 24 h before discharged or declaration of death). a) Inclusion criteria for the analysis. A total of 118 ARDS inpatients were filtered, and the remaining cases were divided into survivor and nonsurvivor groups for further analysis. b) Blood eosinophil cellularity in peripheral blood in healthy volunteers, surviving and nonsurviving ARDS patients. c, d) Corticosteroid administration in ARDS patients: c) daily dose of *i.v.* corticosteroid (calculated as methylprednisolone) and d) daily dose of inhaled corticosteroid (calculated as budesonide). NS: nonsignificant; **: p<0.01; ***: p<0.001.

identified as $CD45^+SiglecF^+F4/80^+CD11b^+CD11c^-$, alveolar macrophages were labelled as $CD45^+SiglecF^+F4/80^+CD11b^-CD11c^+$ according to previous reports [10, 14] and neutrophils were defined as $CD45^+Gr\text{-}1^{hi}CD11b^{hi}$ (figure 2a). The gating strategy was simplified for the assays where neutrophils were not stained (supplementary figure S1) [15]. In agreement with previous findings [13], there existed a notable amount of "resident" eosinophils at the basal condition in the lung (figure 2b). Interestingly, the number of pulmonary eosinophils was comparable with alveolar macrophages and was much higher than that in the circulation (supplementary figure S2). More intriguingly, a rapid but transient increase of eosinophils was observed within 30 min after LPS exposure, while neutrophils were elevated remarkably later after 2 h (figure 2b). Similarly, the total inflammatory cells and the number of neutrophils in bronchoalveolar lavage fluid (BALF) were also significantly increased after 2 h (figure 2c and supplementary figure S3). Also, the early induction of pulmonary eosinophils was validated by *Staphylococcus aureus* injection intratracheally (*i.t.*) in mice (supplementary figure S4).

Surprisingly, eosinophils were not detectable in BALF (figure 2c). Congo red staining (figure 2d) and eosinophil peroxidase immunohistochemistry staining (figure 2e) suggested that lung "resident" eosinophils were located in lung parenchyma. To obtain better visible evidence, CD45.1 wild-type (WT) mice were *i.v.* injected with 1×10^6 CD45.2 eosinophils (from NJ.1638 mice) and the lung sections were


a)
SSC
FSC
FSC-A
FSC-H
SSC
CD45
F4/80
SiglecF
AM
EOS
CD11c
CD11b
NEU
CD11b
Gr-1
b)
Cells per 10^5 CD45+ cells
50 000
40 000
30 000
20 000
10 000
4000
3000
2000
1000
0
***
**
**
p=0.0662
Eosinophils
Neutrophils
c)
BALF cellularity ×10^4 mL^-1
40
30
20
10
0
***
p=0.1214
***
p=0.3867
ND
Total cells
Neutrophils
Eosinophils
0 h
0.5 h
1 h
2 h
3 h
d)
NS
LPS
Lung-infiltrating eosinophils n
20
15
10
5
0
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NS
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10
5
0
**
NS
LPS
f)
Intravascular
Alveoli
Alveoli
Alveoli
DAPI
CD45.2
TIE2
Merge

FIGURE 2 Analysis of homeostatic and early-induced eosinophils in lung parenchyma. SSC: side scatter; FSC: forward scatter; LPS: lipopolysaccharide; BALF: bronchoalveolar lavage fluid; NS: normal saline; DAPI: 4′,6-diamidino-2-phenylindole. a) Gating strategy of alveolar macrophages (AM; $CD45^+SigelcF^+F4/80^+CD11b^-CD11c^+$), eosinophils (EOS; $CD45^+SigelcF^+F4/80^+CD11b^+CD11c^-$) and neutrophils (NEU; $CD45^+Gr\text{-}1^{hi}CD11b^{hi}$) in flow cytometry analysis. b) Influx of eosinophils and neutrophils in mouse lungs upon LPS challenge. c) Total cell, neutrophil and eosinophil counts in BALF after LPS instillation. d) Representative images (left panel; scale bar: 50 µm) and semiquantification (right panel) of Congo red staining of eosinophils (arrows) in mouse lungs 0.5 h after LPS challenge. e) Representative images (left panel; scale bar: 100 µm) and semiquantification (right panel) of eosinophil peroxidase immunohistochemistry staining (arrows) in mouse lungs 0.5 h after LPS challenge. f) Representative images of transferred eosinophils. Blue: DAPI; red: TIE2 (endothelial cells); green: CD45.2 (transferred eosinophils). Upper panel: perivascular structure. Intravascular areas are outlined by white dotted lines. Bottom panel: parenchyma and alveoli. Alveolar structures are outlined by white dotted lines. Transferred eosinophils are marked by white arrows. Scale bar: 5 µm. Sample size is indicated as individual plots in column

collected immediately after the injection for immunofluorescence staining of CD45.2. The results revealed that *i.v.* flushing eliminated the majority of circulating cells (supplementary figure S5) and there were still $CD45.2^+$ eosinophils in the perivascular area or lung parenchyma (figure 2f). These results suggest that circulating eosinophils readily migrate to and reside in lung parenchyma during homeostasis, and could be further recruited during the initiation of ALI.

Lung parenchymal eosinophils originate from bone marrow and are recruited from peripheral blood

Previous study has classified pulmonary eosinophils as "resident" or "inflammatory" [13]. To explore the origin of "resident" and LPS-induced parenchymal eosinophils, we established several chimeric mice. First, WT or PHIL mice received total bone marrow transplant from WT or PHIL mice by tail vein injection following irradiation (figure 3a). PHIL mice are eosinophil deficient due to insertion of diphtheria toxin A in the eosinophil peroxidase promoter (supplementary figure S6). At 1 month after bone marrow reconstitution, eosinophils were uniquely diminished in lung tissue of PHIL→WT mice but were repopulated in lung tissue of WT→PHIL mice (figure 3a). To further verify this phenotype, CD45.2 WT mice were used as recipients, whereas CD45.1 WT mice were used as donors (figure 3b). Within 1 month of bone marrow transfer, in those CD45.1→CD45.2 chimeric mice, pulmonary eosinophils were repopulated as CD45.1 in a time-dependent manner (figure 3b), while lung-resident alveolar macrophages remained predominantly as CD45.2 (supplementary figure S7).

To further clarify whether eosinophils could be recruited during ALI, 5×10^5 eosinophils were injected into PHIL mice through the tail vein and the mice were immediately challenged with LPS (figure 3c). Intriguingly, after LPS administration, eosinophils were notably accumulated in lung tissue and there was a synchronous decrease of eosinophils in peripheral blood (figure 3c). We also validated the rapid recruitment of eosinophils in WT mice. CD45.1 mice received CD45.2 eosinophils injected *via* the tail vein and were subsequently challenged with LPS (figure 3d). Similarly, the amount of CD45.2 eosinophils was significantly increased in the lung after LPS instillation (figure 3d; fluorescence-activated cell sorting and scatter images). Interestingly, the induction of eotaxin was later (2 h) than the increase of eosinophils (30 min) after LPS challenge (supplementary figure S8), suggesting that LPS-induced early recruitment of eosinophils might be eotaxin independent.

