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Direct evidence of secondary necrosis of neutrophils during intense lung inflammation

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ABSTRACT: Several pulmonary inflammatory conditions are characterised by infiltration of neutrophils. Normally, neutrophils are silently removed by apoptosis, followed by phagocytosis. However, if phagocytosis fails, apoptotic cells undergo secondary necrosis. Recent findings of increased levels of the pan-necrosis marker lactate dehydrogenase in bronchoalveolar lavage from lipopolysaccharide-exposed mice implies potential involvement of secondary necrosis. Using a similar model, this study aimed to identify the source of lactate dehydrogenase and to search for direct histological evidence of secondary necrosis.

Lipopolysaccharide (LPS) was administered to the lungs of BALB/c mice, and bronchoalveolar lavage and tissue samples were collected 4, 12, 24, 36, 48, 60 and 72 h after administration. LPS induced a patchy neutrophil-rich lung inflammation, where the numbers of terminal deoxynucleotide transferase-mediated dUTP nick-end labelling-positive neutrophils were increased at 12 h and onwards. Lavage levels of neutrophils and lactate dehydrogenase increased significantly at 4 and 24 h, respectively. Detailed electron microscopic assessment of neutrophil activation and death modes revealed that up to 14% of the neutrophils were undergoing secondary necrosis, whereas apoptotic or primary necrotic structural cells were rarely found.

In summary, this study provides direct evidence that secondary necrosis of neutrophils is a common process during intense lung inflammation. This implies that neutrophil apoptosis may cause rather than resolve airway inflammation.

KEYWORDS: Apoptosis, endotoxin, inflammation, lactate dehydrogenase, neutrophils

Pulmonary neutrophilia is a characteristic of several inflammatory lung conditions. Experimental pulmonary exposure of lipopolysaccharide (LPS) induces a fast and intense neutrophil response [1]. Under normal physiological conditions, neutrophils are silently and swiftly eliminated through apoptosis, followed by phagocytosis by alveolar macrophages [2]. However, if the phagocytosis system fails, apoptotic cells die through secondary necrosis, a pro-inflammatory event associated with cell membrane disruption and extracellular spreading of cell contents [3]. Recently, MEDAN *et al.* [4] suggested that secondary necrosis takes place in inflamed lungs. By using a mouse model of LPS-induced lung inflammation, these authors found the peak of apoptotic cells in bronchoalveolar lavage (BAL) to be followed by an increase in activity of the pan-necrosis marker lactate dehydrogenase (LDH), a phenomenon further aggravated after inhibiting macrophage phagocytosis with phosphatidylserine-containing liposomes [4]. While this important finding suggests secondary necrosis to be a significant feature of LPS-induced lung inflammation, the cell type(s) undergoing secondary necrosis has remained largely unknown. The present study sought to identify the source of LDH in BAL fluid (BALF) *via* a detailed histological analysis of a LPS-induced inflammation. Special focus was given to neutrophil infiltration and clearance, due to the possibility of finding direct evidence for secondary necrosis of neutrophils to be a significant feature of the inflammation. To allow controlled studies of areas with different intensity of inflammation, a patchy neutrophil-rich lung inflammation was induced in mice using a dual-administration regimen of LPS. At seven different time-points, general inflammation as well as the activity and fate of neutrophils were assessed by BAL studies and lung tissue histology, including an electron microscopic analysis. This approach allowed the current authors to follow the fate of cells within patchy areas of intense neutrophil-rich

For editorial comments see page 257.

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inflammation and infiltration (hereafter referred to as inflammatory foci (IF)) at various phases of lung inflammation in detail, and to study the clearance processes following cell death. The data revealed that the extensive number of cells within IF were neutrophils, of which large numbers were undergoing apoptosis and secondary necrosis, suggesting neutrophils to be the primary source of LDH during an intense lung inflammation. Taking the pathogenic potential of necrosis into consideration [5, 6], this study suggests neutrophil secondary necrosis to be a potential pathogenic mechanism during an intense neutrophil-rich lung inflammation.

