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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 characterized 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 lactatedehydrogenase in bronchoalveolar lavage from lipopolysaccharide-exposed mice, implies potential involvement of secondary necrosis. Using a similar model, we sought to identify the source of lactatedehydrogenase, and search for direct histological evidence of secondary necrosis. Lipopolysaccharide 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. Lipopolysaccharide induced a patchy neutrophil-rich lung inflammation, where the numbers of TUNEL-positive neutrophils were increased at 12 h and onwards. Lavage levels of neutrophils and lactatedehydrogenase 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 and this implies that neutrophil apoptosis may cause rather than resolve airway inflammation.

## INTRODUCTION

Pulmonary neutrophilia is a characteristic of several inflammatory lung conditions. Also 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 lactatedehydrogenase (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 by a detailed histological analysis of a LPS-induced inflammation. Special focus was given 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 us to follow the fate of cells within patchy areas of intense neutrophil-rich inflammation and infiltration (hereafter referred to as inflammatory foci, IF), at various phases of a lung inflammation in detail, and study the clearance processes

following cell death. Our data revealed the extensive number of cells within IF to be mainly 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 12 h light-dark cycle and provided food and water *ad libitum*. All protocols were approved by the local ethics committee (Malmö/Lund, Sweden).

### *LPS-Induced Lung Inflammation*

A total dose of 50 μg LPS (*E. coli* (O26:B6) Sigma, St Louis, MO, USA) was administered intranasally 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). BAL were performed on all animals as described previously (7) and tissue samples were obtained for paraffin (immunohistochemistry, IHC) and plastic embedding (TEM) (7). The rationale for analyzing BAL and tissue samples from the same animals was to investigate the total contents of the lung, both what was possible to retrieve by BAL, and also what is left in the lung after BAL.

### *Bronchoalveolar Lavage Fluid (BALF)*

The BALF was centrifuged, the supernatant immediately frozen on dry ice and the cell pellet was resuspended in PBS supplemented with 10% fetal 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 is converting lactate and NAD to pyruvate and NADH, resulting in increased absorbance which is directly proportional to the activity of LDH. The analysis was done using a Hitachi Modular-P unit) in 100 µl 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 H&E stained paraffin sections, co-stained with the DNA-marker Hoechst 33342 (H33342). Neutrophils were identified as cells with polymorph nucleus, apoptotic cells by intensely DNA-stained condensed nuclei, necrotic cells and cell debris as vague and blurry positive DNA staining. The extent of inflammation was determined as 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, where categories 2 and 3 was defined as inflammatory foci (IF).

### *Detection of Apoptosis*

TUNEL technique, performed according to the manufacturers protocol (Intergen Company, NY, USA) as previously described (8), in combination with H33342 was used to identify apoptotic

cells in lung sections, as described before (9). Notably, when using the TUNEL-technique it is important to bear in mind that not only apoptotic cells are labelled, also necrotic cells and free apoptotic cell nuclei stains positive, therefore no separation of these stages were done by this analysis, the differentiation was done by electron microscopy. For quantification high-resolution digital images of the lung parenchyma were randomly obtained (4-6 images / slide), and the total number of TUNEL-positive cells / nuclei in each image was counted (blinded) using the software ImageJ (1.30v, NIH, USA®), and expressed as cells / $mm^2$. For quantification of positivity within areas of intense neutrophil infiltration and inflammation (inflammatory foci, IF), selected areas within sections displaying numerous TUNEL-positive staining, were photographed and analyzed as described above.

### *Transmission Electron Microscopy (TEM)*

TEM analysis was performed for a 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, Figure ~~2~~1 and references 10-13). At least 90 neutrophils / time point were counted, except for controls where a very limited number of neutrophils were found, and the result expressed as percentage of the total number of cells. The areas studied by TEM were consciously chosen to include IF, since we actively aimed to investigate the inflammatory process in these areas.