Several additional experiments were conducted to test whether lung "resident" eosinophils could undergo local replication upon LPS treatment. *In vivo*, WT mice were injected with BrdU. Similarly, pulmonary eosinophils displayed $BrdU^-$ subsets, while circulating monocytes ($CD45^+CD115^+$) exhibited $BrdU^+$ subsets (supplementary figure S9a). Again, very few sorted eosinophils displayed as an EdU^+ cluster, whereas as a positive control, eosinophil cell line Eol-1 was stained isochronously (supplementary figure S9b). Finally, when a colony-forming assay was performed, we did not observe any colonies in the sorted eosinophil group (supplementary figure S9c).

Taken together, these data demonstrate that the "resident" parenchymal eosinophils originate from bone marrow and recruitment of pulmonary eosinophils from peripheral blood occurs during homeostasis or rapidly upon pathogen exposure.

Eosinophil deficiency exacerbates LPS-initiated pulmonary inflammation and ALI

To investigate the role of eosinophils during the initiation of ALI, PHIL mice were challenged with LPS. As shown in figure 4a, neutrophilic inflammation in lung tissue of PHIL mice was evidenced earlier than that of WT mice. Additionally, inflammatory cells and cytokines in BALF (figure 4b and c) were significantly increased in PHIL mice during the initiation of ALI.

To assess the eventual levels of ALI, the inflammatory hallmarks were examined 24 h after LPS administration. Consistently, inflammation in LPS-treated PHIL mice was augmented compared with WT mice, as evidenced by leukocyte infiltration (supplementary figure S10a) and inflammatory mediators in BALF (supplementary figure S10b). Similarly, PHIL mice also displayed an exacerbation of pulmonary

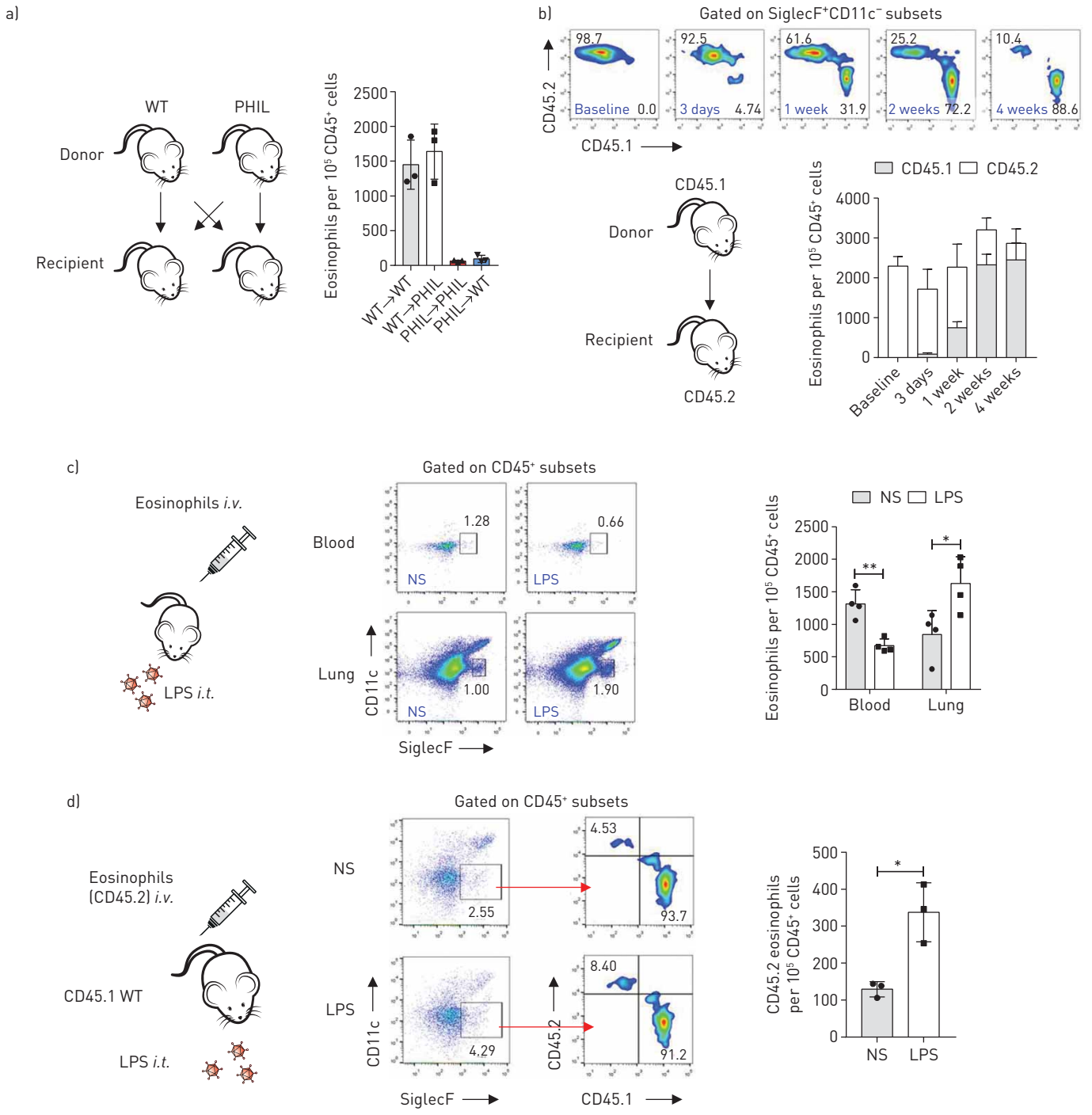

FIGURE 3 Pulmonary eosinophils are derived from bone marrow and are recruited from peripheral blood upon lipopolysaccharide (LPS) challenge. WT: wild-type; NS: normal saline; FACS: fluorescence-activated cell sorting. a) Levels of pulmonary eosinophils in the bone marrow transferred mice. Bone marrow from WT or PHIL mice was transferred into WT or PHIL mice, as shown in the left panel. b) Representative levels of CD45 subtypes of pulmonary eosinophils (FACS plot) and statistical results (column graph) in the bone marrow transferred mice. Bone marrow from CD45.1 mice was transferred into CD45.2 recipients. c) Representative FACS images and quantified results of eosinophils in blood and lungs of PHIL mice receiving eosinophils 0.5 h after LPS challenge. 5×10^5 eosinophils were transferred into PHIL mice by tail vein injection and the mice were immediately challenged with LPS. d) Representative FACS images and quantified results of pulmonary CD45.2 eosinophils in CD45.1 WT mice adoptively receiving CD45.2 eosinophils 0.5 h after LPS challenge. 5×10^5 eosinophils (CD45.2) from NJ.1638 mice were injected into CD45.1 WT mice through the tail vein and the mice were immediately challenged with LPS. Sample size is indicated as individual plots in column graphs. Data are representative of three independent experiments. *: $p<0.05$; **: $p<0.01$.

inflammation when exposed to *S. aureus* (supplementary figure S11). Consistent with these findings, pulmonary oedema (figure 4d), endothelial leakage (figure 4e and f) and elastance (figure 4g) were also more severe in LPS-treated PHIL mice. Consequently, we observed a decrease in both body weight (supplementary figure S10c) and assessment score (supplementary figure S10d) of PHIL mice. Furthermore, PHIL mice still displayed elevated levels of pulmonary inflammation 72 h after LPS instillation (figure 4h) and eventually experienced higher mortality compared with LPS-challenged WT mice (figure 4i).