MATERIAL AND METHODS

Mice

Female BALB/c mice, 6–8 weeks old, were obtained from MoB A/S (Ry, Denmark). Animals were housed in a 12-h light–dark cycle and provided with food and water *ad libitum*. All protocols were approved by the local ethics committee (Malmö/Lund, Sweden).

Lipopolysaccharide-induced lung inflammation

A total dose of 50 μg LPS (*Escherichia coli* (O26:B6); Sigma, St Louis, MO, USA) was administered intra-nasally during a light anaesthesia, using a dual-administration regimen. This administration of LPS has, in preliminary experiments, been shown to produce a patchy lung inflammation with areas of variable inflammatory intensity and neutrophil infiltration. Animals were sacrificed 4, 12, 24, 36, 48, 60 and 72 h after LPS administration (n=5 in each group; controls (n=5) received double administration of saline) by pentobarbital sodium (Pentobarbitalnatrium; Apoteket AB, Umeå, Sweden). BALs were performed on all animals as previously described [7] and tissue samples were obtained for paraffin (*via* immunohistochemistry) and plastic embedding (transmission electron microscopy (TEM)) [7]. The rationale for analysing BAL and tissue samples from the same animals was to investigate the total contents of the lung, both what could be retrieved by BAL and what was left in the lung after BAL.

Bronchoalveolar lavage fluid

The BALF was centrifuged, the supernatant immediately frozen on dry ice and the cell pellet resuspended in PBS supplemented with 10% foetal calf serum. The BALF level of the cytoplasm enzyme LDH was used as a general marker of cell membrane disruption, *i.e.* necrosis. The activity of LDH in BAL was enzymatically determined (LDH converted lactate and nicotinamide adenine dinucleotide to pyruvate and reduced nicotinamide adenine dinucleotide, resulting in increased absorbance that was directly proportional to the activity of LDH; the analysis was performed using a Hitachi Modular-P unit; Roche Diagnostics, Basel, Switzerland) in 100 μl of BALF, by the Laboratory of Clinical Chemistry (Lund University Hospital, Lund, Sweden). Differential cell counting was preformed on May–Grünewald/Giemsa-stained cytospin slides.

Histological assessment of inflammation

Histological observations, such as extent of inflammation, infiltrating cells and occurrence of cell debris, were examined on 6-μm haemotoxylin and eosin-stained paraffin sections, co-stained with the DNA-marker Hoechst 33342 (H33342; Sigma, St Louis, MO, USA). Neutrophils were identified as cells with polymorph nucleus, apoptotic cells by intensely DNA-stained condensed nuclei, and necrotic cells and cell debris as vague and blurry positive DNA staining. The extent of inflammation was determined as the degree of cellular infiltrate in lung parenchyma, classified into three categories: 1) no or mild cellular infiltration; 2) extensive infiltration with partly filled alveoli; and 3) extensive cellular infiltrate with filled alveolar spaces. Categories 2 and 3 were defined as IF.

Detection of apoptosis

The terminal deoxynucleotide transferase-meditated dUTP nick-end labelling (TUNEL) technique, performed according to the manufacturer's protocol (Intergen Company, New York, NY, USA) as previously described [8], in combination with H33342, was used to identify apoptotic cells in lung sections, as discussed in another study [9]. When using the TUNEL technique it is important to consider that not only apoptotic cells are labelled, but also necrotic cells and free apoptotic cell nuclei stain positive. Therefore, no separation of these stages was performed using this analysis; the differentiation was performed with electron microscopy. For quantification, high-resolution digital images of the lung parenchyma were randomly obtained (4–6 images per slide) and the total number of TUNEL-positive cells/nuclei in each image was counted (blinded) using the ImageJ® software (1.30v; National Institutes of Health, Bethesda, MD, USA) and expressed as cells·mm^{-2}. For quantification of positivity within areas of intense neutrophil infiltration and inflammation (IF), selected areas within sections displaying numerous TUNEL-positive staining were photographed and analysed as previously described.