### *Data analysis and statistics*

Analyses were made as described above. The Kruskall-Wallis test was used to assess differences, and when significant the Mann-Whitney test was employed to explore the differences between groups, and all groups were compared to control, using the statistic

program Analyze It™ (Analyze-it Software Ltd, UK). Data were (if not stated otherwise) given as median (range), and $p \leq 0.05$ was considered statistically significant.

## RESULTS

### *Characterization of inflammation*

The number of neutrophils in BALF increased significantly 4 h after LPS administration, peaked at 36h, and returned towards base line 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 patchy neutrophil-rich inflammatory pattern was confirmed by histological analysis of the lung parenchyma. Thus, cell-dense IF was present amidst lung regions with a mild to moderate neutrophilia (Figure 2 A-B). IF (extent of inflammation categories 2 and 3) were characterized 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 (Indicated by *L*) of bronchi and bronchioles in IF (Figure 2 C).

### *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 positive.

### *Cellular Fates within IF*

The most abundant cell type in IF was neutrophils, but also macrophages were 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 small or no signs of 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 (Figure 3A). 24 h after LPS administration a marked increase in apoptosis, primary and secondary necrosis as well as cell debris (i.e. extracellular neutrophil granules, free condensed nuclei and other cell remnants were present) was regularly seen in IF (Figure 3A and B). The proportion of different activation statuses and death modes of alveolar neutrophils varied between time points (Figure 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, however only to few scattered cells, indicating the increased neutrophils derived cell debris to be 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 rupture indicating an ongoing necrosis process (Figure 3 C), and even 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 (figure 3 D).

## DISCUSSION

There is abundant evidence supporting a critical role for apoptosis and subsequent phagocytosis for a swift and silent clearance of peripheral airway neutrophils (2). In this study we investigated the clearance of neutrophils during an LPS-induced lung inflammation seeking to identify the source of the necrosis marker LDH, previously described to increase following endotoxin exposure (4). We found that apoptotic neutrophils in IF were not properly cleared, and therefore underwent 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 PS-containing liposomes (4). However, since LDH is a pan necrosis marker it may be released by any cell undergoing either primary or secondary necrosis. Hence, several fundamental questions remain regarding the nature of secondary necrosis in the lung. For example; it has remained unknown which cell types that actually undergo secondary necrosis, and thereby is 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 regarding when and where in the inflammatory process that secondary necrosis takes place.

This study shows that in IF only scattered pneumocytes displayed ultrastructural signs of apoptosis whereas signs of primary necrosis (for example electron lucid cytoplasm and damaged cell membrane) were more commonly found. Although this finding support the notion that necrosis rather than apoptosis is the usual death mode of structural cells (15), it cannot be excluded tat apoptosis of structural cells occur more frequently in more chronic

situations. We did furthermore find numerous apoptotic neutrophils and neutrophils in the process of secondary necrosis, suggesting that the apoptotic process is not too swift to detect. All together, this implies that apoptotic neutrophils undergoing secondary necrosis is 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, our 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 a swift and silent neutrophil elimination, resulting in sufficient clearance of infiltrating neutrophils. Most likely a similar silent removal process also occurred, to a yet unknown extent, in IF. However, as demonstrated through our TEM analysis, in IF a significant proportion of the neutrophils underwent secondary necrosis, suggesting the clearance to be insufficient. Taken together our data suggests 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 regarding secondary necrosis under relevant clinical situations. It is clear, however, that secondary necrosis occurs extensively in the airway lumen of patients with 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 affect 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), suggested 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 revealed by histopathological and lung imaging techniques, areas with a degree of inflammation similar to the IF examined in the present model, occurs frequently during e.g. common lung infections (18, 19), and probably also during COPD exacerbations. This indicates that similar processes to the one we describe in mouse lungs may in fact be a 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), and similar results have been obtained with LPS stimulated alveolar macrophages from patients suffering from severe asthma (22). It can thus be speculated that the extensive secondary necrosis found in our model is due to impairments in the macrophage clearance system. In support of this, our study revealed that the vast majority of the macrophages within IF to 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 secondary necrosis in the pathogenesis of common airway diseases are highly warranted.