Thus, eosinophil deficiency leads to enhanced inflammation and consequent poorer living status. All the aforementioned results indicate that the lung "resident" and the early-induced eosinophils might be crucial characters in inhibiting the initiation of ALI.

CD101 is a marker to distinguish different subtypes of eosinophils

The aforementioned results suggest a protective role of eosinophils in ALI, which seems not to be allied with the clinical evidence that allergic patients are susceptible to infection [9, 16, 17]. Thus, we established

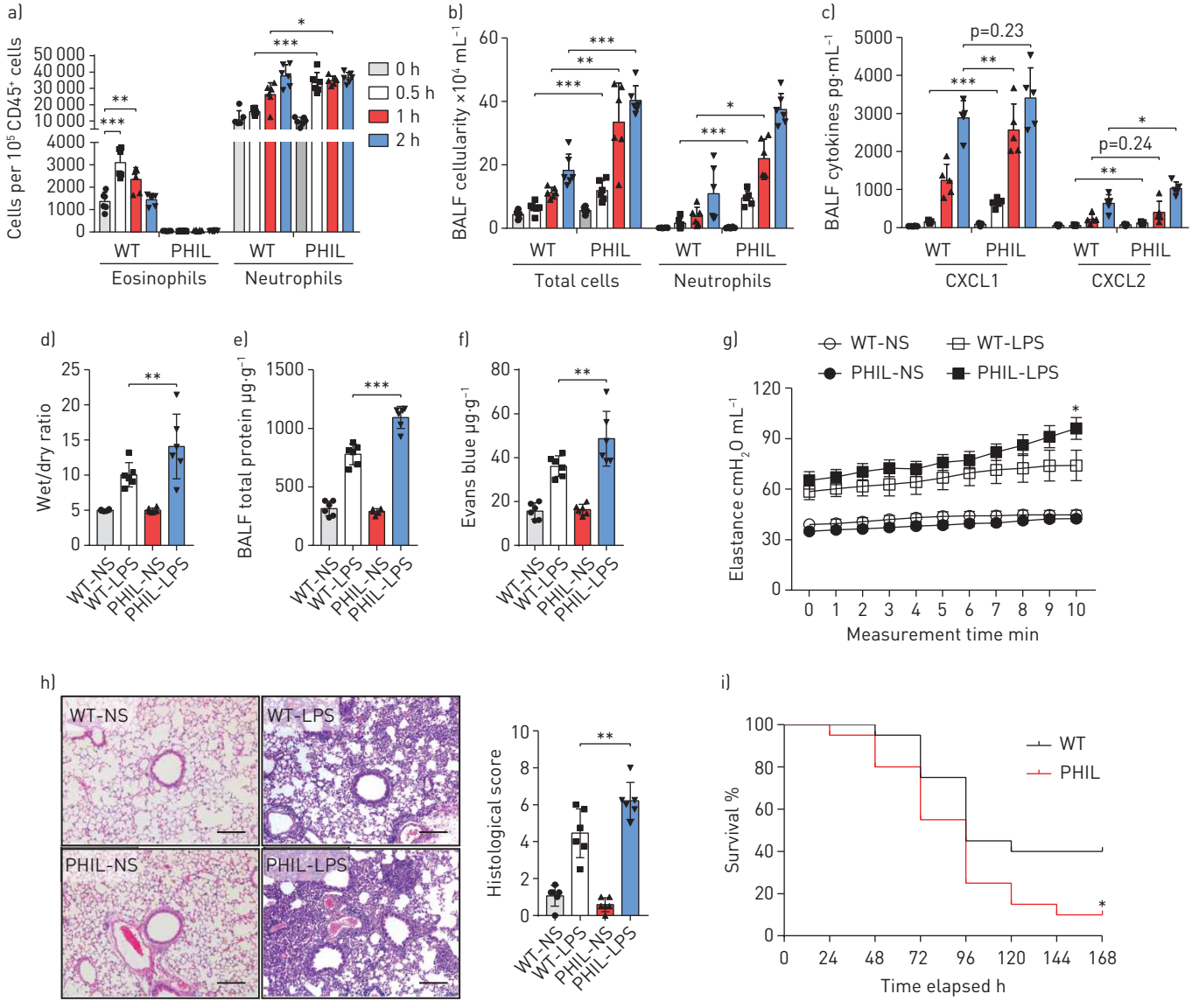

FIGURE 4 Loss of eosinophils augments lipopolysaccharide (LPS)-induced pulmonary inflammation and injury. WT: wild-type; BALF: bronchoalveolar lavage fluid; NS: normal saline. a) Pulmonary eosinophil and neutrophil detection in LPS-treated WT and PHIL mice by fluorescence-activated cell sorting within 2 h after LPS injection. b) Total leukocytes and neutrophils and c) CXCL1 and CXCL2 secretion in BALF of WT and PHIL mice induced by LPS at early time-points. d–g) Levels of d) pulmonary oedema, e, f) endothelial leakage and g) lung elastance were analysed 24 h after LPS injection. h) Pulmonary inflammation 72 h after LPS instillation. Scale bar: 200 µm. i) Eventual mortality of WT and PHIL mice after LPS challenge. Sample size is indicated as individual plots in column graphs. g) n=5; i) n=20. Data are representative of three independent experiments. *: p<0.05; **: p<0.01; ***: p<0.001.

an ovalbumin (OVA)/LPS model as shown schematically in figure 5a. Interestingly, we observed that earlier OVA challenge notably exacerbated LPS-induced infiltration of leukocytes in BALF (figure 5b), regardless of the higher levels of eosinophils in the lung induced by OVA (supplementary figure S12).

To explore the paradoxical effects of eosinophils in the process of ALI pathogenesis, we analysed the heterogeneity of eosinophils and observed two subgroups ($CD101^-$ and $CD101^+$) of eosinophils in lung tissues. Under normal conditions, the majority were $CD101^-$ eosinophils; however, in OVA-challenged lung tissue, the levels of $CD101^+$ eosinophils were higher than in $CD101^-$ eosinophils (figure 5c).