Transmission electron microscopy

TEM analysis was performed to achieve detailed structural analysis. Cells in different modes of activation (*i.e.* neutrophils with granule alterations and/or phagosomes) and death were quantified using set and previously validated criteria (table 1; fig. 1) [10–13]. At least 90 neutrophils per time-point were counted, with the exception being controls for which a very limited number of neutrophils were found, and the results were expressed as percentage of the total number of cells. The areas studied *via* TEM were consciously chosen to include IF, as the aim of the present study was to investigate the inflammatory process in these areas.

Data analysis and statistics

Analyses were made as previously described. The Kruskall–Wallis test was used to assess differences; when significant, the Mann–Whitney test was employed to explore the differences between groups. All groups were compared to control. Unless otherwise stated, data were given as median (range) and $p \leqslant 0.05$ was considered statistically significant.

RESULTS

Characterisation of inflammation

The number of neutrophils in BALF increased significantly 4 h after LPS administration, peaked at 36 h and returned towards baseline levels at 72 h (table 2). The level of the necrosis marker LDH in BALF increased significantly in response to LPS (table 2). The level of LDH peaked at 60 h and correlated with the number of neutrophils in BAL. In the tissue, a

TABLE 1 Criteria used for electron microscopy quantification of activity status following lipopolysaccharide administration

Status	Characterisation features
Resting	Intact cells with normal nucleus, intact cell membrane and normal primary and secondary granules
Activated	One or more of the following features: granule alterations, vacuoles, phagosomes, extensive pseudopodia
Apoptosis	Intact cell membrane, condensed and electron dense nucleus, often with blebs along the nuclear membrane
Primary necrosis	Ruptured cell membrane and normal or chromatolytic nucleus
Secondary necrosis	As apoptosis but with a ruptured cell membrane
Cell debris	Gatherings of neutrophil-derived extracellular material, including the characteristic neutrophil granule