In summary, this study demonstrates that in acutely inflamed lungs insufficient clearance of apoptotic neutrophils results in secondary necrosis, a pro-inflammatory process where intracellular components are released. The increases in LDH recognized following LPS exposure originates mainly from secondary necrosis of apoptotic neutrophils, rather than from

secondary necrosis of structural cells. The process of secondary necrosis may thus have a role in the pathogenesis of neutrophil-rich inflammatory airway conditions such as COPD exacerbations and infectious pneumonia.

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## FIGURE LEGENDS

**FIGURE 1 .** Transmission electron microscopic images exemplifying the different ultrastructural criteria used to determine the fate of lung neutrophils. Resting neutrophil with normal nucleus and intact primary and secondary granules **(A)**, activated neutrophil, exemplified here by occurrence of cytoplasmatic protrusions and phagosomes **(B)**. Apoptotic neutrophil with characteristic chromatin condensation and separation of euchromatin and heterochromatin **(C).** Former apoptotic neutrophil, now in the process of secondary necrosis **(D)**. Note the electron-dense nuclei, ruptured cell membrane and largely dissolved/released cytoplasm. Cluster of neutrophil-derived cell debris **(E)**. For additional details on classification of status, see also Table 1.

**FIGURE 2.** Representative bright field microscopic illustrations of (H&E) sections from a control animal exposed to saline **(A)** and an animal 60 h after LPS administration **(B)**. An inflammatory focus, characterized 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 categories 2 and 3. In areas of intense inflammation (here 60 h after LPS administration), not only the alveolar spaces were filled with cell infiltrates, also the lumen of bronchi and bronchioles (indicated by *L*) were frequently occluded **(C).** Scale bars represent 100 μm in A, B and 50 μm in C.

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

**FIGURE 4.** A detailed transmission electron microscopy analysis was performed to reveal the fates of neutrophils in cell-rich inflammatory foci at different time points following LPS administration. The fates 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 cells at each time point.

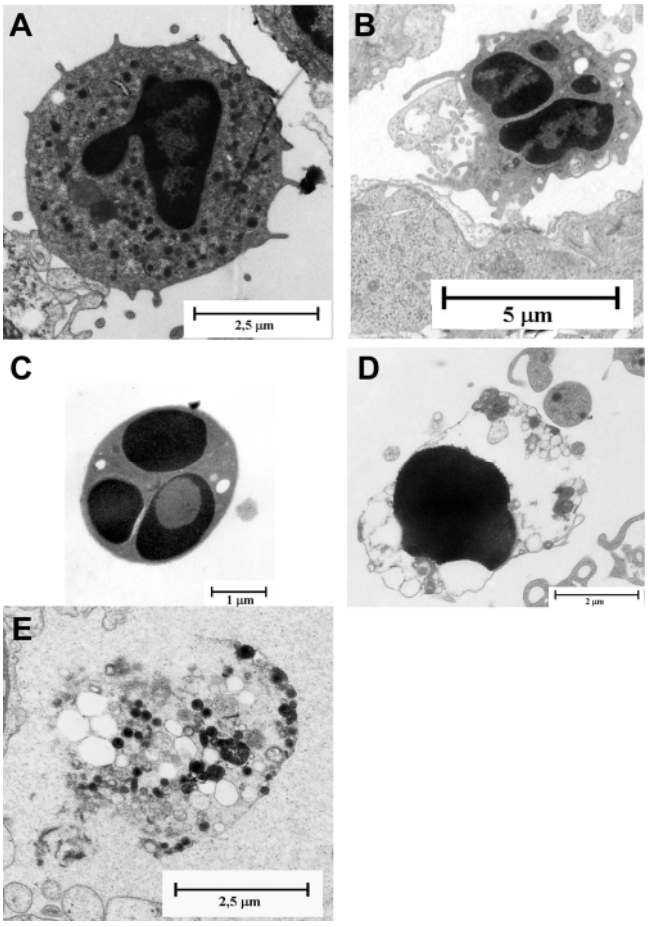
**TABLE 1. Criteria used for electron microscopy quantification of activity status following LPS administration.**

| Status | Characterization 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. |