Similarly, the major subgroup of eosinophils in peripheral blood and bone marrow was again predominantly $CD101^-$ at steady state (figure 5d). OVA challenge significantly induced both $CD101^-$ and

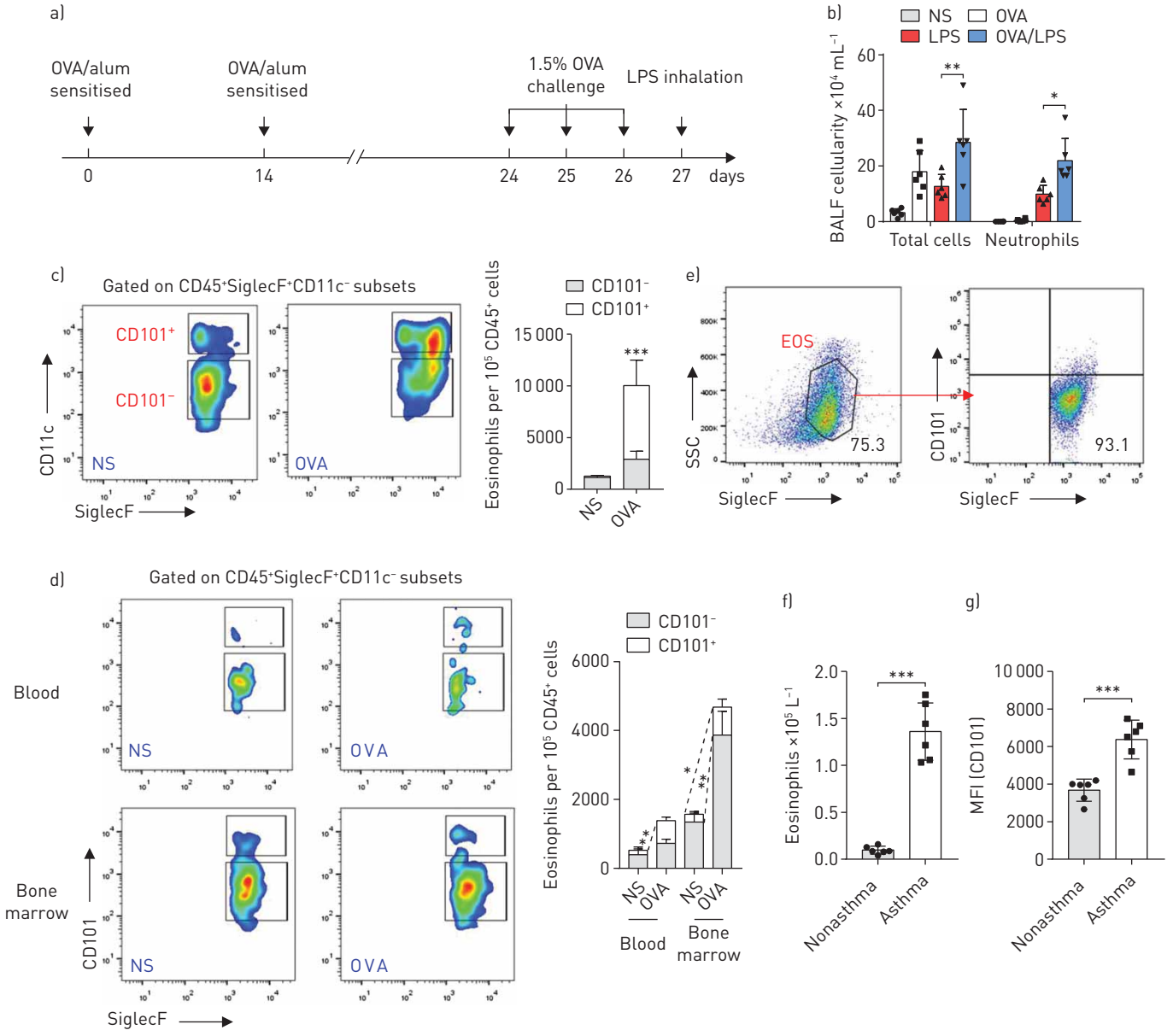

FIGURE 5 CD101 is a marker to distinguish subgroups of eosinophils in both the steady phase and allergic milieu. OVA: ovalbumin; LPS: lipopolysaccharide; BALF: bronchoalveolar lavage fluid; NS: normal saline; SSC: side scatter; MFI: mean fluorescence intensity. a) Scheme of the OVA/LPS overlap model. For studying the initiation of acute lung injury, mice were sacrificed 0.5 h after LPS administration. b) Amounts of total cells and neutrophils in BALF induced by OVA challenge combined with LPS injection. c, d) Eosinophil subgroups in c) lung tissue, d) peripheral blood and d) bone marrow in OVA-challenged mice. Representative plots are shown in c) and d), where eosinophils were gated on $CD45^+SiglecF^+CD11c^-$ singlets. e) Cellularity of bone marrow-derived eosinophils (EOS; left panel) and their CD101 expression (right panel). f) Amount of eosinophils in peripheral blood and g) expression of CD101 in eosinophils from nonasthmatic and asthmatic individuals. In b), sample size is indicated as individual plots in column graphs. In c) and d), every group consists of five individuals. Data are representative of three independent experiments, except for f) and g). *: $p<0.05$; **: $p<0.01$; ***: $p<0.001$.

$CD101^+$ eosinophils in bone marrow and blood; however, the majority were still $CD101^-$ (figure 5d). LPS treatment at early times failed to alter CD101 levels of eosinophils in the periphery (supplementary figure S13). Additionally, we also conducted bone marrow-derived eosinophil differentiation *in vitro* and observed that the differentiated eosinophils induced by interleukin (IL)-5 were predominantly $CD101^-$ (figure 5e). Likewise, eosinophils from peripheral blood of IL-5-transgenic NJ.1638 mice were also predominantly $CD101^-$ and the factors involved in eosinophil differentiation, or house dust mite exposure, could not induce the $CD101^-$ eosinophils to be positive (supplementary figure S14).

To further explore the relevance of our results in humans, we also detected the expression of CD101 in eosinophils from both nonasthmatic and asthmatic patients. Not surprisingly, asthmatic patients displayed higher eosinophil cellularity in peripheral blood (figure 5f). Likewise, the expression of CD101 was significantly elevated in eosinophils from asthmatic patients (figure 5g).

Taken together, these data suggest that CD101 is a marker to distinguish eosinophils between their naive and allergy-induced status, and that molecules other than IL-5 and granulocyte–macrophage colony-stimulating factor might drive $CD101^-$ eosinophils to be positive in an allergic microenvironment.