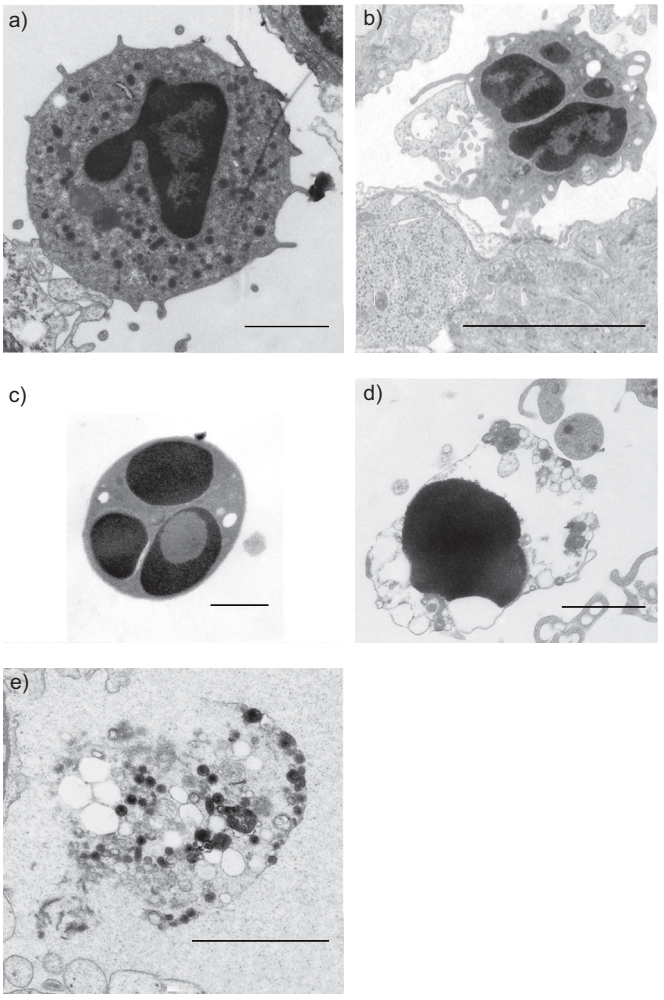

FIGURE 1. Transmission electron microscopic images exemplifying the different ultrastructural criteria used to determine the fate of lung neutrophils. a) Resting neutrophil with normal nucleus and intact primary and secondary granules. b) Activated neutrophil, exemplified here by occurrence of cytoplasmatic protrusions and phagosomes. c) Apoptotic neutrophil with characteristic chromatin condensation and separation of euchromatin and heterochromatin. d) Former apoptotic neutrophil, in the process of secondary necrosis. The electron-dense nuclei, ruptured cell membrane and largely dissolved/released cytoplasm should be noted. e) Cluster of neutrophil-derived cell debris. For additional details on classification of status, see table 1. Scale bars=2.5 μm (a and e), 5 μm (b), 1 μm (c) and 2 μm (d).

patchy neutrophil-rich inflammatory pattern was confirmed by histological analysis of the lung parenchyma. Thus, cell-dense IF were present amidst lung regions with a mild-to-moderate neutrophilia (fig. 2a and b). IF (extent of inflammation categories 2 and 3) were characterised by extensive cellular infiltrate that completely or partly filled the alveolar spaces and by plugs in the luminal spaces of bronchi and bronchioles. The most common cell type in IF was neutrophils, followed by macrophages. Neutrophils were also the most abundant cell type in cell-rich plugs found in the luminal spaces of bronchi and bronchioles in IF (fig. 2c).

Focal accumulation of TUNEL positivity

Whereas the total number of TUNEL-positive cells increased dramatically after LPS administration, the augmentation was most prominent in IF (table 2). Based on cell-specific morphological criteria the TUNEL-positive cells were identified as being primarily neutrophils, but also scattered macrophages and structural cells stained positively.

Cellular fate in IF

The most abundant cell type in IF was the neutrophil, but macrophages were also present in relatively large numbers. Generally, in IF, neutrophils inside the alveolar wall or the subepithelial tissue surrounding bronchi and bronchioles were at rest showing little or no activation. Scattered neutrophils in these tissue compartments did undergo apoptosis, but no apoptotic neutrophils were found inside pulmonary capillaries. In contrast, resting neutrophils were rarely observed in the alveolar lumen; the vast majority of the neutrophils were activated with granulae alterations and/or intracellular phagosomes (fig. 3a). A marked increase in apoptosis, and primary and secondary necrosis as well as cell debris (*i.e.* extracellular neutrophil granules, free condensed nuclei and other cell remnants) was regularly seen in IF 24 h after LPS administration (fig. 3a and b). The proportion of different activation statuses and death modes of alveolar neutrophils varied between time-points (fig. 4). From 4 h after LPS administration, the majority of the neutrophils were displaying an activated phenotype. At later time-points, the number of apoptotic neutrophils increased, as did the number of neutrophils undergoing secondary necrosis. Neutrophils undergoing primary necrosis were found, though only a few scattered cells, indicating that the increase in neutrophil-derived cell debris was mainly due to secondary necrosis. The TEM analysis further revealed that scattered structural cells (*i.e.* type I and II pneumocytes) exhibited signs of cell damage, such as a pale and swollen cytoplasm and cell membrane

TABLE 2 Effects of lipopolysaccharide on neutrophil infiltration, lactate dehydrogenase (LDH) activity and terminal deoxynucleotide transferase-mediated dUTP nick-end labelling (TUNEL)-positive cells in whole sections and inflammatory foci (IF)