**Table 2. Effects of LPS on neutrophil infiltration, LDH-activity and TUNEL-positive cells in whole sections and inflammatory foci.**

| Parameters | Control | 4h | 12h | 24h | 36h | 48h | 60h | 72h |
|---|---|---|---|---|---|---|---|---|
| **PMN** *Cells/ml 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** *Inflammatory foci* | 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). PMN: Neutrophils. LDH: Lactatedehydrogenase. TUNEL; Terminal deoxy RNase nick end labelling. *:p <0.05 and **:p<0.01 versus control.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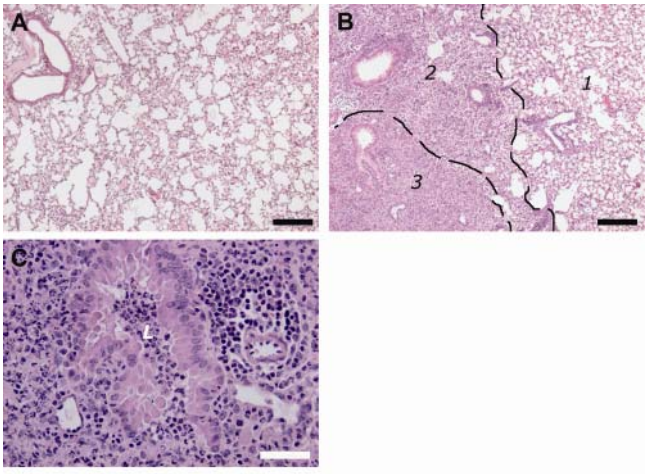
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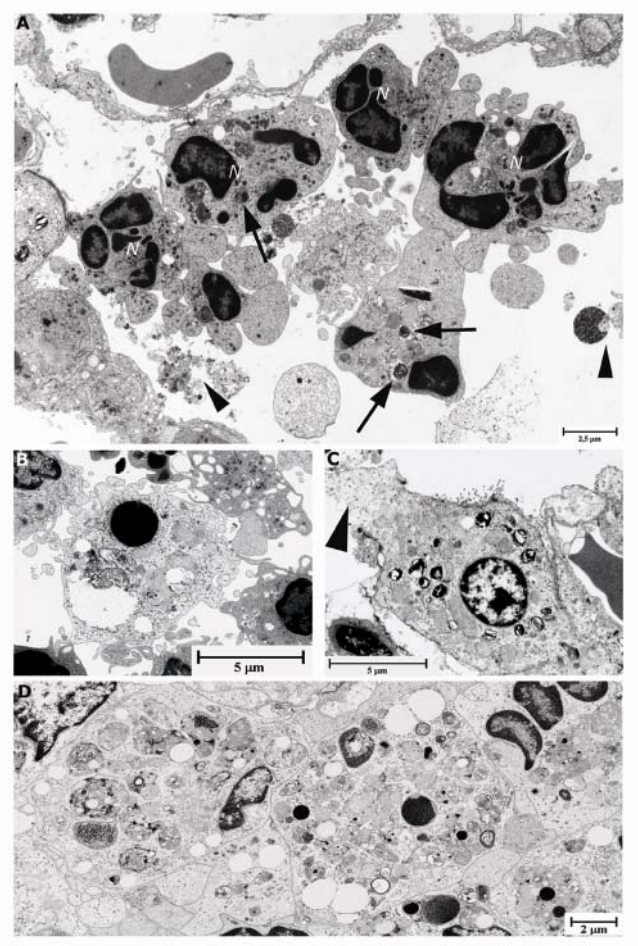
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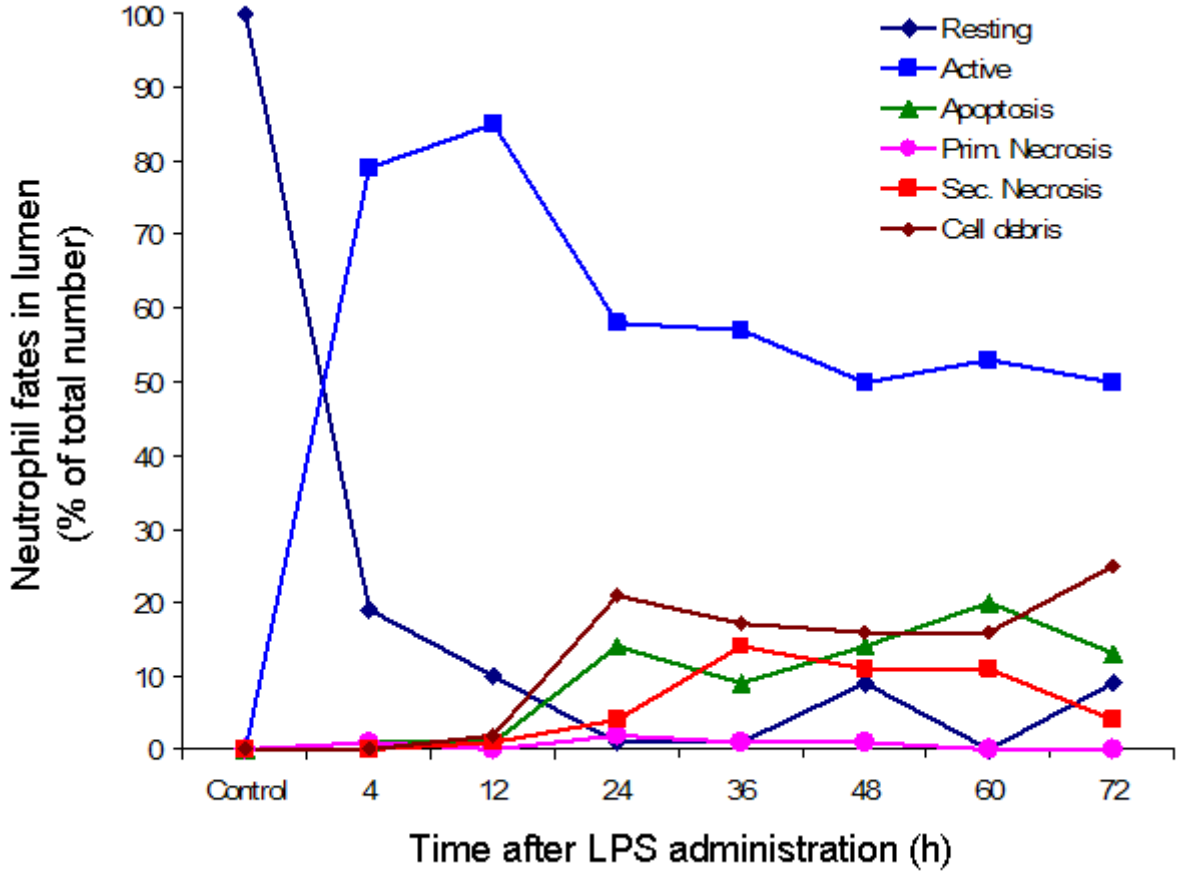
Neutrophil fates in lumen
(% of total number)
100
90
80
70
60
50
40
30
20
10
0
Control
4
12
24
36
48
60
72
Time after LPS administration (h)
Resting
Active
Apoptosis
Prim. Necrosis
Sec. Necrosis
Cell debris