$CD101^-$ eosinophils ameliorated, while $CD101^+$ eosinophils exacerbated, the neutrophilic inflammation induced by LPS

We next examined the possible different functions of the subtypes of eosinophils during the process of initiation of ALI. Interestingly, most of the increased eosinophils upon LPS challenge in the lungs were $CD101^-$ (figure 6a) and, similarly, the amount of $CD101^-$ eosinophils showed isochronous aggregation in *S. aureus* infection (supplementary figure S15), suggesting that the $CD101^-$ and $CD101^+$ eosinophils may play distinct roles in ALI pathogenesis. To address this, $CD101^-$ or $CD101^+$ eosinophils were isolated from OVA-induced allergic mice and were adoptively transferred into PHIL mice, followed by LPS challenge. The efficacy of adoptive transfer is presented in supplementary figure S16. Intriguingly, $CD101^-$ eosinophils relieved, while $CD101^+$ eosinophils exacerbated, LPS-induced early leukocyte aggregation (figure 6b) and cytokine production (figure 6c). Similar effects were observed in these mice 24 h after LPS challenge. Following adoptive transfer, $CD101^-$ eosinophils decreased, yet $CD101^+$ eosinophils augmented, the eventual inflammation, as evidenced by leukocyte amounts (figure 6d), expression of certain cytokines and chemokines (figure 6e), and histological scores (figure 6f). Unlike the high expression of CD101 in eosinophils from asthmatic patients (as a positive control), the levels of CD101 in eosinophils from ARDS patients were much lower and comparable to healthy subjects (figure 6g). To test whether these two subsets of eosinophils exhibited differential expression of critical signalling in response to LPS challenge, we detected the levels of Toll-like receptor-4-related molecules and inflammatory cytokines in each subset of eosinophils, and found that there was no difference in these eosinophils between the control and LPS-treated groups (supplementary figure S17).

Moreover, we examined the effects of eosinophils in both "prevention" and "resolution" models. The prevention model is shown schematically as supplementary figure S18a. As predicted, transferred $CD101^-$ eosinophils prevented ALI inflammation, whereas $CD101^+$ eosinophils exacerbated LPS-induced inflammation (supplementary figure S18b and c). The resolution model is shown schematically as supplementary figure S18d. To our surprise, transferred $CD101^-$ eosinophils after LPS injection only showed transitory protection (supplementary figure S18e and f).

Nonetheless, these data altogether demonstrate that $CD101^-$ eosinophils exhibit an anti-inflammatory effect and eventually attenuate, while $CD101^+$ eosinophils further promote, the neutrophilic inflammation and ALI.

Alox15 and Protectin D1 are associated with the anti-inflammatory effect of $CD101^-$ eosinophils in ALI

Since $CD101^-$ and $CD101^+$ eosinophils exerted distinct effects on inflammation, we next explored the possible dissimilarities of these subtypes of cells *via* RNA sequencing. By comparing the transcriptomes between $CD101^-$ and $CD101^+$ eosinophils, we noticed that Alox15 was more abundant in the $CD101^-$ eosinophils (figure 7a) and this result was confirmed by quantitative PCR (figure 7b). Interestingly, the expression of Alox15 was tightly associated with emergent eosinophils in ALI. In lung homogenates, Alox15 was also increased 30 min after LPS instillation (figure 7c) and LPS failed to induce Alox15 expression in PHIL mice (figure 7d). By comparing Alox15 expression in eosinophils from NJ.1638 mice ($CD101^-$) with that of lung homogenates, we illustrated that $CD101^-$ eosinophils expressed much higher levels of Alox15 than other pulmonary cells (figure 7e).

Alox15 (also referred as 12/15-LO in mice or ALOX15/15-LOX in humans) is the key enzyme known to convert docosahexaenoic acid into anti-inflammatory lipid mediators like Protectin D1