Parameters	Control	4 h	12 h	24 h	36 h	48 h	60 h	72 h
Neutrophil cells·mL^{-1} BAL	0.28 (0.55–0.21)	0.3 (0.43–0.12)**	0.71 (1.15–0.23)**	1.26 (1.41–0.87)**	2.94 (4.37–2.49)**	4.24 (6.47–2.09)**	4.54 (5.21–3.23)**	2.51 (3.54–0.75)
LDH activity	0.00 (0.01–0.00)	3.31 (4.04–2.90)	6.33 (7.28–2.33)	6.83 (9.00–4.75)**	7.49 (12.29–6.12)**	7.82 (9.66–2.96)**	3.59 (5.40–2.16)**	0.83 (1.18–0.06)**
TUNEL whole sections	1.28 (6.27–0.89)	31.62 (47.01–17.09)*	129.81 (210.90–66.67)*	290.00 (585.64–71.79)**	347.33 (767.58–147.14)*	361.00 (489.58–253.91)*	193.15 (552.56–159.62)*	227.56 (453.33–48.72)*
TUNEL IF	0.00 (0.00–0.00)	0.00 (0.00–0.00)	223.08 (350.00–125.64)*	703.85 (747.44–419.23)*	647.14 (997.40–335.94)*	842.45 (878.91–395.83)*	392.31 (624.79–280.77)*	325.64 (757.69–164.10)*

Data are presented as median (range). BAL: bronchoalveolar lavage. *: $p<0.05$; **: $p<0.01$ *versus* control.

rupture indicating an ongoing necrosis process (fig. 3c); fewer displayed an apoptotic phenotype. The vast majority of the alveolar macrophages in IF contained multiple large phagosomes, and occasional macrophages displayed signs of necrosis, revealed by chromatolytic nucleus- and electron-lucent cytoplasm.

DISCUSSION

There is abundant evidence supporting a critical role for apoptosis and subsequent phagocytosis in swift and silent clearance of peripheral airway neutrophils [2]. This study investigated the clearance of neutrophils during LPS-induced lung inflammation and sought to identify the source of the

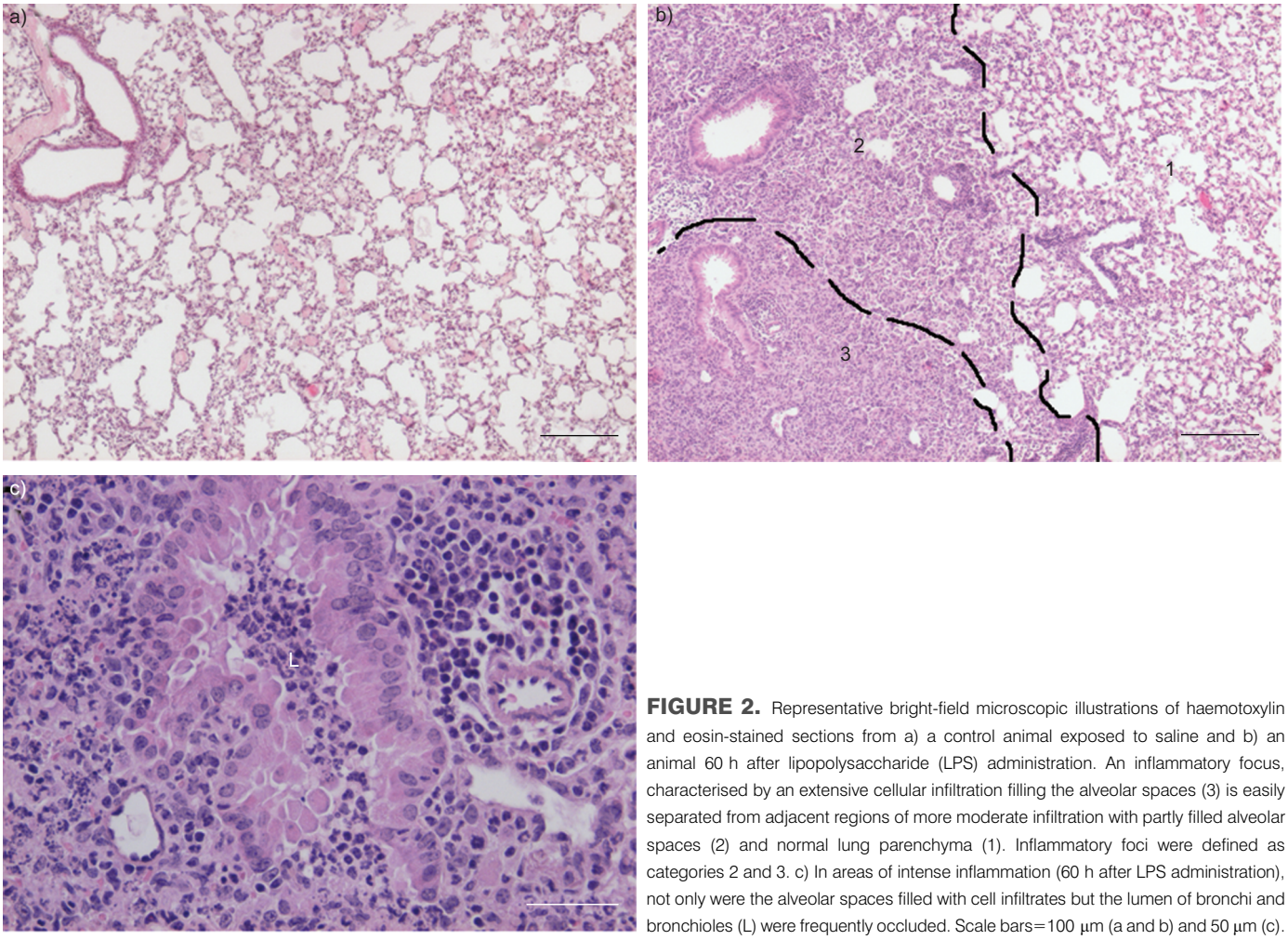

FIGURE 2. Representative bright-field microscopic illustrations of haemotoxylin and eosin-stained sections from a) a control animal exposed to saline and b) an animal 60 h after lipopolysaccharide (LPS) administration. An inflammatory focus, characterised by an extensive cellular infiltration filling the alveolar spaces (3) is easily separated from adjacent regions of more moderate infiltration with partly filled alveolar spaces (2) and normal lung parenchyma (1). Inflammatory foci were defined as categories 2 and 3. c) In areas of intense inflammation (60 h after LPS administration), not only were the alveolar spaces filled with cell infiltrates but the lumen of bronchi and bronchioles (L) were frequently occluded. Scale bars=100 μm (a and b) and 50 μm (c).