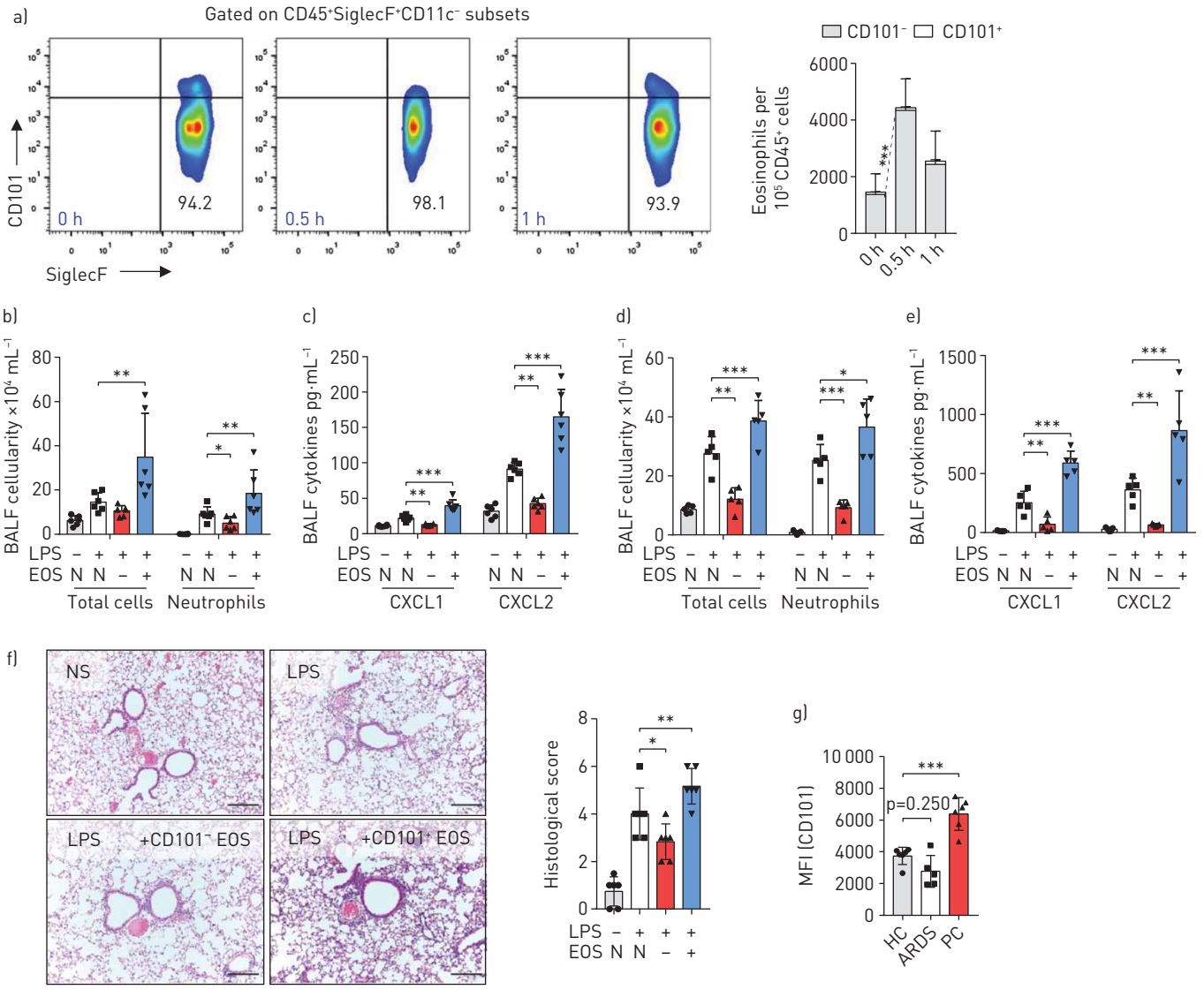

FIGURE 6 CD101$^-$ eosinophils ameliorated, while CD101$^+$ eosinophils exacerbated, the neutrophilic inflammation induced by lipopolysaccharide (LPS). BALF: bronchoalveolar lavage fluid; N: null; NS: normal saline; MFI: mean fluorescence intensity. a) CD101 expression of the aggregated eosinophils in mouse lungs after LPS administration. Left panels: representative images; right panel: quantification results. b, c) Levels of b) leukocytes and neutrophils, and c) inflammatory cytokines in BALF of PHIL mice receiving CD101$^-$ or CD101$^+$ eosinophils (EOS) 0.5 h after LPS challenge. d–f) Levels of d) leukocytes and neutrophils in BALF, e) BALF inflammatory cytokines, and f) representative images of haematoxylin/eosin staining (left panel; scale bar: 200 µm) and semiquantitative results (right panel) of PHIL mice receiving CD101$^-$ or CD101$^+$ eosinophils (EOS) at 24 h after LPS challenge. g) CD101 expression of blood eosinophils (EOS) in human subjects: healthy controls (HC), patients with acute respiratory distress syndrome (ARDS) and positive control asthma patients (PC). Sample size is indicated as individual plots in column graphs. Results are representative of three independent experiments, except for g). *: p<0.05; **: p<0.01; ***: p<0.001.

(10*R*,17*S*-dihydroxy-docosa-4*Z*,7*Z*,11*E*,13*E*,15*Z*,19*Z*-hexaenoic acid) [18, 19]; hence, we reasoned that CD101$^-$ eosinophils might attenuate the initiation of inflammation of ALI by influencing lipidomic profiles in the lung. Liquid chromatography/mass spectroscopy-based lipid analysis was performed with CD101$^-$ and CD101$^+$ eosinophils, as well as lung tissues from both PHIL and WT mice to detect Protectin D1 abundance (figure 7f–h). As predicted, Protectin D1 was more abundant in CD101$^-$ eosinophils compared with CD101$^+$ eosinophils and WT lung homogenates had more Protectin D1 than PHIL mice. These results were congruent with Alox15 expression at the mRNA level, suggesting that CD101$^-$ eosinophils might influence inflammation initiation through Alox15-mediated production of Protectin D1.

Treatment of Protectin D1 isomer (PDx) is known to mimic the effect of Protectin D1 administration [20]. As expected, intraperitoneal injection of PDx ameliorated the onset of LPS-induced inflammation in

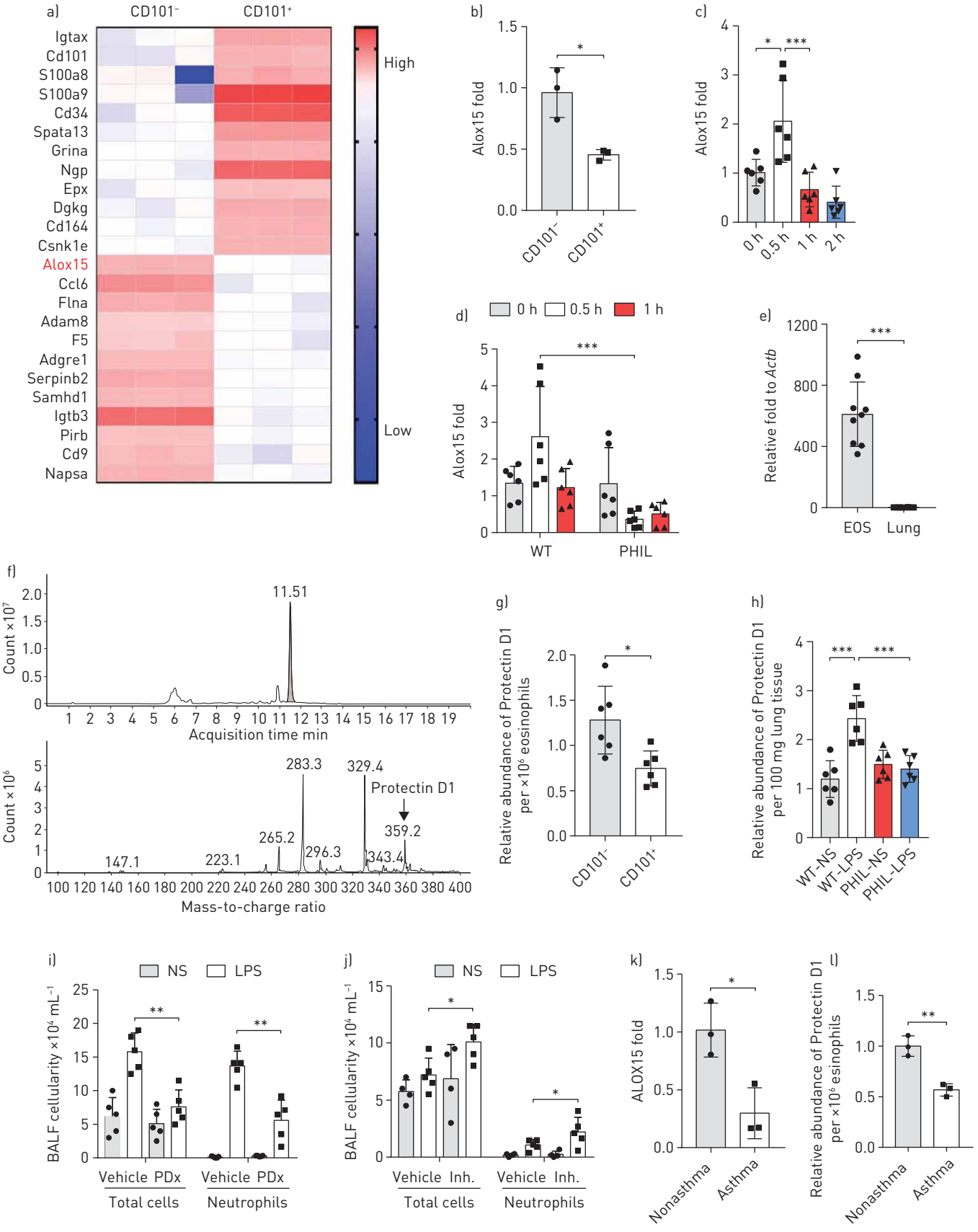
a)
CD101⁻
CD101⁺
Igtax
Cd101
S100a8
S100a9
Cd34
Spata13
Grina
Ngp
Epx
Dgkg
Cd164
Csnk1e
Alox15
Ccl6
Flna
Adam8
F5
Adgre1
Serpinb2
Samhd1
Igtb3
Pirb
Cd9
Napsa
High
Low
b)
Alox15 fold
CD101⁻
CD101⁺
c)
Alox15 fold
0 h
0.5 h
1 h
2 h
d)
0 h
0.5 h
1 h
Alox15 fold
WT
PHIL
e)
Relative fold to Actb
EOS
Lung
f)
Count ×10⁷
11.51
Acquisition time min
Count ×10⁶
283.3
329.4
Protectin D1
359.2
265.2
296.3
147.1
223.1
343.4
Mass-to-charge ratio
g)
Relative abundance of Protectin D1 per ×10⁶ eosinophils
CD101⁻
CD101⁺
h)
Relative abundance of Protectin D1 per 100 mg lung tissue
WT-NS
WT-LPS
PHIL-NS
PHIL-LPS
i)
NS
LPS
BALF cellularity ×10⁴ mL⁻¹
Vehicle
PDx
Total cells
Neutrophils
j)
NS
LPS
BALF cellularity ×10⁴ mL⁻¹
Vehicle
Inh.
Total cells
Neutrophils
k)
ALOX15 fold
Nonasthma
Asthma
l)
Relative abundance of Protectin D1 per ×10⁶ esinophils
Nonasthma
Asthma