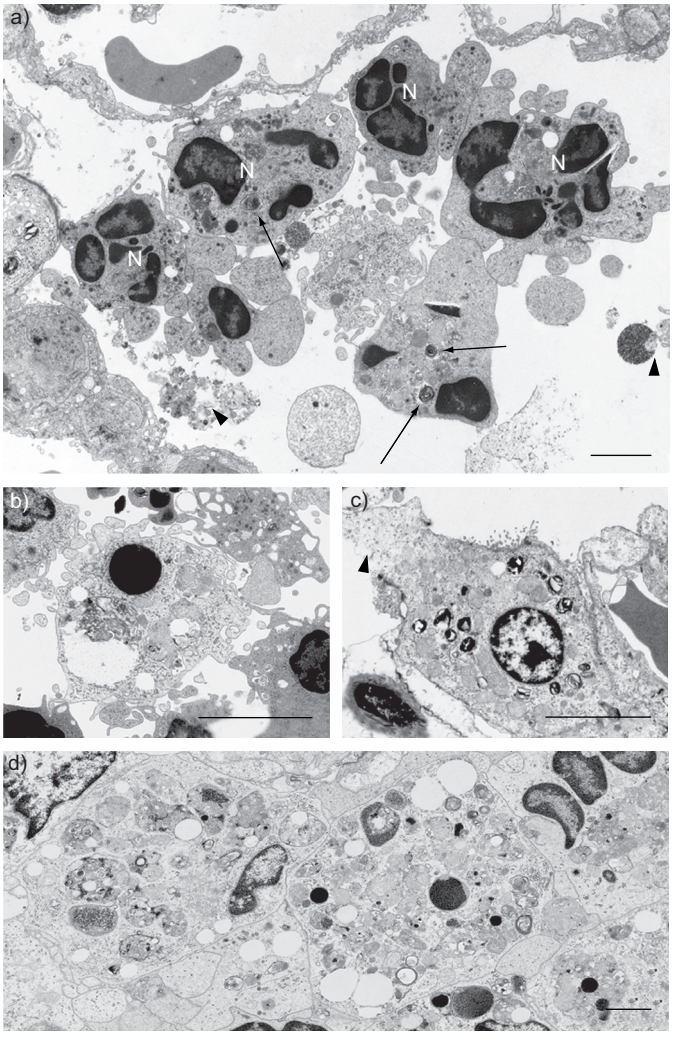

FIGURE 3. Transmission electron micrographs demonstrating characteristic features of neutrophils, structural cells and macrophages in inflammatory foci (IF). a) Neutrophils (N) were primarily activated with granulae alterations or phagosomes (indicated by arrows) in lumen amidst cell debris (arrowheads), such as free apoptotic nuclei and neutrophil-derived granulae. b) Secondary necrosis of neutrophils (cells with condensed and electron-dense nuclei and ruptured cell membrane) were extensively seen in IF. c) Only scattered dying structural cells were found, illustrated by a type-II pneumocyte undergoing primary necrosis, with early signs of chromatolysis and rupture of the cell membrane (arrowhead). d) In IF, macrophages commonly contained multiple phagosomes. Scale bars=2.5 μm (a), 5 μm (b and c) and 2 μm (d).

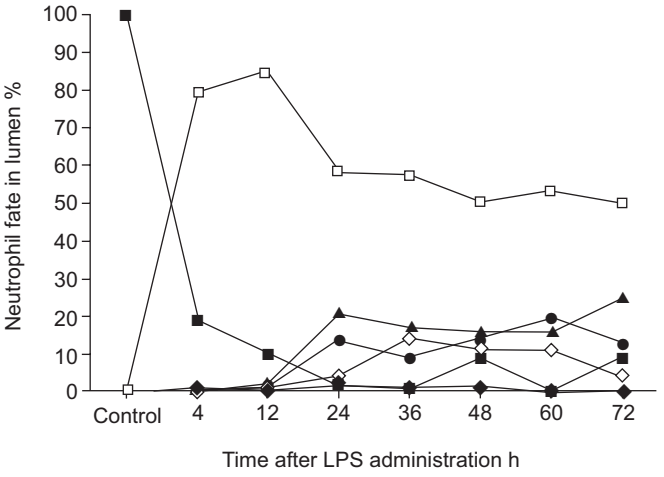

FIGURE 4. A detailed transmission electron microscopy analysis was performed to reveal the fate of neutrophils in cell-rich inflammatory foci at different time-points following lipopolysaccharide (LPS) administration. The fates of the neutrophils were divided into six categories depending on the following morphological characteristics: resting (■; intact cells, normal nucleus and granulae), activated (□; granulae alterations, vacuoles, phagosomes and/or extensive pseudopodia), apoptosis (●; intact cell membrane, condensed nucleus, often blebs along the nuclear membrane), primary necrosis (♦; ruptured cell membrane and normal/chromatolytic nucleus), secondary necrosis (◇; as apoptosis but ruptured cell membrane) and cell debris (▲; extracellular gatherings of neutrophil-derived material). The data are given as percentages of different categories of total number of neutrophils at each time-point.

necrosis marker LDH, previously suggested to increase follwing endotoxin exposure [4]. The present study found that apoptotic neutrophils in IF are not properly cleared and therefore undergo secondary necrosis in large numbers.

In light of the pro-inflammatory potential of secondary necrosis [4, 6, 14], surprisingly little is known about its occurrence and role in inflamed lungs. A relationship between apoptosis and LDH levels has been shown in BAL samples of LPS-treated mice, a phenomenon further increased following interference with macrophage phagocytosis by phophatidylserine-containing liposomes [4]. However, as LDH is a pan-necrosis marker it may be released by any cell undergoing either primary or secondary necrosis. Several fundamental questions therefore remain regarding the nature of secondary necrosis in the lung. For example, it is still unclear which cell types actually undergo secondary necrosis, and are therefore the source of the LDH found in BAL, and if/how this release relates to modes of cell death, such as primary necrosis [13]. Furthermore, until now, virtually nothing has been known about when and where in the inflammatory process secondary necrosis takes place.

This study shows that in IF, only scattered pneumocytes displayed ultrastructural signs of apoptosis, whereas signs of primary necrosis (electron-lucid cytoplasm and damaged cell membrane, for example) were more commonly found. Although this finding supports the notion that necrosis rather than apoptosis is the usual death mode of structural cells [15], it must not be forgotten that apoptosis of structural cells occurs more frequently in more chronic situations. The study also found numerous apoptotic neutrophils and neutrophils in the process of secondary necrosis, suggesting that the apoptotic process is not too swift to be detected. This implies that apoptotic neutrophils undergoing secondary necrosis are the primary source of LDH, and the fact that secondary necrotic neutrophils occurred amidst apoptotic neutrophils and extracellular neutrophil-derived cell debris suggests a rapid turnover of neutrophils.

In general, the present data support the notion of apoptosis and phagocytosis as a highly effective neutrophil-elimination mechanism [3, 16]. As previously described, LPS induced a patchy inflammation [1], with regions of no or mild

inflammation amidst areas of extensive inflammation. In the regions of mild and moderate inflammation, apoptosis and subsequent macrophage phagocytosis carried out swift and silent neutrophil elimination, resulting in sufficient clearance of infiltrating neutrophils. A similar silent removal process may also have occurred in IF, to an as yet unknown extent. However, as demonstrated through TEM analysis, in IF a significant proportion of the neutrophils underwent secondary necrosis, suggesting the clearance to be insufficient. Taken together, the present data suggest secondary necrosis to be a rare phenomenon in large areas of the lung parenchyma (with mild-to-moderate neutrophil infiltration) but a common fate of lung neutrophils in IF.

Little is known about secondary necrosis in relevant clinical situations. It is clear, however, that secondary necrosis occurs extensively in the airway lumen of patients with chronic obstructive pulmonary disease (COPD), as assessed by TEM analysis of directly fixated sputum samples [17]. Hence, secondary necrosis seems to be a common fate of senescent and apoptotic cells trapped in airway mucus plugs. To what extent such luminal necrosis affects the underlying airway mucosa is currently unknown, although it has recently been demonstrated *in vitro* that neutrophils undergoing secondary necrosis have the capacity to damage airway epithelial cells [6], suggesting a potentially pathogenic role of luminal secondary necrosis. Human data on secondary necrosis in the more fragile peripheral airways are lacking. From the present study, it can be concluded that the most likely site for secondary necrosis to occur is in areas of intense inflammation and neutrophil infiltration, IF. As demonstrated by histopathological and lung-imaging techniques, areas with a degree of inflammation similar to the IF examined in the present model occur frequently during common lung infections [18, 19], for example, and probably during COPD exacerbations. This indicates that similar processes to the one currently described in mouse lungs may in fact be part of several clinical conditions.

Interestingly, macrophages exposed to smoke or collected from COPD patients have been shown to exhibit an impaired phagocytic capacity [20, 21]; similar results have been obtained with LPS-stimulated alveolar macrophages from patients suffering from severe asthma [22]. It can therefore be speculated that the extensive secondary necrosis found in the present model is due to impairments in the macrophage clearance system. In support of this, the present study revealed that the vast majority of the macrophages within IF contain multiple large phagosomes and had occasionally started to disintegrate in necrosis. However, to clarify whether the macrophage clearance system is impaired or overwhelmed, further investigations on this subject and the role of secondary necrosis in the pathogenesis of common airway diseases are required.

In summary, this study demonstrates that in acutely inflamed lungs insufficient clearance of apoptotic neutrophils results in secondary necrosis, a pro-inflammatory process in which intracellular components are released. The increases in lactate dehydrogenase seen following lipopolysaccharide exposure originate mainly from secondary necrosis of apoptotic neutrophils, rather than from secondary necrosis of structural cells. The process of secondary necrosis may thus play a role in the pathogenesis of neutrophil-rich inflammatory airway conditions, such as chronic obstructive pulmonary disease exacerbations and infectious pneumonia.

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