FIGURE 7 Alox15-mediated Protectin D1 is responsible for the anti-inflammatory effect of CD101$^-$ eosinophils in acute lung injury (ALI). WT: wild-type; NS: normal saline; LPS: lipopolysaccharide; BALF: bronchoalveolar lavage fluid; PDx: Protectin D1 isomer. a) Representative heatmap of RNA sequencing analysis of differential expression in both CD101$^-$ and CD101$^+$ eosinophils (n=3). b–e) Quantitative PCR analysis of Alox15: b) expression of Alox15 in CD101$^-$ and CD101$^+$ eosinophils, c) expression of Alox15 in lungs during the initiation of LPS-induced ALI, d) comparison of Alox15 expression between WT and PHIL mice in the initiation of ALI, and e) relative abundance of Alox15 in eosinophils (EOS) compared with lung tissue (β-actin). f–h) Liquid chromatography/mass spectroscopy analysis of Protectin D1: f) identification of Protectin D1, g) Protectin D1 in CD101$^-$ and CD101$^+$ eosinophils, and h) Protectin D1 in WT and PHIL mice after 0.5 h LPS challenge. PDx (0.05 mg·kg^{-1}) was injected into PHIL mice for 3 continuous days before LPS treatment. i) Amounts of leukocytes and neutrophils in BALF of PHIL mice administrated with PDx 0.5 h after LPS treatment. j) Alox15 inhibitor PD146176 (Inh.) was injected intraperitoneally into WT mice at the dose of 10 mg·kg^{-1} 24 h before LPS administration. Amounts of total leukocytes and neutrophils in BALF 0.5 h after LPS challenge. k, l) Detection of k) ALOX15 and l) Protectin D1 in human eosinophils from both nonasthmatic and asthmatic patients. Sample size is indicated as individual plots in column graphs. Data are triplicates of individual experiments, except for k) and l). *: p<0.05; **: p<0.01; ***: p<0.001.

PHIL mice (figure 7i). Furthermore, Alox15 inhibitor PD146176 administration exacerbated initiative neutrophil accumulation in WT LPS mice (figure 7j).

For further validation, we also detected the expression of ALOX15 and Protectin D1 in human eosinophils of both asthmatic and nonasthmatic individuals. Congruent with the results in mice, eosinophils of the nonallergic subjects displayed elevated expression of ALOX15 (figure 7k) and increased secretion of Protectin D1 (figure 7l) compared with eosinophils of asthmatic subjects.

These findings further substantiate that Alox15-mediated Protectin D1 from CD101$^-$ eosinophils is a predominant signature that attenuates neutrophil infiltration in the initiation of endotoxin-induced ALI.

Discussion

In our study, we demonstrate that the "resident" eosinophils in lung parenchyma are predominantly CD101$^-$, which are derived from bone marrow. LPS challenge causes a rapid accumulation of eosinophils into lungs from the peripheral circulation. Loss of eosinophils augments LPS-induced neutrophilic inflammation and the eventual levels of ALI. Homeostatic CD101$^-$, but not allergic CD101$^+$, eosinophils exert anti-inflammatory function in ALI, likely *via* the Alox15-mediated production of Protectin D1 (figure 8).

Eosinophil accumulation in nonallergic diseases is easily overlooked; in fact, eosinophils have been shown to be involved in tumorigenesis [21], bowel inflammatory diseases [20, 22] and haematopoietic stem cell homeostasis [23]. In ALI patients, survivors displayed an increase in the number of eosinophils in lung compared with nonsurvivors [8]. We also observed increased levels of eosinophils in peripheral blood of surviving ARDS patients, which suggests that eosinophils may exert a protective effect on ALI. Our current study provides more experimental evidence and mechanistic details involving how homeostatic and early-recruited CD101$^-$ eosinophils exhibit an anti-inflammatory character in endotoxin-induced ALI.

Mechanistically, the protective effect of CD101$^-$ eosinophils might be associated with the Alox15-mediated production of Protectin D1. Lipid synthesis is involved in granulocyte development [24], recruitment [25] and other inflammation. In inflamed tissues, synthesised Protectin D1 could block neutrophil recruitment [20] and simultaneously stimulate noninflamed removal of neutrophil debris [26]. It has been shown that eosinophils express higher levels of Alox15 compared with neutrophils [27], which suggests that eosinophils might be the main originating cells involved in the biosynthesis of Protectin D1. Furthermore, Protectin D1 is downregulated in both asthmatic patients and mice [18, 27], which corroborates our findings in asthmatic and nonasthmatic eosinophils in humans, and also CD101$^+$ eosinophils and CD101$^-$ eosinophils in mice. Exogenous administration of either Protectin D1 or its isomer PDx attenuates recruitment of inflammatory cells [20, 26, 28] and decreases inflammatory cytokine expression [28], thereby limiting inflammatory processes, which is consistent with our current observations.

Intriguingly, for the data shown in the "prevention" and "resolution" models (supplementary figure S18), the efficacy of CD101$^-$ eosinophils in limiting LPS-induced pulmonary inflammation in the prevention model was superior to that in the resolution model. The detailed mechanisms for such a difference were not clear. We hypothesised that CD101$^-$ eosinophils might take effect only after they "are located in their position" in lung parenchyma during homeostasis; however, large amounts of neutrophils destroyed the functional microenvironment of CD101$^-$ eosinophils after the inflammation outburst.

On the contrary, adoptive transfer of CD101$^+$ eosinophils deteriorated LPS-induced ALI. CD101$^+$ eosinophils are mainly enhanced in asthmatic inflammation, highly implying that asthmatic patients might not benefit from eosinophil infiltration in lung injury [9]. Based on the results from RNA sequencing, it is plausible to hypothesise that the pro-inflammatory function of CD101$^+$ eosinophils is due to the overexpression of alarmins, such as S100A8 and S100A9 (figure 7a), since these alarmins have been shown to enhance the development and influx of monocyte and neutrophils [25, 29].

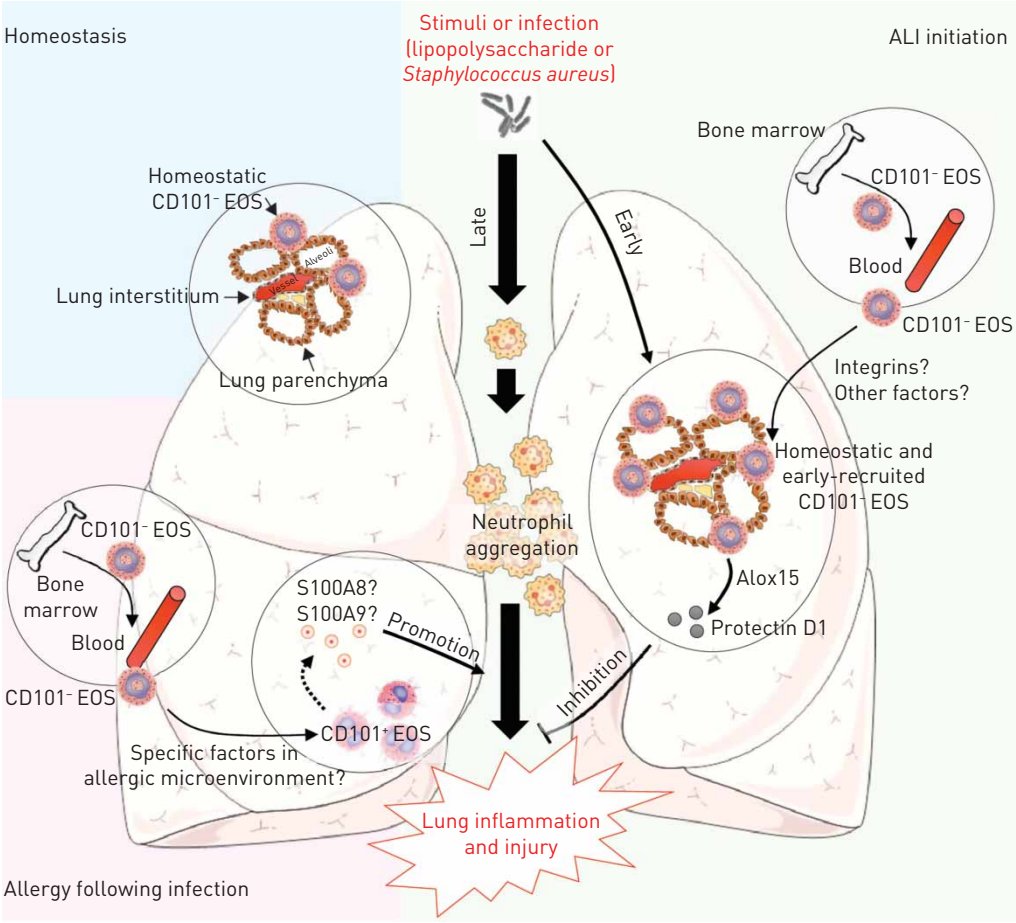

FIGURE 8 Differential functions of CD101^{-} and CD101^{+} eosinophils (EOS) in acute lung injury (ALI) pathogenesis. Homeostatic eosinophils, mainly CD101^{-}, are localised in lung parenchyma. Upon stimulation (*e.g.* with lipopolysaccharide or *Staphylococcus aureus*) more CD101^{-} eosinophils are rapidly recruited into the lung, while the factors mediating this early eosinophil recruitment are not clear. These accumulated CD101^{-} eosinophils attenuate lung inflammation and injury *via* Alox15-mediated Protectin D1 secretion. On the other side, in the allergic microenvironment, CD101^{-} eosinophils are turned to CD101^{+} upon stimulation with certain unknown factors and the CD101^{+} eosinophils promote ALI pathogenesis likely through S100A8 or S100A9.

CD101^{+} eosinophils are noticed in asthmatic mice accompanied with a few CD101^{-} eosinophils. However, in asthmatic patients, eosinophils display CD101^{hi} eosinophils only. This discrepancy is likely due to either the diversity in duration of asthma or the difference between human eosinophils and murine eosinophils. Furthermore, CD101^{+} eosinophils frequently accompany CD62L shedding [13]. However, we observed that the expression of CD62L was very low in pulmonary eosinophils, irrespective of being either CD101^{-} or CD101^{+}, or homeostatic or allergy induced (data not shown). Therefore, we used CD101, but not CD62L, as a marker to distinguish the different eosinophil subgroups in this study.

In both asthma and endotoxin-induced ALI, the transformation of CD101^{-} to CD101^{+} in eosinophils is of great significance. We observed that the expression of CD101 in eosinophils is independent of the process of eosinophil development-related cytokines or house dust mite exposure (supplementary figure S13). A recent study demonstrates a crucial role of IL-18 in the transformation of eosinophils [30]; however, the molecular spectrum which promotes the transformation of eosinophils is still unclear.

Tissue-resident immune cells are known to have prominent effects in maintaining homeostasis of a certain milieu [31]. In our study, we demonstrated that the lung was an important organ for eosinophil residence and also observed that the parenchymal eosinophil population was bone marrow dependent. These data strongly suggest that lung eosinophils are rather "homeostatic" than "resident". Although we demonstrate a recruitment of eosinophils during ALI initiation, the mechanisms mediating the recruitment are not clear. At the early time-points after LPS challenge, we failed to observe any induction of eotaxins before eosinophil aggregation. Previously, it has been reported that LPS induces eosinophil migration in pleurisy independent of eotaxin [32]. Likewise, the amount of pulmonary eosinophils at steady state remained

intact in $CCR3^{-/-}$ mice [32]. Considering the high expression of the integrin family, such as Igtax and Igtae, in eosinophils [33], it is plausible to hypothesise that eosinophils migrate to lung tissues during homeostasis or upon pathogen exposure *via* an eotaxin–CCR3-independent axis, although this needs to be clarified in future studies.

It is of great interest to note that circulating eosinophils are increased in surviving ARDS patients, suggesting that the levels of peripheral eosinophils could indeed serve as a new biomarker and a target of ALI therapy. Augmented eosinophils could display antipathogen activity in host defence by releasing their cationic proteins [34] and even extracellular traps [35]. Besides, eosinophils also accumulate and participate in prompting the process of tissue repair after damage [12]. Clinically, therapy with an anti-IL-5 monoclonal antibody, such as mepolizumab, is widely used for both asthmatic [36] and eosinophilic chronic obstructive pulmonary disease patients [37], resulting in reduced eosinophil levels in peripheral blood [36–38]. Although there is a lack of experimental evidence, it is likely that anti-IL-5 antibody would reduce both eosinophil subgroups. Theoretically, the reduction of $CD101^{+}$ eosinophils will benefit both asthma and ALI patients; however, the reduction of homeostatic $CD101^{-}$ eosinophils might increase the risks and levels of ALI. Thus, our study suggests that the scheme of anti-IL-5 therapy for eosinophilic airway inflammation should be neatly controlled to reduce the risks for infection.

In summary, our findings uncover a function of homeostatic and rapidly recruited $CD101^{-}$ eosinophils in lung parenchyma for the suppression of endotoxin-induced ALI *via* the Alox15–Protectin D1 axis, and suggest a new biomarker and a potential therapeutic target for ALI.

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